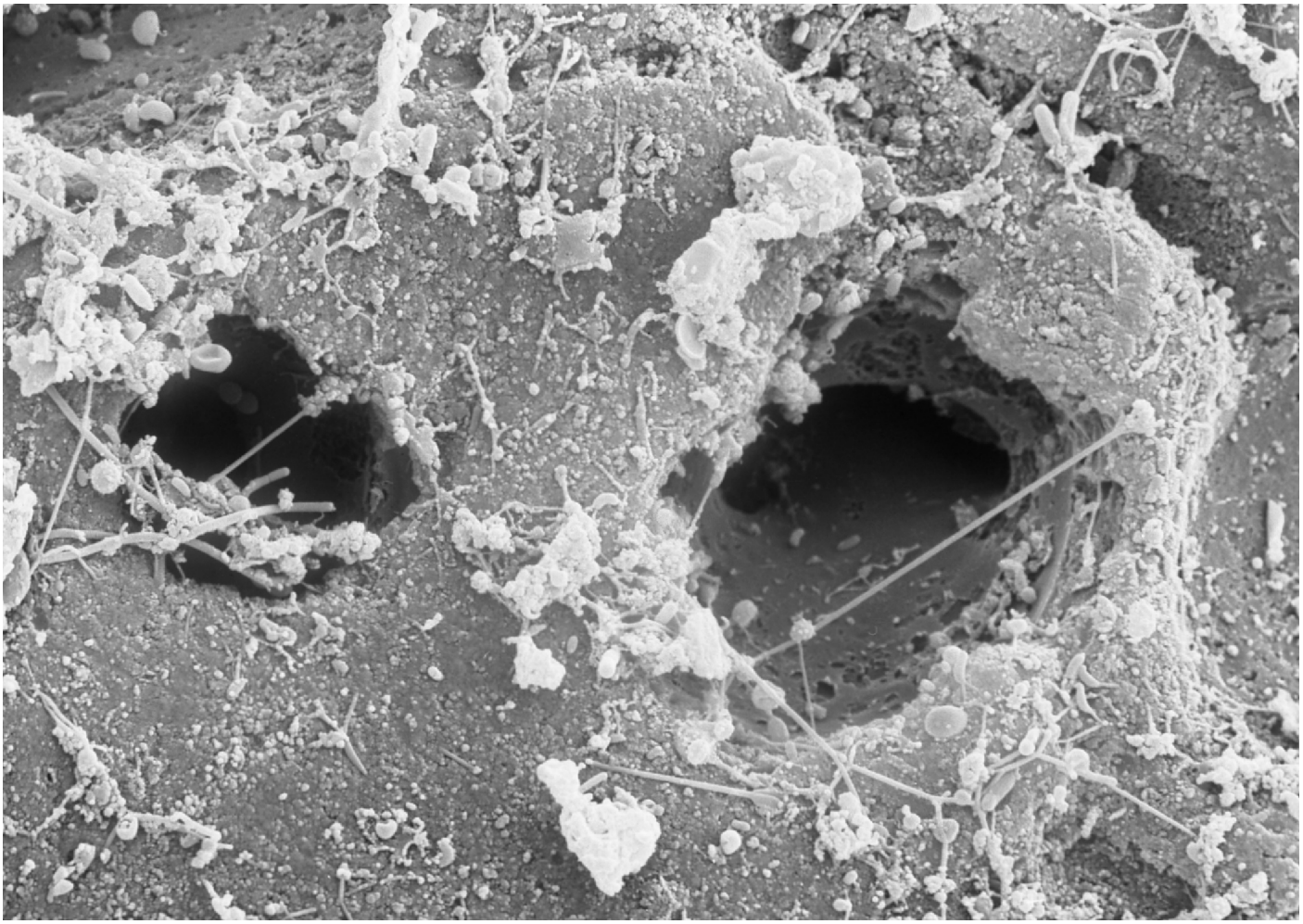

Biologische stabiliteit, biofilms en biofouling

Publicaties over onderzoek in 2000 - 2010

BTO 2011.015(s)
Februari 2011

KWR Watercycle Research Institute

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Voorwoord

De bepaling en beoordeling van de biologische stabiliteit van drinkwater vormt een belangrijk onderwerp in het BTO-programma omdat groei van micro-organismen in het distributiesysteem ('nagroei') kwaliteitsproblemen kan veroorzaken. Bacteriën die lage concentraties aan afbreekbare stoffen benutten spelen een centrale rol bij de biofilmvorming in distributiesystemen maar ook bij biologische processen in de zuivering, bijvoorbeeld in actieve koolfilters, en bij biofouling van membranen. Methoden voor de bepaling van aantallen bacteriën in water en in biofilms en van concentraties afbreekbare stoffen in het water zijn verbeterd. Daarnaast zijn moleculaire methoden voor detectie en identificatie van micro-organismen in water en biofilms ontwikkeld en/of verbeterd en toegepast, omdat hiermee in korte tijd selectieve en kwantitatieve informatie wordt verkregen over de identiteit van de betreffende micro-organismen en de mogelijke oorzaken van groei en biofilmvorming. De resultaten van het onderzoek, grotendeels uitgevoerd in BTO-verband, zijn ondermeer gepubliceerd in wetenschappelijke tijdschriften en in het vakblad H_2O. De voorliggende bundeling van publicaties heeft tot doel om de resultaten van het BTO-onderzoek toegankelijker te maken voor medewerkers van de waterleidingbedrijven en de waterlaboratoria.

Samenvatting

Beperking van de groei van micro-organismen in distributiesystemen ('nagroei') is noodzakelijk om vermindering van de drinkwaterkwaliteit te voorkomen. In biofilms en sediment kunnen zich ongewenste bacteriën vermeerderen en ook vrijlevende protozoa die dienen als gastheer voor *Legionella pneumophila.* Waterpissebedden, draadwormen en andere ongewervelde dieren voeden zich eveneens met biofilm en sediment. Een sterke vermeerdering van deze dierlijke organismen kan tot klachten van de consument leiden. Biofilmvorming speelt ook een centrale rol bij biologische processen in de zuivering, bijvoorbeeld in actieve koolfilters en bij vervuiling (biofouling) van membranen. Dit betekent dat kennis over bacteriegroei en biofilmvorming bij lage concentraties van afbreekbare stoffen nodig is voor het beoordelen van de biologische stabiliteit van drinkwater, het optimaliseren en beheersen van biologische processen bij de waterbehandeling en het beperken van nagroei in het distributiesysteem.

De concentratie van gemakkelijk afbreekbare laagmoleculaire stoffen, die dienst doen als voeding voor bacteriën, kan worden bepaald met de AOC-methode. Deze methode is gebaseerd op het meten van het groeimaximum van twee reincultures van bacteriën. Een stam van de bacteriesoort *Flavobacterium johnsoniae,* geïsoleerd uit drinkwater en geïdentificeerd op basis van DNA-analyse, bleek snel te kunnen groeien bij lage concentraties van hoogmoleculaire stoffen (koolhydraten, eiwitten) en kan mogelijk worden gebruikt voor optimalisatie/uitbreiding van de AOC-test.

De lage detectiegrens van de analyse van het gehalte adenosinetrifosfaat (ATP) maakt deze methode geschikt voor de bepaling van de concentratie van actieve bacteriën in drinkwater en biofilms. Het ATP-gehalte van het drinkwater ligt meestal tussen 1 en 10 ng per liter, is duidelijk gerelateerd aan het totale aantal bacteriën en is afhankelijk van de watersamenstelling, de watertemperatuur en de afstand tot het pompstation. ATP-metingen worden ook toegepast bij het bepalen van de biofilmvormingssnelheid met behulp van de biofilmmonitor en in de biomassaproductiepotentie (BPP)-test voor de bepaling van de groeibevorderende eigenschappen van materialen die in contact komen met drinkwater.

Gebruik van de ATP-analyse voor de bepaling van de biologische activiteit in actieve koolfilters vereist een serie ultrasone behandelingen van monsters van actieve kool (AK) om de bacteriën vrij te maken voor analyse. In AK-filters die worden toegepast bij de drinkwaterbereiding bleken bacteriën van de geslachten *Polaromonas* en *Hydrogenophaga* te domineren. *Polaromonas* kan groeien bij enkele microgrammen afbreekbare carbonzuren per liter. Uit doseerproeven met acetaat bleek dat een concentratie van enkele microgrammen acetaat per liter voedingswater al biofouling van spiraalgewonden membranen kan veroorzaken. Bij een concentratie van 20 µg acetaat-C per liter voedingswater was de snelheid van biofouling 50% van de maximum snelheid. Bij het optreden van operationele problemen met spiraalgewonden membranen (nanofiltratie en omgekeerde osmose) kan na autopsie van een membraanelement met ATP-analyse worden vastgesteld of biofouling de oorzaak is van de problemen.

Steeds meer moleculaire methoden komen beschikbaar voor de detectie en identificatie van micro-organismen. Voordelen van het gebruik van moleculaire methoden zijn: snelheid, selectiviteit en detectie van micro-organismen die met kweekmethoden niet worden waargenomen. Voor de detectie van ammoniumoxiderende micro-organismen zijn selectieve en kwantitatieve methoden (qPCR) ontwikkeld. Bij toepassing van deze methoden bleek dat naast ammoniumoxiderende bacteriën, ook ammoniumoxiderende archaea aanwezig waren in snelfilters en (drink)water. Moleculaire methoden zijn ook toegepast voor de identificatie van de *Flavobacterium johnsoniae* en de dominante bacteriën in AK-filters. Verdere ontwikkeling en toepassing van moleculaire methoden bij het onderzoek naar bacteriën die groeien in drinkwater en biofilms is van belang voor het analyseren van de oorzaken en gevolgen van nagroei en biofilmvorming.

Inhoud

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Threshold concentrations of biomass and iron for pressure drop increase in spiral-wound membrane elements

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ABSTRACT

In a model feed channel for spiral-wound membranes the quantitative relationship of biomass and iron accumulation with pressure drop development was assessed. Biofouling was stimulated by the use of tap water enriched with acetate at a range of concentrations (1–1000 $\mu g\,C\,l^{-1}$). Autopsies were performed to quantify biomass concentrations in the fouled feed channel at a range of Normalized Pressure Drop increase values (NPD_i). Active biomass was determined with adenosinetriphosphate (ATP) and the concentration of bacterial cells with Total Direct Cell count (TDC). Carbohydrates (CH) were measured to include accumulated extracellular polymeric substances (EPS). The paired ATP and CH concentrations in the biofilm samples were significantly ($p < 0.001$; $R^2 = 0.62$) correlated and both parameters were also significantly correlated with NPD_i ($p < 0.001$). TDC was not correlated with the pressure drop in this study. The threshold concentration for an NPD_i of 100% was 3.7 ng ATP cm^{-2} and for CH 8.1 μg CH cm^{-2}. Both parameters are recommended for diagnostic membrane autopsy studies. Iron concentrations of 100–400 $mg\,m^{-2}$ accumulated in the biofilm by adsorption were not correlated with the observed NPD_i, thus indicating a minor role of Fe particulates at these concentrations in fouling of spiral-wound membrane.

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1. Introduction

Microbial growth ('biofouling') in high pressure spiral-wound (SW) membranes for nanofiltration (NF) or reverse osmosis (RO) has been identified as a major cause of operational problems such as increased feed channel pressure drop (PD), decreased mass transfer coefficient (MTC) and product quality decline. First studies on biofouling date from the 1970s and 1980s of the twentieth century (Bailey and Jones, 1974; Potts et al., 1981; Ridgway et al., 1985) and a number of reviews on this issue have been published in the 1990s (Flemming, 1997; Flemming et al., 1993a; Ridgway and Flemming, 1996) because of the increasing application of membrane processes in water treatment and desalination. Destructive membrane sampling (autopsies) has been used to analyze the composition and structure of accumulated biofilms in order to elucidate the fundamentals of the biofouling process in spiral-wound membranes. With different microscopic techniques membrane foulants have been detected and identified as bacterial matter (Ridgway and Flemming, 1996). Still there is a lack of information on the quantitative relationship between biomass concentrations and the resulting operational problems in

Abbreviations: AOC, assimilable organic carbon; ATP, Adenosinetriphosphate; CH, carbohydrates; EPS, extracellular polymeric substances; FS, feed spacer; HPC, heterotrophic colony plate count; IPC, ion chromatography; NF, nanofiltration; NPD, normalized pressure drop; MFS, membrane fouling simulator; MTC, mass transfer coefficient; PD, pressure drop; R_f, exponential fouling rate constant; RO, reversed osmosis; SW, spiral-wound; TDC, total direct cell count.

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NF/RO membranes. Such information is not only needed for diagnostic purposes and improvement of pretreatment but also for assessing the efficacy of cleaning procedures to control biofouling.

Microbial analysis such as Heterotrophic Plate Counts (HPC) and Total Direct Cell counts (TDC), and physical and (bio) chemical analysis including total wet weight of deposits, adenosinetriphosphate (ATP), extracellular polymeric substances (EPS) and proteins have been used to measure the amount of biomass on SW membranes (Flemming and Schaule, 1988; Griebe and Flemming, 1998; Ridgway et al., 1983; Schaule et al., 1993; Vrouwenvelder et al., 1998; Vrouwenvelder et al., 2008). Only few studies have tried to establish quantitative relationships between these biomass parameters and the pressure drop or flux decline. Flemming et al. (1993b) observed an MTC decline of 25% at total cell coverage of the membrane surface of 5×10^7–2×10^8 cells cm^{-2} and suggested a 'pain level' of bacterial cells of 10^8 per cm^2; no correlation with pressure drop was presented. This 'pain level' corresponds with the amount of bacterial cells observed in SW membranes with operational problems related to biofouling (Griebe and Flemming, 1998; Hijnen et al., 2009; Schaule et al., 1993; Vrouwenvelder et al., 1998). Measuring active bacterial biomass with ATP in cell cultures or biomass samples is attractive because the analytical method is rapid, cheap and simple to perform and has a low detection level; a concentration of 1 ng l^{-1} ATP can be detected without concentration techniques. The proportional relationship between ATP and TDC (Magic-Knezev and Van der Kooij, 2004; Vrouwenvelder et al., 2008) indicates that ATP is a potential parameter to quantify the amount of accumulated biomass. Furthermore, autopsy results from full-scale SW membrane installations showed that the increase of the Normalized Pressure Drop (NPD) was related to ATP concentrations (Vrouwenvelder et al., 2008). However, establishment of a causal relationship between ATP and NPD requires more defined conditions to exclude effects of other deposits (dead biomass, EPS and other organic or inorganic substances). Quantification of carbohydrates (CH) with the Dubois method (Dubois et al., 1956) in autopsy studies enables to estimate biomass concentrations based on EPS which consists of polysaccharides with a large water-retention capacity resulting in voluminous deposits. The Dubois method is commonly used in membrane autopsy studies (Gabelich et al., 2004; Griebe and Flemming, 1998; Ridgway et al., 1983) and correlated with flux decline (Fonseca et al., 2007). Biofouling rarely occurs without mineral deposition (Ridgway and Flemming, 1996) and Fe was identified as a predominating foulant in SW elements (Baker and Dudley, 1998), but a causal relationship between Fe and PD increase was not reported. Hence, evaluation of the use of ATP, TDC and CH as quantitative biomass parameters in diagnostic autopsies and cleaning studies as well as elucidation of the role of Fe in pressure drop problems requires biofouling studies under well defined conditions.

In a recent laboratory study using a Membrane Fouling Simulator (MFS) (Vrouwenvelder et al., 2006) a quantitative relationship between acetate as a model substrate and the pressure drop increase was demonstrated (Hijnen et al., 2009). Samples of the biofouled membranes were available for autopsy studies. The objectives of the current study were: (i) elucidation of the quantitative relationship between biomass parameters ATP, TDC and CH and the extent of the PD increase and (ii) determination of the threshold concentrations of these parameters for a 100% increase of the normalized pressure drop (NPD) and (iii) to investigate the role of iron as the major mineral in the water under the experimental conditions. Such information enables the selection of proper biomass parameter(s) in autopsies to assess the cause of PD in membrane elements.

2. Materials and methods

2.1. Biofouling of an NF membrane

The Membrane Fouling Simulator (MFS) loaded with sheets (7×30 cm) of a "virgin" nanofiltration membrane sheet (Trisep 4040-TS80-TSF) was supplied with non-chlorinated tap water after filtration (10 and 1 μm poly-propyleen cartridge filtration; Van Borselen Ltd.) to exclude accumulation of suspended solids and spiked with low amounts of acetate-C to initiate biofouling. These experiments have been described in detail (Hijnen et al., 2009). Briefly, the MFS is a small scale continuous flow model of an SW feed channel (0.8x4x22.cm) filled with the matching Trisep feed spacer ($0.8 \times 4 \times 20$ cm; front 2 cm without feed spacer FS) and operated at a constant feed water flow of 16 l h^{-1} (cross-flow velocity of 0.14 m s^{-1}) at a constant pressure of 1 bar without permeation. Fig. 1 depicts the experimental set up. The rate of clogging of the feed channel was measured by monitoring the pressure drop normalized (NPD) to a moderate environmental temperature of 12.5 °C in the feed channel (Hijnen et al., 2009). The extent of biofouling, given by the relative NPD increase (NPD_i), is calculated from the final NPD (NPD_f) and the initial NPD (NPD_o) by

$$\%NPD_i = \frac{NPD_f - NPD_o}{NPD_o} \cdot 100\% \quad (1)$$

The MFS units were supplied with pre-filtered tap water spiked with acetate-C at concentrations (S_{ac}) of 1, 3, 5, 10, 25, 100, 500 and 1000 μg l^{-1}. Four blank MFS units with no acetate supply were operated with either filtered tap water or unfiltered tap water.

2.2. Feed water quality

The feed water was non-chlorinated tap water produced from anaerobic groundwater using aeration and rapid sand filtration. The pH of the water was 7.98 ± 0.05, dissolved organic carbon content was 2.0 ± 0.1 mg C l^{-1}, assimilable organic carbon concentration (AOC) was 3–5 μg acetate-C eq l^{-1}, NO_3^- and PO_4^{3-} content was 0.12 ± 0.04 and 0.02 ± 0.02 mg l^{-1} respectively. The iron content (ion chromatography; ICP method with a lower detection limit of 0.005 mg l^{-1}) of the filtered tap water was 0.008 ± 0.014 mg l^{-1} and 0.32 ± 0.24 mg l^{-1} in the unfiltered tap water. Iron was the major mineral in the tap water and visually (brown deposits) accumulated in the biofilms. The ambient water temperature was daily monitored during the experiments and ranged from 13.5 to 16.8 °C (average of 15.9 ± 0.7 °C) and was 19.4 ± 2.0 °C in one experiment.

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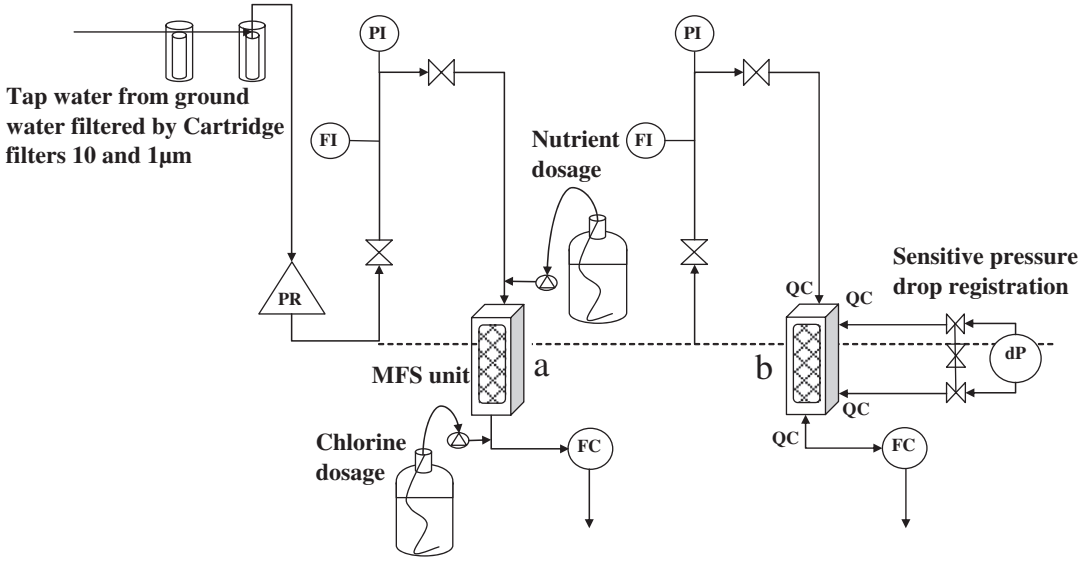

Fig. 1 – Experimental set up for the experiments with a total of five MFS units. Unit on the left with dosage equipment and unit on the right with the mobile pressure drop monitor; PI and FI = pressure and flow indicator; FC = flow controller; QuC = quick connector.

2.3. *Autopsy of the membrane sheets*

Two samples (1.5 × 2 cm) were cut from the membrane at the inlet section of the feed channel without spacer (no FS) and five samples (2 × 2 cm) were cut from the membrane with spacer. The samples were transferred to sterile glass tubes with 20 ml of autoclaved tap water and sonicated with High Energy Sonication (HES, #100) using a BRANSON Digital Sonifier (Model 250 D) at optimized conditions established in a previously published study (Magic-Knezev and Van der Kooij, 2004). The sonifier tip (size 6.5 mm) was inserted into the tube (1–2 cm) containing 20 ml of autoclaved water and the membrane/spacer sample. This tube was placed in melting ice and sonicated for one minute at an amplitude of 45% (15–20 watt) to separate the biomass from the membrane/spacer samples. This treatment was repeated in 20 ml fresh sterile tap water and both suspensions were mixed to obtain a total sample volume of 40 ml. The biomass samples of the experiments with S_{ac} of 25, 3 and 1 $\mu g\,l^{-1}$ were collected by additional swabbing to enlarge the biomass recovery. The swab was treated with HES for 1 min in 20 ml autoclaved tap water and subsequently mixed with the 40 ml HES suspension.

2.4. *Microbial parameters*

ATP was measured to determine the amount of active bacterial biomass in the biofilm samples. The analysis is based on measuring the amount of light produced by an enzymatic reaction using the luciferine–luciferase assay in a luminometer (Celcis Ltd.) and has a lower limit of detection of 1 $ng\,l^{-1}$, which corresponds with 0.01 $ng\,cm^{-2}$ of membrane surface. The method has been described in detail previously (Magic-Knezev and Van der Kooij, 2004). The total direct cell count (TDC) was based on counting of fluorescing cells using epifluorescence microscopy (Hobbie et al., 1977) and the analytical procedure was described in detail before (Hijnen et al., 2009). The detection limit is 180 cells ml^{-1}, which corresponds to 720 cells cm^{-2}.

2.5. *Carbohydrate analysis*

The CH concentration in the biofilm samples was analyzed with the method described by Dubois et al. (1956) using glucose as the reference carbohydrate. The extinction/adsorption at 490 nm was measured directly in the biomass suspension after hydrolysis and complexation with sulphuric acid and phenol, respectively and expressed in glucose equivalent concentration. The detection limit of this parameter was approximately 5–10 $\mu g\,cm^{-2}$ depending on the sampled membrane area.

2.6. *Iron content*

The iron (Fe) content of the obtained biomass suspension was assessed with Atomic Absorption Spectrometry resulting in a lower limit of detection of approximately 1 $mg\,cm^{-2}$ of membrane surface.

2.7. *Correlation analysis and statistics*

Correlation analyses was done by determining Pearson's correlation coefficient between paired values of ATP, TDC, CH and Fe using SPSS 17.0 software with a significance level of $p \leq 0.01$. For the correlation of the biomass and Fe accumulated in the MFS units with the NPD increase (%), the weighted average concentrations ($\overline{C}_{avg}$) were calculated from the concentrations observed in the samples at different locations in the MFS units using

$$\overline{C}_{avg} = \frac{\sum_{i=1}^{n} (C_i + C_{i+n})/2 * A_{i+n}}{A_{tot}} \quad (2)$$

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where and C_i, C_{i+n} and A_{i+n} (cm^{-2}) are the concentration and the surface area at the ith part of n parts of the feed channel surface ($n = 3–7$) and A_{tot} is the total surface area of the MFS unit. The linear regression analysis of the correlation between the $\overline{C}_{avg}$-values of the biomass parameters and Fe concentrations obtained from membrane autopsy and the NPD_i was performed with Excel software. For the correlation of the biomass parameters and Fe concentrations with the NPD_i the non-parametric Spearman's rank correlation coefficient was calculated and multi-regression analysis was conducted with SPSS 17.0 software.

3. Results

3.1. NPD increase

In the MFS units supplied with acetate-enriched tap water biofouling was observed at each concentration (Fig. 2) and the NPD increase (NPD_i) was characterized as a first order process (Hijnen et al., 2009). The blank MFS unit supplied with pre-filtered tap water without added acetate showed no NPD_i during 28 days of operation (Fig. 2a), whereas in units supplied with 1 μg of acetate-C/l biofouling was observed (Fig. 2b). Also no fouling was observed within 100 days of operation in the two blank units supplied with unfiltered water (Fig. 2c). After 100 days the pressure drop started to increase in these units. The accumulated biofilm in the feed channels was colourless at high biofouling rates and short operation times (<20 days). At lower biofouling rates and operational times of ≥25 days the feed channel showed accumulation of brown coloured deposits. These observations initiated the analysis of the Fe concentrations in the fouled membrane samples.

3.2. Spatial distribution of biomass and Fe

The units were sampled for biomass and Fe concentrations at different fouling conditions with relative NPD_i values ranging from 71 to 3390% (Table 1). The MFS units supplied with acetate showed high ATP concentrations at the inlet section without spacer (no FS), further elevated concentrations in the first part of the section with feed spacer (0–2 cm) and a decline of concentrations in the subsequent parts of the channel (Fig. 3). In the units supplied with acetate the percentage of the total amount of ATP at the inlet section (no FS) was 1.5–9%, in the first 2 cm with feed spacer 8–16% and in the last part (18–20 cm) 3–10% (Fig. 3c). At acetate concentrations of 1000, 500 and 25 μg C l^{-1} the ATP concentrations were higher than in the units supplied with the lower acetate concentrations (10, 5, 3 and 1 μg C l^{-1}). In the blank MFS units without acetate dosing a lower ATP concentration was observed (Fig. 3b). The spatial distribution of parameters TDC and CH and also of Fe in the channels was similar to the distribution of ATP; a declining concentration in the section with feed spacer (no figures presented; weighted average concentrations presented in Table 1).

3.3. Correlation analysis of biomass parameters and iron

The operational periods with acetate dosing, the final NPD_i values and the exponential fouling rate constant R_f and the weighted average values ($\overline{C}_{avg}$) of the biomass parameters and Fe for the correlation analysis are presented in Table 1. The correlation analysis of the paired biomass parameters showed that the log value of the ATP concentration in the MFS units was significantly ($p < 0.01$) correlated with the log value of the TDC and the CH concentrations, respectively (Table 2). The linear regression equation for the relationship with TDC was Log [ATP] = 0.79 (95% CI 0.69–0.89) Log [TDC] + 4.1 (95% CI 3.6–4.6) with a goodness of fit (R^2) of 0.75. Based on this correlation 1 ng of ATP equals 3×10^6 (95%CI 5.3×10^5–1.7×10^7) TDC cm^{-2}. The CH concentration ranged between 10 and 100 μg cm^{-2} at ATP concentrations of 10–100 ng cm^{-2}, but the linear regression fit of paired ATP and CH values was poor ($R^2 = 0.39$). A better fit ($R^2 = 0.62$; $p < 0.0001$) was observed for the values of the units operated under acetate limitation conditions where the fouling rate R_f was below $R_{f,max}$ ($S_{ac} \leq 10$ μg l^{-1}). ATP and Fe concentrations were not correlated when the results of the MFS units operated at high S_{ac} values with relatively short operation times (≤20 days) were included. For the MFS units operated at S_{ac} values ≤10 μg l^{-1} with longer operational periods ATP and Fe concentrations were significantly correlated ($p < 0.001$; Table 2). TDC did not correlate with CH and Fe. The latter two parameters correlated significantly ($p < 0.001$) with a better

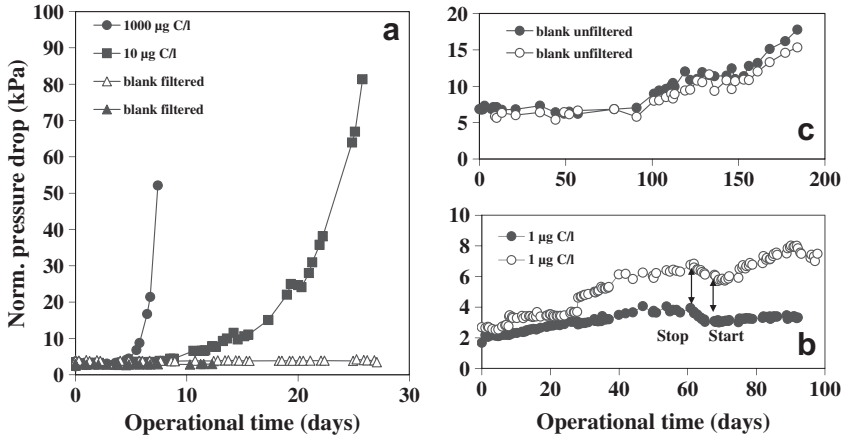

Fig. 2 – The development of the Normalized pressure drop (NPD) in the MFS units supplied with filtered tap water enriched with different acetate concentrations (a,b) and (c) supplied with unfiltered tap water (lines in b and c are duplicates).

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Table 1 – The fouling conditions of the MFS experiments and the weighted average concentration ($\overline{C}_{avg}$) of the biomass parameters and iron measured by autopsies.

Acetate S_{ac} (μg C/l)	Operational time (days)	Final pressure drop (kPa)	% NPD increase	R_f [b] (ln NPD_i d^{-1})	ATP (ng cm^{-2})	TDC (cell × 10^8 cm^{-2})	CH (μg gluc. eq cm^{-2})	Fe (mg m^{-2})
0 pre-filtered	20	2.9	6.0	<0.001	0.8	0.02	9.4	0.32
0 unfiltered	184; 184	17.8; 15.3	156; 157	0.015; 0.016	3; 2	0.3; 0.4	9.6; 10.0	394; 298
1	146; 98	3.3; 7.2	71; 119	0.063; 0.027	6; 3	Nd[d]; 0.1	Nd[e]	96; 129
3	34; 152	18.6; 24.5	306; 526	0.102; 0.109	20; 18	2.0; 2.3	Nd[e]	83; 173
5	39; 46	15.7; 37.9	376; 919	0.128; 0.245	16; 13	0.3; 0.4	11.7; 11.9	149; 212
10	35; 28	7.9[c]; 81.4	234[c]; 2369	0.205; 0.224	28; 46	0.3; 0.6	13.0; 44.6	201; 426
25	35; 33	37.8; 54.7	1352; 993	0.766; 0.696	175; 158	1.4; 0.5	Nd[e]	82; 79
500	20; 15	61.3; 22.2	3390; 507	0.859; 1.144	200; 91	2.2; 3.1	52.4; 20.6	11; 1
1000	15; 14; 8	52.1; 18.4; 7.9	1820; 445; 182	1.126; 1.475; 1.097	118; 58; 184	1.0; 1.2; 1.4	21.1; 14.4; 47.1	1; nd; 7
S1[a]	31	46.1	725	100: 1.123	37	0.5	21.3	334
S2[a]	53	45.4	1231	1000: 1.160	37	0.8	37.3	154

a Starvation experiments with variable acetate dosages and starvation periods (S1 = 100-5 and S2 = 1000-1000-10) (Hijnen et al., 2009).
b First order fouling rate R_f values from Hijnen et al. (2009) modified as submitted in an erratum (Hijnen et al., in press).
c Low NPD_i caused by preferential flow path in the feed channel.
d Nd = not determined.
e Unreliable CH data due to the use of cotton swab.

goodness of fit for the units with acetate-C concentrations of ≤10 μg l^{-1} (Table 2).

3.4. Correlation with NPD_i

The study aimed at assessing the relationship between the biomass concentration and Fe with the NPD_i at the time of the autopsy. The weighted average ATP and CH concentrations in the MFS units were both significantly ($p < 0.01$) correlated to the NPD_i (%) as evaluated with the non-parametric Spearman's rank correlation coefficient (R^2 of 0.71 and 0.91, respectively). The linear regression analysis also showed a significant ($p < 0.001$) correlation with a high correlation coefficient for ATP and CH (0.52, 0.70 and 0.82; Table 2 and Fig. 4). No significant correlation was observed for TDC with NPD_i (Fig. 4c). The variability of ATP and CH concentrations in the MFS units is presented in Fig. 4 with the standard deviation (s.d.; $n = 3–7$). The bars show an increased variability of both parameters at increased NPD_i values which was caused by increased heterogeneity of the concentrations in the feed channel (Fig. 3).

No correlation was found between the concentrations of Fe and the NPD_i values (Fig. 4c), but the regression plots of ATP and CH with NPD_i revealed that the low ATP and CH concentration at relatively high NPD_i values contained Fe concentrations of >100–200 mg m^{-2}. However, a multi-regression analysis in combination with either ATP or CH again showed

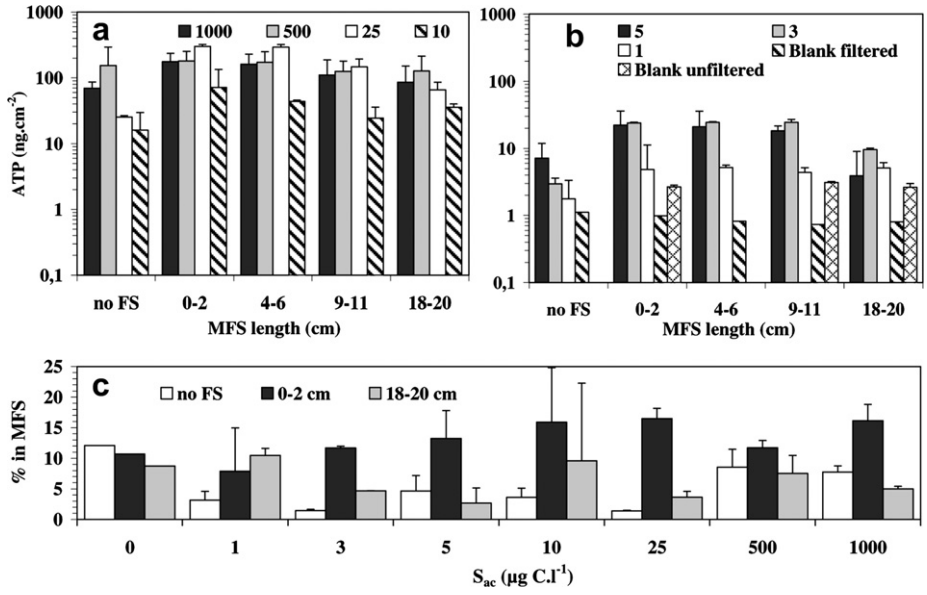

Fig. 3 – Distribution of ATP concentrations (error bar is s.d.) in the MFS units supplied with filtered tap water enriched with different acetate-C concentrations (μg l^{-1}) and with unfiltered tap water (a,b) and (c) % of the total ATP amount in the membrane feed channel without feed spacer (no FS) and after 0–2 and 18–20 cm with feed spacer.

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Table 2 – Correlation matrix of the different biomass parameters, Fe and NPD_i measured in the standard MFS experiments; presented are the square of the Pearson correlation coefficient R^2, for all correlations *p*-values were < 0.001 except for values indicated by * (p-value < 0.01), the number of observations (n).

	S_{ac} values ($\mu g\,C\,L^{-1}$)	TDC (cells cm^{-2})	CH ($\mu g\,cm^{-2}$)	Fe ($mg\,m^{-2}$)	NPD_i[c] (%)
ATP ($ng\,cm^{-2}$)	1–1000	0.75; 85[a]	0.39; 49	nc[b] ($p = 0.09$)	0.52; 19
	≤10	0.66; 43[a]	0.62; 30	0.56; 42	0.70*; 9
TDC (cells cm^{-2})	1–1000	1	nc	nc	nc
CH ($\mu g\,cm^{-2}$)	1–1000		1	0.34; 30	0.82; 13
	≤10			0.65; 16	
Fe ($mg\,m^{-2}$)	1–1000			1	nc

a Log transformed values.
b Nc = no correlation.
c Correlation with the weighted average concentrations of the parameters.

no significant correlation between Fe and NPD_i. This indicates that the contribution of Fe accumulation in the MFS units to the pressure drop increase was limited. The minor effect of the Fe concentrations on the NPD_i was also demonstrated by the Fe content in the MFS units supplied with unfiltered tap water (unfiltered blanks; Fig. 4c). The Fe concentrations in these unfiltered blanks with a limited NPD_i of 156% were 298 and 394 $mg\,m^{-2}$, whereas ATP and CH concentrations were low (2–3 $ng\,cm^{-2}$ and 9.6–10 $\mu g\,cm^{-2}$, respectively; Table 1). In the MFS units at S_{ac} value of 10 $\mu g\,L^{-1}$ considerably higher NPD_i values (234–2369%) were observed at comparable Fe content of 201–426 $mg\,m^{-2}$ and higher biomass concentrations of 28–46 ng ATP cm^{-2} and 13–44.6 μg CH cm^{-2}. Similar observation was recorded for the MFS unit supplied with 100 and 5 $\mu g\,l^{-1}$ acetate and intermediate starvation period; Fe, ATP and CH content was 334 $mg\,m^{-2}$, 37 $ng\,cm^{-2}$ and 21.3 $\mu g\,cm^{-2}$, respectively at an NPD_i of 725%.

The pressure drop increase was due to a decrease of the open pore volume of the feed channel which in turn was a result of biomass accumulation. The relationship between the biofilm thickness and the NPD_i has been described with hydraulic equations (Schock and Miquel, 1987) and is linear in the initial stage of biofouling but exponential in the subsequent stage (Hijnen et al., 2009). Assuming that ATP and CH concentrations were linearly related with the biofilm thickness the correlations with NPD_i were also tested for an exponential relationship. Both exponential fits were significant ($p < 0.01$), but the goodness of fit was lower compared to the linear regression (Fig. 4).

3.5. *Threshold concentrations*

The ATP concentration in the feed channels of the MFS units for an NPD_i of 100% was 3.7 $ng\,cm^{-2}$ (±95% CI = 1.3–10.9), calculated from the equation presented in Fig. 4a. For CH the threshold concentration for this criterion was calculated from the equation given in Fig. 4b at 8.1 $\mu g\,cm^{-2}$ (±95% CI of 6.1–11.7). This was around the detection limit of the analysis of 5–10 $\mu g\,cm^{-2}$. For TDC and Fe no threshold concentration was calculated because of the lack of correlation with NPD_i.

3.6. *Fouling and accumulation rate*

The fouling rate in the feed channel of the MFS units could be described with the exponential fouling rate constant R_f (Table 1). Formation of biofilms on surfaces initially is an exponential process that is rapidly followed by a linear phase due to diffusion limitation of the substrate flux into the

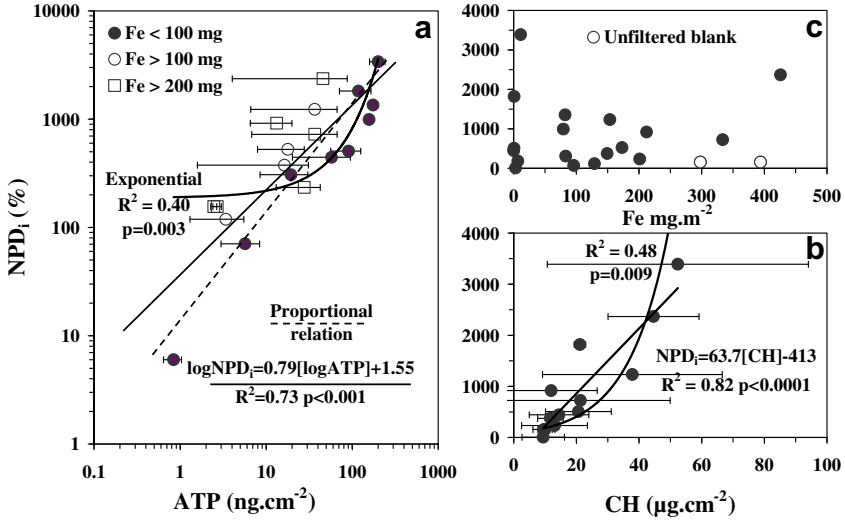

Fig. 4 – The relationship between the accumulated biomass measured with ATP and CH (a,b) and (c) the accumulated mass of Fe with the NPD_i (%); error bar is s.d. (n = 4–7).

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biofilm (Rittmann, 1995). This was clearly demonstrated for ATP and Fe accumulation on glass (Van der Kooij et al., 2003). On base of the concentrations of ATP, CH and Fe measured in the MFS units after the different dosing periods the linear accumulation rate of these parameters was calculated and correlated with the S_{ac} in the influent and R_f (Fig. 5a). Correlations of both biomass parameters with S_{ac} showed a similar saturation curve and relationship with S_{ac} as described for R_f (Fig. 5a). The ATP and CH accumulation rates were strongly correlated ($p < 0.0001$) with R_f with R^2 of 0.91 for ATP and 0.90 for CH. This clearly demonstrates the proportional relationship of both biomass parameters with the porosity decline in the feed channel.

4. Discussion

4.1. ATP and TDC as biomass parameters in autopsies

In full-scale SW membrane filtration installations where operation is hampered by fouling problems, it is common practice to carry out an autopsy to verify the cause of the fouling process. Only few studies have been published on the quantitative correlation between biomass parameters and operational problems in membranes such as pressure drop increase and flux decline (Flemming et al., 1993b; Fonseca et al., 2007; Vrouwenvelder et al., 2008). The results of the present study show that ATP is a suitable parameter to elucidate the role of biofilm formation in the pressure drop increase in such membranes. This conclusion was based on the correlation with the observed NPD_i and supported by the good correlation between the biofilm formation rate (ng ATP cm^{-2} d^{-1}) in the feed channel of the MFS with the acetate concentration (Fig. 5a) and the exponential fouling rate constant R_f (Fig. 5b). Additionally, this clearly shows that the assessment of the biofilm formation rate for the feed water of SW membranes which is also based on ATP measurements (Van der Kooij et al., 2003) is an appropriate parameter to assess the biofouling potential of the feed water.

The choice of a 100% NPD_i in the current study to assess a threshold biomass concentration was based on a commonly used NPD_i cleaning criterion of 15% over one stage of a series of six successive membrane elements (Graham et al., 1989; Hickman, 1991; Speth et al., 1998). The NPD_i is not evenly distributed over the elements and usually is mainly located in the first element. Consequently, the NPD_i in this element is higher (Vrouwenvelder et al., 2009a) and may be close to 100%. The threshold ATP concentration for 100% NPD_i in the MFS units was 3.7 ng cm^{-2}. A higher threshold ATP concentration for 100% NPD_i of 30 ng cm^{-2} was reported for SW elements operated under field conditions (Vrouwenvelder et al., 2008). However, one would expect this the other way around: lower for the same NPD_i in the field elements because of differences in biofilm conditions. MFS units of the present study contained relatively young biofilms whereas biofilms in field elements were more aged with a lower ratio between active (ATP) and total biomass (including EPS and dead cell material). This difference between threshold values might be caused by the difference in 100% NPD_i over SW elements and the MFS of the current study. It can also be caused by correlating on one hand the maximum ATP concentration with the NPD_i in field elements with a length of 1 m (Vrouwenvelder et al., 2008) and on the other hand the weighted average ATP concentration with the NPD_i in a 0.2 m feed channel of the MFS as done in the present study. Consequently, despite the positive correlations ATP results in field autopsies must be interpreted with care and additional parameters which are more related to the total amount of the accumulated biomass are needed.

The present study and also the mentioned field study (Vrouwenvelder et al., 2008) revealed that in contrast to ATP, TDC was not correlated with NPD_i. The range of TDC values of 1×10^7–3.1×10^8 corresponds with a biofilm thickness of 0.1–1.6 μm (assumed bacteria cell volume of 0.5 μm^3; diameter of 1 μm). Theoretically for the 100% NPD_i a biofilm thickness of 60 μm was estimated (Hijnen et al., 2009) thus indicating that microscopic cell count (TDC) is not an accurate parameter for total biomass and more importantly biofilms consist of more than bacterial cells.

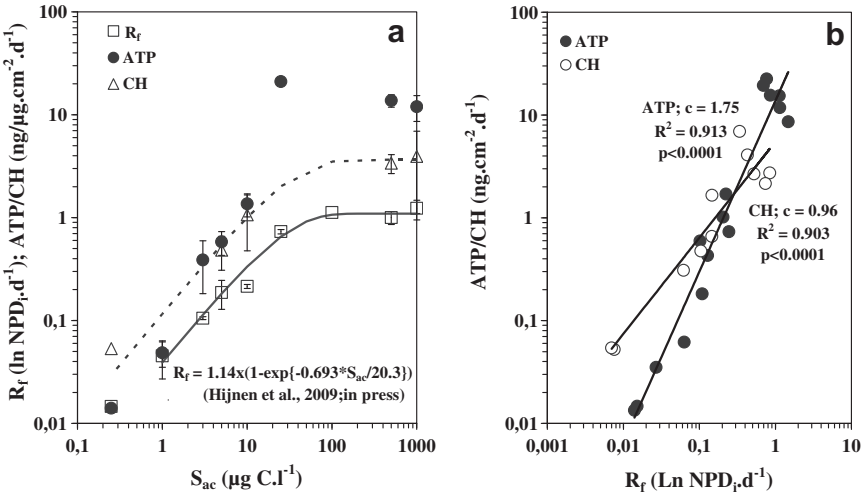

Fig. 5 – The correlation of the exponential fouling rate R_f and the ATP and CH accumulation rates with the acetate concentrations S_{ac} (a) and (b) the correlation of the biomass (ATP/CH) accumulation rates with R_f.

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4.2. Carbohydrates as a biomass parameter

Biofilms are established by adsorption and adherence of bacteria followed by growth due the supply of nutrients. Extracellular polymeric substances (EPS) excreted by bacteria to anchor themselves to the surface and to each other play a key role in the development of biofilms (i.e. protection against environmental stress, nutrient availability; Or et al., 2007; Flemming and Wingerden, 2010). CH are important components of EPS (Sutherland, 1999). The method of Dubois et al. (1956) is commonly applied to quantify the CH concentration in field SW elements (Gabelich et al., 2004; Griebe and Flemming, 1998; Ridgway et al., 1983, 1984) and their reported CH concentrations were in the same order of magnitude as measured in the current study. The positive correlation between the CH concentration and pressure drop in the feed channel of the MFS (Fig. 4b) and the proportional correlation of the CH accumulation rate ($\mu g\,cm^{-2}\,d^{-1}$) to the exponential fouling rate the R_f, (Fig. 5b) confirms that the CH concentration in SW membranes is a valuable parameter in diagnostic autopsies. The threshold CH concentration of 8.1 $\mu g\,cm^{-2}$ for the defined NPD_i was around the current detection limit of the analysis, but the analysis can be optimized by sampling larger surface areas. More recently the CH parameter was correlated with flux decline in NF membranes (Fonseca et al., 2007) and they reported a decline of 30–80% when the CH concentrations increased to 50 $\mu g\,cm^{-2}$. Another study presented a >50% flux decline and 100% NPD increase in SWM elements at a CH concentration of 12.4 $\mu g\,cm^{-2}$ (Gabelich et al., 2004). Consequently, we propose the use of the parameters ATP and CH in membrane autopsy studies: the ATP method which is cheap and fast and reveals information on the accumulated active biomass in the feed channel and CH which represents the total amount of active and inactive biomass. Inclusion of the analysis of CH is especially of interest for studies on the effect of membrane cleaning with chemicals (Cornelissen et al., 2009).

4.3. Fe accumulation and pressure drop increase

In the flat sheet MFS units without permeate production the process of particulate accumulation on the membrane surface was not influenced by vertical forces and particle settling which normally occur in SW elements with permeate production (Belfort and Nagata, 1985; Belfort, 1988). Thus, the observed accumulation of Fe particles in the MFS was a result of adsorption of these particles onto biomass produced on the membrane surface during the short cross-flow contact time. The results of the current study show that the accumulation of biomass has a far greater effect on NPD_i in the feed channel than the accumulation of Fe particulates (Fig. 4c). In the units supplied with unfiltered tap water lower biofilm concentrations were observed than in the units supplied with 1 μg acetate-C L^{-1} (Fig. 3) but the Fe content was much higher at similar NPD_i values (71–157%; Table 1). The significant correlation between CH and Fe (Table 2) indicates that EPS plays a substantial role in the adsorption of Fe onto charged biopolymers which is related to the presence of negatively charged carboxylic and phosphate groups (Wuertz et al., 2001). The dominant role of biomass in the NPD_i is explained by the high water-retention capacity of EPS. Water-retention curves show that certain polysaccharides hold more than 50–70 g of water per gram while maintaining structural coherence (Or et al., 2007; Chenu, 1993). No studies on the role of Fe particulates in SW membranes on pressure drop are known to the authors. A recent study presented the correlation of the mass deposit of Fe micro- and nanoparticles in a porous sand column (208 cm^3; empty space volume of 102 cm^3 and porosity of 0.49) with the pressure drop increase (Vecchia et al., 2009). In this study an Fe concentration of 2.6 $mg\,cm^{-3}$ resulted in a PD_i in the sand column of 1 kPa. The Fe concentration in the MFS supplied with unfiltered water was 300–400 $mg\,m^{-2}$ (Table 2) which equals a volumetric concentration of 0.41–0.54 $mg\,cm^{-3}$ (channel height of 0.0008 m and porosity of 92%). The $\%NPD_i$ in this MFS was 156–157% with higher ATP and TDC values than in the pre-filtered blank (Table 1). These calculations show that Fe accumulation in the feed channel of SW elements at a level of $\leq$100 $mg\,m^{-2}$ (10 $\mu g\,cm^{-2}$) has no effect on the NPD. Further studies under field conditions are required, however, to collect additional data on the relationship of particulate accumulation and biofouling.

4.4. Feed spacer enhances biofilm accumulation

The spacer in the feed channel enhanced biomass accumulation (Fig. 3) which is consistent with observations in other studies (Picioreanu et al., 2009; Vrouwenvelder et al., 2009b). Possible explanations for this observation are: an increase in attachment area or/and enhanced mass transfer of nutrients to the biofilm due to increased turbulence. Based on a specific surface area of the feed spacer of 7700 $m^2\,m^{-3}$ (Picioreanu et al., 2009) it can be estimated that the spacer contributes with around 25% to the attachment surface in the feed channel. An earlier autopsy study on SWM elements from field locations showed more accumulation of biomass (ATP) on the membrane (38–90%) than on the feed spacer (5–62% of the total amount) (Vrouwenvelder et al., 2008). Preferential flow paths shown by Computational Fluid dynamics and filamentous streamers at the spacer junctions (Picioreanu et al., 2009; Vrouwenvelder et al., 2009b) were not observed in the present study. Verification of the role of the feed spacer in biofouling of SWM elements requires further research.

5. Conclusions

The effect of biomass accumulation in spiral-wound membranes on pressure drop increase can be elucidated by measuring concentrations of active biomass with adenosine-triphosphate (ATP) and of total biomass with carbohydrates (CH; Dubois, method) in membrane autopsies. There was a significant correlation ($p < 0.001$) between these parameters in the current study. This study also showed a significant ($p < 0.001$) and causal relationship between both parameters and the NPD_i in a model feed channel. Furthermore, the calculated ATP and CH accumulation rates were highly correlated with the observed exponential fouling rate. Threshold concentrations for 100% NPD_i were 3.7 ng ATP cm^{-2} and 8.1 μg CH cm^{-2}. Because ATP is related to active biomass and CH to the total biomass, monitoring both parameters in autopsies will reveal further information on the metabolic

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state of the accumulated biofilm. Iron accumulation in the feed channel was enhanced by the biofilm growth as demonstrated by the significant correlation between CH and Fe concentrations ($p < 0.001$). Iron concentrations of $\leq 100\,mg\,m^{-2}$ ($10\,\mu g\,cm^{-2}$) of membrane surface did not contribute to pressure drop increase in spiral-wound membranes. The high impact of accumulation of low biomass concentrations on pressure drop increase is attributed to the high water-retention characteristics of polysaccharides in biofilms.

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ADDITION/CORRECTION

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Threshold Concentration of Easily Assimilable Organic Carbon in Feedwater for Biofouling of Spiral-Wound Membranes [*Environmental Science & Technology* **2009**, *43*, 4890]. Wim A.M. Hijnen,* David. Biraud, Emile R. Cornelissen, and Dick van der Kooij

The values calculated for first-order fouling rate constant, R_f, published in Hijnen et al. *Environ. Sci. Technol.* **2009**, *43*, 4890−4895 are incorrect because of the use of an incorrect formula. The following formula was used:

$$\mathrm{NPD}_i = \mathrm{NPD}_t - \mathrm{NPD}_o = e^{R_f t} \text{ and } \ln \frac{\mathrm{NPD}_t}{\mathrm{NPD}_o} = R_f t$$

The values for R_f were calculated with linear regression analysis of the increase of NPD_i in time, and ln NDP_i was derived from $\ln(\mathrm{NPD}_t) - \ln(\mathrm{NPD}_o)$, which is not correct. The correct formula for the calculation of ln NDP_i should use $\ln(\mathrm{NPD}_t - \mathrm{NPD}_o)$ because

$$\mathrm{NPD}_i = (\mathrm{NPD}_t - \mathrm{NPD}_o) = e^{R_f t} \text{ and } \ln(\mathrm{NPD}_t - \mathrm{NPD}_o) = R_f t$$

The corrected R_f values are presented in Table 1. The relationship between the acetate concentration and the corrected R_f is

Table 1. Recalculated Exponential Fouling Rate Constant R_f Values

S_{ac} (μg C·L^{-1}) (temp °C)	MFS I; R_f (ln NPD$_i$.d^{-1}; 95% CI; r^2)	MFS II; R_f (ln NPD$_i$.d^{-1}; 95% CI; r^2)
1000 (13.5 ± 0.3)	1.475 (1.421−1.528; 0.99)	1.126 (1.057−1/194; 0.99)
500 (13.5 ± 0.3)	0.859 (0.756−0.962; 0.99)	1.144 (1.041−1.247; 0.99)
1000 (14.3 ± 0.4)	1.097 (0.954−1.240; 0.99)	1.116 (1.007−1.312; 0.99)
100 (15.2 ± 0.5)	1.123 (0.917−1.330; 0.96)	
10 (16.0 ± 0.9)	0.205 (0.197−0.214; 0.99)	0.224 (0.206−0.242; 0.96)
5 (16.1 ± 0.9−19.4 ± 2.0)[a]	0.128 (0.116−0.140; 0.95)	0.245 (0.221−0.269; 0.93)
25 (16.8 ± 0.5)	0.696 (0.597−0.795; 0.96)	0.766 (0.697−0.835; 0.98)
3 (14.9 ± 0.6)	0.102 (0.094 − 0.110; 0.94)	0.109 (0.099−0.119; 0.93)
1 (15.5 ± 0.8)	0.063 (0.055 − 0.071; 0.82)	0.027 (0.024−0.031; 0.89)

[a] Temperature during test MFS I and II, respectively.

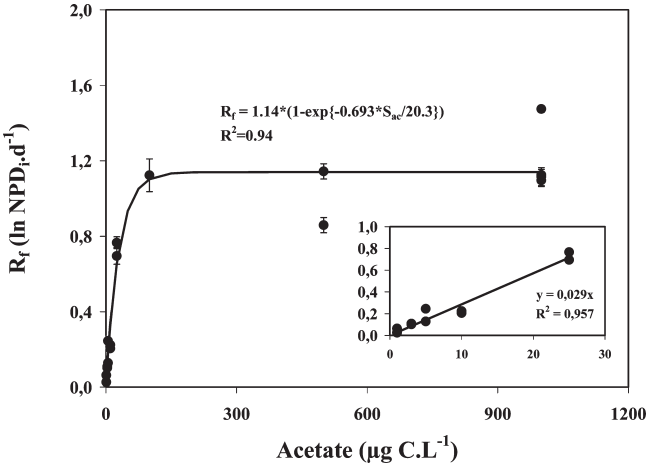

Figure 1. Relationship between the concentration of acetate in the feedwater and the fouling rate constant R_f (error bar = SD).

recalculated (Figure 1). This calculation gives a higher maximum R_f value of 1.14 d^{-1}, and the half saturation constant k_f calculated from the new data is 20.3 $\mu g\ C \cdot L^{-1}$, which is also higher than the value of 15 $\mu g\ C \cdot L^{-1}$ presented in the original publication. The major conclusion of the study that the threshold concentration for biofouling of the feed channel is about 1 μg acetate $C \cdot L^{-1}$ remains unaffected by these corrections.

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Effect of water composition, distance and season on the adenosine triphosphate concentration in unchlorinated drinking water in the Netherlands

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ABSTRACT

The objective of our study was to determine whether water composition, distance to the treatment plant and season significantly affect the adenosine triphosphate (ATP) concentration in distributed drinking water, in order to resolve the suitability of ATP as an indicator parameter for microbial regrowth. Results demonstrated that the ATP concentration in distributed water averaged between 0.8 and 12.1 ng ATP L^{-1} in the Netherlands. Treatment plants with elevated biofilm formation rates in treated water, showed significantly higher ATP concentrations in distributed drinking water and ATP content was significantly higher in the summer/autumn compared to the winter period at these plants. Furthermore, transport of drinking water in a large-sized distribution system resulted in significantly lower ATP concentrations in water from the distal than the proximal part of the distribution system. Finally, modifications in the treatment significantly affected ATP concentrations in the distributed drinking water. Overall, the results from our study demonstrate that ATP is a suitable indicator parameter to easily, rapidly and quantitatively determine the total microbial activity in distributed drinking water.

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1. Introduction

Regrowth of microorganisms in drinking water is undesired because multiplication of potentially pathogenic bacteria like *Legionella pneumophila* (Lawrence et al., 1999), nontuberculous mycobacteria (Falkinham et al., 2001; Torvinen et al., 2004), or *Pseudomonas aeruginosa* (Edberg et al., 1996; Lee et al., 2006) might occur. In addition, regrowth might result in esthetic problems, e.g. biofilm formation (van der Kooij, 2003), deteriorated taste and odor (Hoehn, 1988), growth of invertebrates (Levy et al., 1986; van Lieverloo et al., 2004), and/or technical problems, e.g. corrosion of pipe material (Lee et al., 1980). Growth of microorganisms in the drinking water distribution system can be prevented by maintaining a disinfectant residual in drinking water or by limiting the concentration of growth-promoting compounds in water that enters the distribution system.

Bacterial concentrations and microbiological changes in treated and distributed drinking water are mainly monitored by determining heterotrophic plate counts (HPC) on solid agar media (Bartram et al., 2003). The HPC method was originally developed by Robert Koch in 1881, and soon after introduction of the method, information became available about HPCs in drinking water (Frankland and Frankland, 1894). Nowadays, a wide diversity of methods is used to determine HPC values, which makes it difficult to compare results obtained from different countries (van der Kooij, 2003). Although HPC has proven its value as an indicator for the microbiological quality in drinking water (Bartram et al., 2003), there are some limitations related to the use of HPC. The microorganisms that are

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obtained with HPC represent only a small fraction (<1%) of the total microbial community in drinking water that is enumerated with microscopic or flow cytometric methods (Maki et al., 1986; Siebel et al., 2008). In addition, HPC methods can be time-consuming and usually require 3–10 days before results are available.

Adenosine triphosphate (ATP) is an energy-rich metabolic compound that is produced in all active organisms and, therefore, can be used as a parameter to determine the active microbial biomass in water (Holm-Hansen and Booth, 1966). An early study reporting ATP values in drinking water demonstrated that unchlorinated drinking water samples contained ATP-levels between 1 and 23 ng ATP L^{-1}, with free ATP concentrations being negligible (van der Kooij, 1992). In addition, it was also observed that ATP values correlated only weakly with HPC in drinking water (van der Kooij, 1992). Furthermore, a reference database with ATP concentrations of drinking water from 241 different treatment plants in the Netherlands is available (van der Kooij, 2003). Recently, ATP measurements in drinking water has regained interest (Berney et al., 2008; Deininger and Lee, 2001; Delahaye et al., 2003; Siebel et al., 2008), and results from most of these studies confirmed the absence of a strong relationship between HPC values and ATP concentration. Moreover, a strong relationship between total cell concentration and ATP was observed in drinking water samples (Deininger and Lee, 2001; Siebel et al., 2008). Although these studies provided valuable information on ATP in drinking water, there were some limitations in the different studies. Sampling methods, sample storage and transport conditions were poorly described (Siebel et al., 2008), sampling method was inaccurate to determine ATP reliably (Delahaye et al., 2003), a low number of drinking water samples was analyzed (Berney et al., 2008) or drinking water samples from only one facility or treatment plant were analyzed (Berney et al., 2008; Siebel et al., 2008).

The objectives of our study were to determine (i) the effect of water composition on the ATP concentration in unchlorinated distributed drinking water from six treatment plants, (ii) the effect of distance to the treatment plant on the ATP concentration in unchlorinated drinking water in the distribution systems, (iii) the effect of season on ATP concentrations in distributed drinking water and (iv) the relationship between ATP concentration, total cell numbers, HPC and *Aeromonas* plate count in distributed drinking water samples.

2. Materials and methods

2.1. Sample locations

The selection of water types analyzed in this study was based on source water used for drinking water production, total organic carbon (TOC) content in finished water, and size of the distribution system (Table 1). The unchlorinated distributed drinking water of four treatment plants that used groundwater (plant A, B, E and F) and of two plants that used surface water (plant C, D) were analyzed. The TOC content, easily assimilable organic carbon (AOC) concentration and biofilm formation rate in treated water of plants A, B, and C are low for drinking water in the Netherlands. In contrast, these concentrations in treated water of plants D, E and F are considered high in the Netherlands. The distribution systems of these six drinking water treatment plants were sampled during the periods June 2006, October/November 2006 and February/March 2007. In June 2006 nine treated water samples were taken at each treatment plant, together with drinking water samples taken from the tap of 18 different houses connected to the distribution system of the treatment plant. Nine houses were connected to the central part and nine houses were connected to the distal part of the distribution system. In October/November 2006 and February/March 2007, 27 different houses connected to the distribution system were sampled: nine to the proximal part, nine to the central part and nine to the distal part. Besides these 27 samples, three samples of the treated water were taken at each plant. The average distance of the proximal, central and distal part of the distribution system to each of the production plants is shown in Table 1. Before samples were taken for analysis of ATP, HPC, *Aeromonas*, total cell count and temperature, the tap was flushed until the water temperature remained stable for 30 s. Water samples were transported and stored at 4 °C and analyses were performed within 24 h after water sample collection.

Table 1 – The water source used for drinking water production and the biofilm formation rate and AOC level in the finished drinking water of six treatment plants.

Treatment plant	Water source	TOC (mg C L^{-1})	AOC (μg C L^{-1})	BFR (pg ATP cm^{-2} day^{-1})	Distribution system[b]		
					Proximal (km)	Central (km)	Distal (km)
A	Oxic groundwater	< 0.5	1.9	0.21	1.1 ± 0.4	4.0 ± 0.8	10.4 ± 1.5
B	Suboxic groundwater	1.0	ND[a]	ND	0.4 ± 0.4	3.3 ± 1.6	9.6 ± 1.1
C	Surface water	2.4	6.0	0.64	1.0 ± 0.5	5.7 ± 1.6	8.1 ± 1.9
D	Surface water	2.9	14.2	4.5	5.4 ± 2.2[c]	6.8 ± 1.3	13.7 ± 2.1
E	Anoxic groundwater	8.0	10.6	33.1	5.3 ± 0.9	17.3 ± 2.1	41.2 ± 2.4
F	Anoxic groundwater	3.8	8.0	17.6	3.0 ± 0.6	4.8 ± 0.4	11.2 ± 0.4

a ND is not determined.
b The average distance of the ten sampling locations in the proximal, central and distal part to the production plant.
c The difference in distance between proximal and central is not significant, because it was not possible to sample the distribution system near the treatment plant (distance < 2.0 km).

2.2. ATP measurement

The ATP concentration was determined in all drinking water samples by measuring the amount of light produced in the luciferin-luciferase assay. A nucleotide-releasing buffer (NRB, Celsis) was added to the water sample to release ATP from the cells. For the water samples from plant D, E and F, the intensity of the emitted light was measured in a Celsis AdvanceTM luminometer calibrated with solutions of free ATP (Celsis) in autoclaved tap water according to the suppliers' protocol. The emitted light of the water samples from plant A, B and C were measured on a Turner Glomax luminometer calibrated with solutions of free ATP (Celsis) according to the procedure described by the manufacturer of the luminometer. The detection limit of the Celsis AdvanceTM luminometer was 1 ng ATP L^{-1}, whereas the detection limit of the Turner Glomax luminometer was 0.05 ng ATP L^{-1}. A more detailed description of the method to measure ATP in drinking water associated environments (including calibration curves and the use of standards) is given in van der Kooij et al. (2006).

2.3. Total cell count

The total number of microbial cells in water samples collected in October/November 2006 and February/March 2007 was determined by filtering 13 mL over a 0.22 μm polycarbonate filter. Thereafter, microbial cells were stained for 5 min with acridine orange as described by Hobbie et al. (1977) and enumerated using epifluorescence microscopy (1000×, Leica DM RXA). In total, ten random fields with each 20–200 cells were analyzed.

2.4. Heterotrophic plate counts

The drinking water samples that were obtained in February/March 2007 were ten-fold diluted in sterile drinking water and 50 μL of the appropriate dilution was spread in triplicate over the surface of R2A agar plates (Oxoid Ltd.). Subsequently, agar plates were incubated at 25 °C and after 10 days the number of colony forming units (cfu) was determined.

2.5. Aeromonas

Drinking water samples taken in October/November 2006 were analyzed for *Aeromonas* by filtering 100 mL water over a 0.45 μm filter. Subsequently, the filter was incubated on *Aeromonas* dextrin agar (Merck) and agar plates were incubated for 24 h at 30 °C before the number of cfu was determined (Havelaar et al., 1987).

2.6. Statistical analyses

The obtained data were statistically analyzed to determine whether ATP concentrations were significantly different (i) between distributed water from the six treatment plants, (ii) between distributed water in the proximal, central and distal end of the water supply system of each treatment plant and (iii) between distributed water sampled in June 2006, October/November 2006 and February/March 2007. Results from the Kolmogorov–Smirnov test demonstrated that ATP data were normally distributed ($p > 0.05$). Therefore, ANOVA using General Linear Models with Bonferonni post-hoc testing was used to determine the differences in ATP concentration. Differences were considered significant when the p-value was lower than 0.01. Correlation analyses were done by determining Pearson's (to test for linear correlation) and Spearman's rho (to test for non-linear correlation) correlation coefficient between paired values of ATP and HPC, ATP and *Aeromonas* plate counts and ATP and total cell counts. All statistical calculations were performed with SPSS 17.0.

3. Results

3.1. Effect of water composition on ATP levels in distributed drinking water

The average ATP concentration in unchlorinated distributed drinking water of each treatment plant at three different periods in the year ranged from 0.8 to 12.1 ng ATP L^{-1} (Fig. 1; minimum 0.32 ng ATP L^{-1}, maximum 28.0 ng ATP L^{-1}). The average ATP concentration in the distributed drinking water of plants E and F was between 9.3 and 12.1 ng L^{-1} in the periods June 2006 and October/November 2006, which was significantly higher than in drinking water samples from the other treatment plants ($p < 0.01$; Fig. 1). In addition, the average ATP concentration in the distributed drinking water of plant A was always below 1 ng L^{-1}, which was significantly lower than the ATP concentration in water samples from the supply system of plant D (June 2006 and February/March 2007) and plant B (February/March 2007).

3.2. Effect of distance to the plant on ATP levels in distributed drinking water

The differences in the ATP concentration in the distributed water sampled from the proximal, central and distal part of the distribution system of plants C, D, and F (Fig. 2), were not statistically significant ($p > 0.01$). Drinking water sampled from the distal part of the distribution system of plant A and B

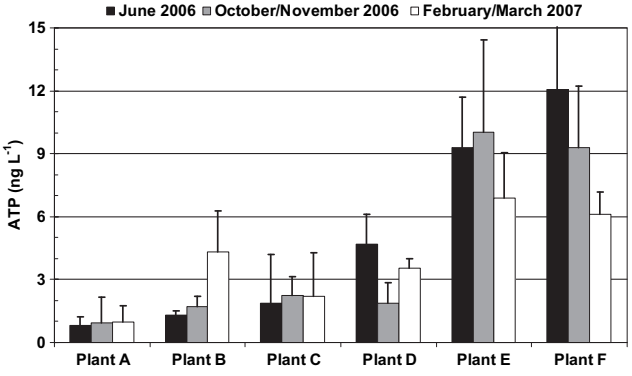

Fig. 1 – Average ATP concentration and standard deviations in drinking water sampled at 27 different locations in the distribution system of six different water treatment plants sampled in June 2006, October/November 2006 and February/March 2007.

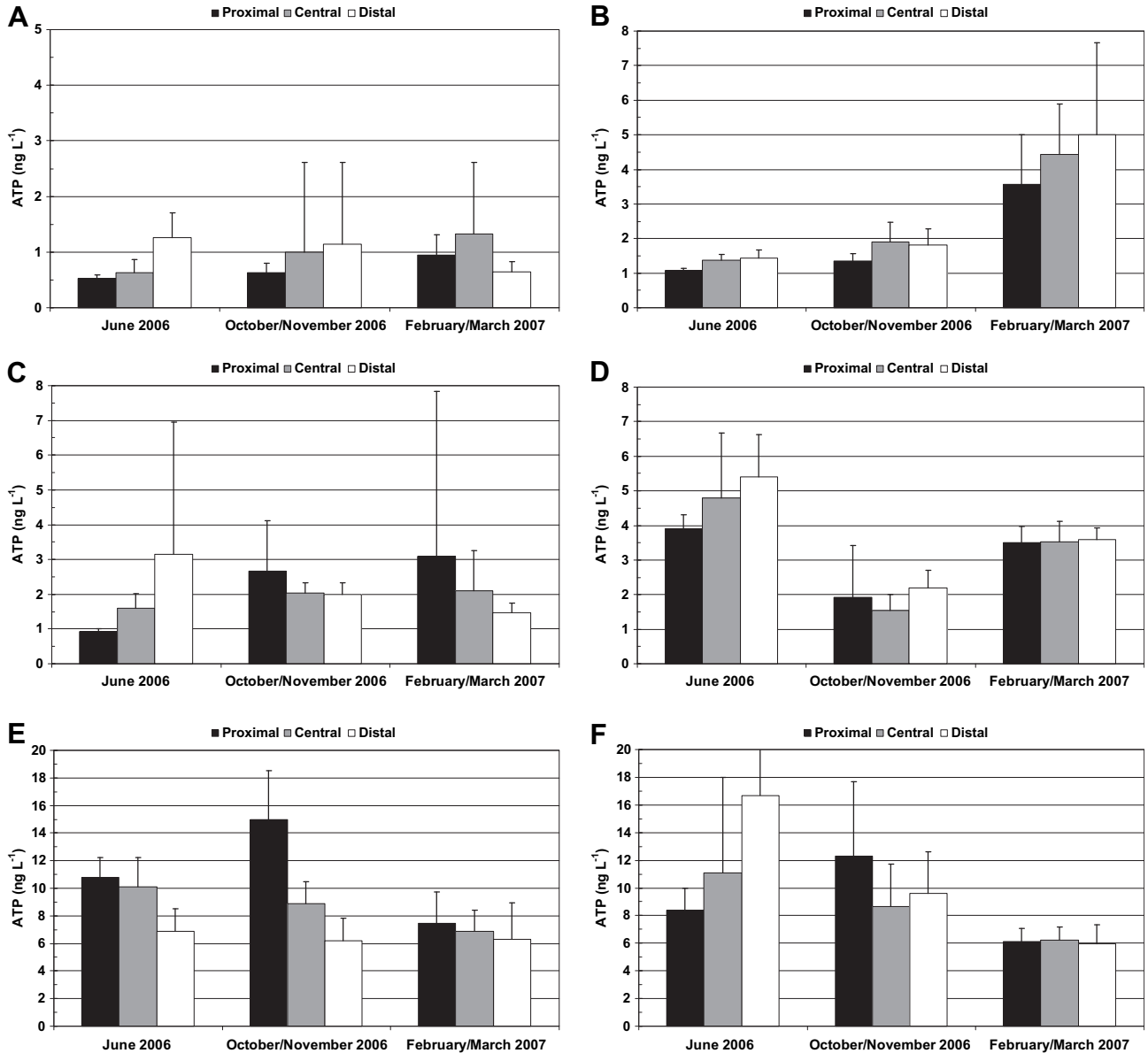

Fig. 2 – Average ATP concentration and standard deviations in drinking water sampled from nine locations at the proximal, central and distal part of the distribution system of treatment plant A, B, C, D, E and F. The distance of the proximal, central and distal part to each treatment plant is given in Table 1.

contained higher ATP concentrations than water sampled from the proximal part (Fig. 2). However, only in June 2006 were the ATP concentrations in water from the distal part of the distribution system significantly higher than in the proximal part, although the concentration never exceeded 2.0 ng L^{-1} ATP. The average ATP concentration in water sampled from the distal part of the water supply system of plant E was 6.9 ± 1.6 ng L^{-1} and 6.2 ± 1.6 ng L^{-1} in June 2006 and October 2006, respectively (Fig. 2E). These ATP concentrations were significantly lower than the concentrations in the proximal part of the distribution system (10.8 ± 1.4 and 15.0 ± 3.6 ng ATP L^{-1} in June 2006 and October 2006, respectively). In contrast, the lower ATP concentration in water sampled from the distal end of the distribution system of plant E in February was not significantly different from the ATP content in the water sampled from the proximal and central part of the supply system.

3.3. *Effect of season on ATP levels in distributed drinking water*

The ATP concentration in the distributed drinking water of plants A and C was relatively constant throughout the year (Fig. 1). In the water sampled from the distribution system of plant B, a significantly higher ATP concentration was observed in March 2007 (4.3 ± 2.0 ng L^{-1}) compared to June 2006 (1.3 ± 0.2 ng L^{-1}) or October 2006 (1.7 ± 0.5 ng L^{-1}; $p < 0.01$). In contrast, the ATP concentration in the distributed drinking water of treatment plants E and F was significantly lower in February 2007 compared to the other two periods, with

average ATP concentrations ranging from 6.1 to 6.9 ng L^{-1} in February 2007 to 9.3–12.1 ng L^{-1} in the investigated periods of 2006 (Fig. 1). The ATP concentration of drinking water sampled from the distribution system of plant D differed significantly between June 2006 (4.7 ± 1.4 ng L^{-1}), November 2006 (1.9 ± 1.0 ng L^{-1}) and March 2007 (3.5 ± 0.5 ng L^{-1}; Fig. 1).

3.4. *Relationship between ATP and other microbiological parameters*

Forty-eight of the 150 water samples taken in October/November 2006 in the distribution system of five treatment plants were randomly chosen to be analyzed for both ATP and total cells. A correlation analysis showed that both parameters were significantly linearly related with a high R^2 (Table 2; supplementary information), demonstrating that ATP can be used as a measure for the total cell concentration in drinking water. However, for water samples taken in February/March 2007 a significant but weaker linear relationship was observed between ATP and total cell counts. These results imply that the relationship between ATP and total cells might be affected by the period of the year in which samples were taken.

HPC values showed a significant correlation with ATP concentrations in distributed drinking water samples collected from six different plants in February/March 2007. The relationship seemed to be non-linear since the R^2 of Spearman rho was higher than the R^2 of Pearson's (Table 2; supplementary information), but the non-linear relationship was weak. The amount of ATP in drinking water samples taken from the water supply system of the different treatment plants in October/November 2006 had a significant but weak linear or non-linear correlation with the *Aeromonas* plate counts (Table 2; supplementary information).

4. Discussion

4.1. *ATP concentrations in drinking water*

HPCs on agar media have been used for more than 100 years to determine the level of regrowth in distributed drinking water (Bartram et al., 2003), but HPC methods are time-consuming and have not been developed to reflect the total cell concentration in drinking water. It has been shown that ATP might be a useful additional parameter to determine the active biomass in drinking water (Deininger and Lee, 2001; Delahaye et al., 2003; Siebel et al., 2008; van der Kooij, 1992), although the methods used and number of samples analyzed were limited in most of these studies. The results from our study demonstrate that ATP concentrations in unchlorinated distributed drinking water can vary between treatment plants, between parts in the distribution system and between seasons. Consequently, representative information on the amount of active biomass in distributed drinking water from a treatment plant is obtained when finished water at the treatment plant and distributed water on locations in the proximal, central and distal part of the water supply system are analyzed for ATP in different periods of the year. The ATP concentration in unchlorinated distributed drinking water of six different treatment plants in the Netherlands ranged from 0.3 to 28 ng ATP L^{-1}. These levels were two times lower than ATP concentrations measured in unchlorinated drinking water sampled from a distribution system in Switzerland (Berney et al., 2008; Siebel et al., 2008). This might indicate that regrowth occurred to a lesser extend in drinking water investigated in our study compared to the drinking water samples analyzed in the Swiss studies, although different sampling strategies and/or applied methods might be responsible as well for the lower ATP concentrations observed in our study.

A recent study indicated that Swiss drinking water samples contained relatively high concentrations of free ATP (Hammes et al., 2010), which is in contrast to results from the Netherlands (van der Kooij, 1992). It is unlikely that free ATP is a stable non-degradable molecule in drinking water without chlorine, because it was shown that even under cold conditions (<3.5 °C), ATP is rapidly biodegraded (Cowan and Casanueva, 2007). The high concentrations of free ATP in Swiss drinking water samples might have been a result of analyzing public fountain drinking water samples, which are probably chlorinated due to health risks of public fountains (e.g. *L. pneumophila*). Another possible explanation is that the method applied by Hammes et al. (2010) stressed the bacterial cells, because of the filtration and heat shock steps in the protocol. Stressing bacterial cells before ATP-measurements can result in increasing levels of free ATP (Lundin, 2000). Because it was demonstrated in the past that unchlorinated drinking water samples in the Netherlands do not contain significant amounts of free ATP, only total ATP concentrations were measured in our study.

The distributed water of both plant E and F had significantly higher ATP concentrations than water from the supply systems of the other treatment plants, which can be attributed to higher concentrations of biodegradable compounds in the treated water of plant E and F. The biofilm formation rate is used as a measure for the regrowth potential of drinking water (van der Kooij, 2003). The finished water of treatment plants E and F had relatively high biofilm formation rates compared to the finished water of the other plants (Table 1)

Table 2 – Results from the correlation analysis between ATP and other microbiological parameters.

Relationship	Period	Pearson's			Spearman rho	
		N	*p*	R^2	*p*	R^2
ATP – Total cell count	October/November 2006	48	< 0.01	0.82	< 0.01	0.74
ATP – Total cell count	February/March 2007	42	< 0.01	0.55	< 0.01	0.55
ATP – Heterotrophic plate count	February/March 2007	56	< 0.01	0.20	< 0.01	0.50
ATP – *Aeromonas* plate count	October/November 2006	112	< 0.01	0.25	< 0.01	0.37

and is consistent with the high concentration of active biomass observed in the distributed drinking water samples of plant E and F. These results might implicate that higher level of biofilm formation in the distribution network results in increased ATP concentrations in drinking water flowing along the biofilm on the distribution pipes, but water samples from more treatment plants have to be analyzed, before such a conclusion can be made.

4.2. *Effect of distance to the treatment plant on ATP concentration*

The ATP concentration in drinking water did not change during transport to the distal part of most of the distribution systems. The ATP concentration in water from the distal part of the distribution system of plant E was significantly lower than in water from the proximal part during the warmer periods. The decrease of the ATP concentration in water during transport suggests that the biodegradable organic matter content was lower in distributed water entering the distal part of the water supply system. A possible lower biodegradable organic matter concentration in the distal part of the distribution system might have been caused by biological processes (e.g. biofilm growth) and sedimentation in the proximal and central part of the distribution system. The distribution system size of plant E is three to four times larger than the distribution system size of the other treatment plants and this explains why the ATP concentration in the small distribution systems of the other plants remained relatively constant.

4.3. *Effect of season on ATP concentration*

The ATP concentration in the distributed water of plant E and F was significant lower in February 2007 than in June or October/November 2006. In contrast, the ATP concentration of the finished water was not different in these three periods (data not shown), indicating that the decrease in ATP concentrations in February/March was not caused by a change in water quality of the water entering the water supply system. In February 2007, drinking water temperatures were between 6 and 12 °C, whereas in the other two periods drinking water temperatures varied between 13 and 18 °C (Fig. 3). Apparently, the lower drinking water temperatures in February 2007 limited the microbial activity in the drinking water, which resulted in a lower ATP concentration in this period compared to the ATP concentration in late spring and autumn of 2006. Delahaye et al. (2003) did not observe a relationship between temperature and ATP concentration in drinking water samples, but this is probably caused by the relatively high drinking water temperatures (11–15 °C) and the presence of free chlorine in the drinking water that affects the activity of microorganisms.

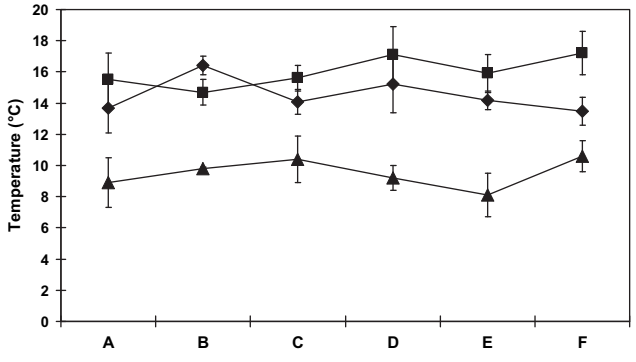

Fig. 3 – Average temperature in drinking water samples from 27 different locations in the distribution system of treatment plant A till F, sampled in June 2006 (■), October/November 2006 (◆) and February/March 2007 (▲).

In contrast to plants E and F, the ATP concentration in distributed water of plant B was 2.5–3.5 times higher in March 2007 compared to June and October 2006. Concomitantly, ATP concentrations in the finished water of treatment plant B were 2–2.5 times higher in March 2007 as well (data not shown). The higher ATP content in the finished and distributed water in March 2007 can be explained by changes in the treatment train. In December 2006 an extra dosing step of $FeCl_3$ was implemented in the treatment train of plant B. Apparently, this modification affected the microbial processes and growth in the water, resulting in higher ATP concentrations in finished and distributed water. The ATP concentration in the distributed and finished water of treatment plant D differed significantly as well between the three periods of the year. Also in this case, the difference in ATP concentration was related to modifications in the treatment train, since ozone concentrations in one of the treatment steps varied considerably in this period. These results demonstrate that ATP is a suitable parameter to monitor for changes in microbial activity of distributed and finished water when treatment trains of drinking water treatment plants are modified.

4.4. *Significance of ATP analysis*

A weak correlation was obtained between paired values of HPC and ATP as was observed by others, although different methods to measure HPC were used in the different studies (Berney et al., 2008; Deininger and Lee, 2001; Siebel et al., 2008). Similarly, a weak relationship between paired values of *Aeromonas* plate counts and ATP concentration was observed as well. The absence of a strong direct relationship between ATP concentration and HPC can be explained by our observation that only 0.1–4% of the bacteria enumerated with fluorescence microscopy were able to grow on R2A medium (data not shown), as has been observed by others (Maki et al., 1986; Siebel et al., 2008). In addition, growth of *Aeromonas* is influenced by different factors that do not affect the total microbial community. For instance, we observed increased *Aeromonas* numbers towards the distal end of the distribution systems, whereas ATP concentrations remained constant or decreased in the distal part of the supply systems. These results suggest that residence time influence the growth of *Aeromonas*. Besides residence time, other factors like temperature, sediment concentration and pipe material probably affect growth of *Aeromonas* in drinking water distribution systems as well. Consequently, ATP cannot be used as a surrogate parameter for heterotrophic and *Aeromonas* plate counts, parameters that have to be measured in drinking water according to the drinking water decree in the Netherlands (Anonymous, 2001). The results show that ATP is a better indicator for total (active) biomass in drinking water than plate counts and might, therefore, be preferred over HPC to determine microbial

drinking water quality. However, because HPC are currently the 'golden standard' to determine drinking water quality and because large databases are available for HPC, we suggest to use ATP as an additional parameter to determine the biomass concentration in drinking water. Maybe in the future, when a more comprehensive database of ATP concentrations in drinking water is available, ATP can replace HPC measurements in drinking water.

3.4% of drinking water samples from the distribution system of plant F and in 2.4% of the samples taken from the supply system of plant D in the period June 2006 till April 2007 exceeded the maximum concentration of *Aeromonas* (1000 cfu 100 mL^{-1}) defined in the Dutch water decree. In contrast, none of the samples taken from plant A, B and C exceeded this *Aeromonas* standard during June 2006 till April 2007. Thus, exceeding of the *Aeromonas* standard in the Netherlands occurred in drinking water with elevated ATP concentrations (plant D and F). Still, the HPC standard in the Netherlands (geometric year mean of 100 cfu mL^{-1}) was never exceeded in the water samples analyzed. Hence, we conclude that drinking water with an average ATP concentration below 15 ng L^{-1} has low plate counts, which indicates acceptable levels of (culturable) heterotrophic bacteria in the drinking water samples analyzed in this study.

The results obtained in this study showed a strong correlation between the paired values of ATP and total cell concentration in the distributed drinking water sampled in October/November 2006. Previous studies have also shown a strong relationship between ATP and total cell concentration in drinking water (Deininger and Lee, 2001; Siebel et al., 2008). However, the results from our study demonstrated that the relationship between ATP and total cell count is weaker for samples obtained at low temperature in February/March 2007. The ATP to cell ratio did not differ significantly between October/November 2006 ($1.7 \pm 1.4 \times 10^{-17}$ g ATP $cell^{-1}$) and February/March 2007 ($1.8 \pm 0.95 \times 10^{-17}$ g ATP $cell^{-1}$). Thus, the lower water temperatures in February/March 2007 did not result in lower microbial activity per cell. The ATP content per cell in the drinking water samples are low compared to the ATP content for microorganisms in groundwater, on membranes used in drinking water treatment and in sand filters used in water treatment, but are comparable to values reported for microorganisms in granular activated carbon used in water treatment and in Swiss drinking water (Berney et al., 2008; Eydal and Pedersen, 2007; Magic-Knezev and van der Kooij, 2004; Siebel et al., 2008; Vrouwenvelder et al., 1998).

Our results show that ATP cannot replace conventional HPC measurements. However, the results from our study and previous studies have shown that ATP is a supplementary parameter to determine the concentration of active biomass in drinking water. Especially the short analysis time and strong correlation with total cell concentration is advantageous compared to the conventional HPC methods (Siebel et al., 2008; van der Kooij, 2003). In addition, results from our study show that ATP concentrations in distributed drinking water relate to the biofilm formation rate of the water and to non-compliance of the *Aeromonas* standard in the Netherlands. Finally, the effects of water transport from the proximal to distal part of the distribution system on the active biomass in the distributed water, can be determined by measuring the ATP concentration in drinking water.

5. Conclusions

- ATP can be used to quantitatively measure the concentration of active biomass and, thus, is a good indicator for the total microbial activity in drinking water. In addition, ATP is determined fast and easy and the method to measure ATP has a low detection limit.
- A decrease in the ATP concentration in the distal part of a large-sized water supply system demonstrates that the biofilm in the proximal and central part of the distribution network utilizes significant amounts of biodegradable compounds from the along flowing drinking water.
- There is no direct relationship between ATP and HPC or *Aeromonas* numbers in drinking water, but distribution systems with relatively high ATP concentrations showed exceeding of the *Aeromonas* standard (1000 cfu mL^{-1}). Thus, ATP is a suitable and fast method for screening and detecting distribution systems that might have regrowth problems.
- The relation between the average ATP concentration in the distributed water and the biofilm forming potential of the water implies that ATP in water is related to biofilm formation and vice versa.
- ATP is a suitable parameter to detect changes in the level of microbial activity of drinking water when the treatment train is modified.

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Appendix. Supplementary information

Supplementary information associated with this article can be found, in the online version, at doi:10.1016/j.watres.2010.07.016

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Threshold Concentration of Easily Assimilable Organic Carbon in Feedwater for Biofouling of Spiral-Wound Membranes

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One of the major impediments in the application of spiral-wound membranes in water treatment or desalination is clogging of the feed channel by biofouling which is induced by nutrients in the feedwater. Organic carbon is, under most conditions, limiting the microbial growth. The objective of this study is to assess the relationship between the concentration of an easily assimilable organic compound such as acetate in the feedwater and the pressure drop increase in the feed channel. For this purpose the membrane fouling simulator (MFS) was used as a model for the feed channel of a spiral-wound membrane. This MFS unit was supplied with drinking water enriched with acetate at concentrations ranging from 1 to 1000 μg C·L^{-1}. The pressure drop (PD) in the feed channel increased at all tested concentrations but not with the blank. The PD increase could be described by a first order process based on theoretical considerations concerning biofilm formation rate and porosity decline. The relationship between the first order fouling rate constant R_f and the acetate concentration is described with a saturation function corresponding with the growth kinetics of bacteria. Under the applied conditions the maximum R_f (0.555 d^{-1}) was reached at 25 μg acetate-C·L^{-1} and the half saturation constant k_f was estimated at 15 μg acetate-C·L^{-1}. This value is higher than k_s values for suspended bacteria grown on acetate, which is attributed to substrate limited growth conditions in the biofilm. The threshold concentration for biofouling of the feed channel is about 1 μg acetate-C·L^{-1}.

Introduction

The use of high pressure spiral-wound membranes, nanofiltration (NF), or reverse osmosis (RO) has increased rapidly over the past decade (*1*). Major fields of current application are (i) desalination of seawater, (ii) reclamation of wastewater, (iii) production of industrial waters, and (iv) treatment of surface water or groundwater for drinking water production. Biofouling is one of the major impediments in operation of these membranes, causing clogging of the feed channel and fouling of the membrane surface with increased pressure drop and decreased flux as results. The evident effect of attached microbial growth (biofilms) on the pressure drop in spiral-wound membranes is well documented in the literature (*2, 3*). Recently the effect of biofilms on the mass transfer coefficient (MTC) has been demonstrated and related to the occurrence of cake-enhanced osmotic pressure (*4, 5*).

The different mechanisms involved in biofouling, viz. bacterial adhesion, growth, and sloughing and aspects such as biofilm composition and structure have been reviewed extensively (*2, 3*). Only a few studies reported on the relationship between the nutrient level in the feedwater and biofouling. Under most environmental conditions, organic carbon compounds are the growth limiting nutrients for biomass production (assimilation) and energy supply. A wide range of organic compounds, e.g., carboxylic acids, amino acids, proteins, and carbohydrates promote bacterial growth in the aquatic environment. The affinity of bacteria for these substances can be high which is demonstrated by a low half saturation constant k_s derived from the Monod growth kinetics. For aquatic bacteria grown on acetate, benzoate, oxalate, oleate, and starch at 15 °C in drinking water k_s-values ranging from 0.4 to 15.4 μg C·L^{-1} have been established (*6, 7*). In spiral-wound membranes with turbulent flow the substrate concentration in the bulk water is the most important growth-determining parameter. Biological clogging of the feed spacer of a spiral-wound membrane at acetate concentrations below 0.1 mg C·L^{-1} was demonstrated with a model system, the membrane fouling simulator (MFS *8, 9*). In these studies the lowest acetate concentrations tested (25 μg C·L^{-1}) is much higher than the k_s values mentioned above. Studies of the effects of low substrate levels on biofouling do not seem available. Hence, the threshold concentration of easily assimilable organic compounds such as acetate to induce biological clogging of the feed channel and the kinetics of the biological clogging process are not clear yet.

The objective of the current study was to assess the relationship between the concentration of acetate in the feedwater as a model for easily assimilable organic compounds, and the rate of clogging of the feed channel caused by biofouling under well-defined conditions. Extended acetate dosing studies showed that (i) biological clogging follows the kinetics of biofilm formation and (ii) the threshold concentration for biofouling in the feed channel is in the order of a few micrograms.

Materials and Methods

Model Membrane System. The membrane fouling simulator MFS as presented by Vrouwenvelder et al. (*8*) was used for the experiments. The MFS consists of a stainless steel housing divided in an upper and lower part with a sheet of a membrane fixed in between. To simulate the feed channel of a spiral-wound membrane in the upper and lower part of this MFS, a feed spacer (height, width, and length of 0.08, 4, and 20 cm, respectively) is placed on top of the membrane and a product spacer of similar size below the membrane. The MFS was operated without a vertical flux (permeate production). Under the turbulent flow conditions in the feed channel of the MFS mass transfer of acetate in a laminar layer is of minor importance and vertical fluxes are usually ≤10% of the total cross-flow flux. The feedwater is introduced into the feed spacer channel at a flow of 4.44×10^{-6} m^3·s^{-1} and a cross-flow velocity of 0.14 m·s^{-1}. A "virgin" nanofiltration membrane (Trisep 4040-TS80-TSF) was used in the MFS as the standard membrane (feed spacer height and filament thickness 0.066 and 0.034 cm; porosity ε of 0.92). The normalized pressure drop or initial NPD$_o$ (kPa) of a

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"virgin" membrane at a volumetric flow rate of 4.44×10^{-6} $m^3 \cdot s^{-1}$, calculated from the formula presented in the Supporting Information (SI), is 2.8 kPa. Five MFS units were used and fitted into an experimental system (Figure 2, SI). The MFS units were supplied with prefiltered tap water (10 and 1 μm polypropylene cartridge filtration; Van Borselen Ltd.) at a constant feedwater flow of 16 $L \cdot h^{-1}$ and at a reduced and constant overpressure of 1 bar in the system (using a Teflon pressure regulator, PR). The cartridge filters were replaced once a week. The water was supplied to the units with black Nylon tubing (1/4 in. tube) and the flow was regulated by a flow indicator (FI; Adinco) at the front and a flow controller (Brooks, FC 8800) after the MFS. The MFS unit is connected to the system with quick connectors (QC) as part of the MFS housing. The use of these quick connectors enabled maintaining over-pressure in the MFS unit during manipulations of the system (e.g., renewal cartridge filters, nutrient solutions). To prevent biofouling of the flow controller 1−2 mg $Cl_2 \cdot L^{-1}$ was dosed to the outlet of the MFS prior to the flow controller (see SI).

Feed Water Quality. The feedwater is non chlorinated tap water produced from anaerobic groundwater using aeration and rapid sand filtration. The pH of the water was 7.98 ± 0.05, dissolved organic carbon content was 1.98 ± 0.10 mg $C \cdot L^{-1}$, AOC was 3−5 μg acetate-C eq $\cdot L^{-1}$, NO_3^-, and PO_4^{3-} content was, respectively, 0.123 ± 0.04 and 0.017 ± 0.02 mg L^{-1} and iron content (ICP) was 0.008 ± 0.014 mg L^{-1}. The ambient water temperature (no temperature control, daily monitored) during the experimental periods ranged from 13.5 to 16.8 °C (average of 15.9 ± 0.7 °C) and was 19.4 ± 2.0 °C in one experiment.

Acetate Dosing. Sodium acetate was used as a model nutrient and dosed from a 5 L autoclaved solution (121 °C, 15 min.) into the feedwater with a pump (Masterflex L/S variable speed digital standard drive type 77201-60 with a Masterflex Easy Load II pump head and Masterflex norprene food tubing 6402-13 type L/S). The dosage point was located approximately 10 cm before the inlet of the MFS. The pump was calibrated and set by means of the weight loss of the nutrient bottle to ensure a flow of 0.04 (±0.004) L.h^{-1}.

Experimental Set-Up. Initially, acetate concentrations (S_{ac}) of 0, 500, and 1000 μg $C \cdot L^{-1}$ were tested in duplicate experiments, and 100 μg $C \cdot L^{-1}$ in a single test. Subsequently, S_{ac} values of 10 and 25 μg $C \cdot L^{-1}$ were tested in duplicate experiments. In the experiments at acetate concentrations of 5, 3, and 1 μg $C \cdot L^{-1}$ the effect of an acetate-free blank period (t_b; days) on the pressure drop development was examined. The effect of variation in nutrient supply and starvation on the pressure drop was studied in two runs with an initial S_{ac} of 1000 and 100 μg $C \cdot L^{-1}$.

Pressure Drop Monitoring and Calculations. The feed channel pressure drop (PD) was measured with a sensitive (0.2 mBar) pressure drop transmitter (PDT; Pascal CI ΔP, Diff transmitter CI 1330, range 0/1000 mbar, Labom Industriel Messtecknick). The pressure drop (dP in mBar) was registered digitally (Comline.Hart software) every two seconds during 3−5 minutes (90−150 values). The average value was calculated (relative standard deviation 0.9%), normalized to a temperature of 12.5 °C, and converted to kPa (1 mBar = 0.1 kPa). The normalization for temperature was done with the following equation:

$$\mathrm{NPD} = \mathrm{PD}\left(\frac{\eta_{T,\mathrm{ref}}}{\eta_{T,\mathrm{actual}}}\right)^{c}$$

where PD is the observed pressure drop, $\eta_{T,\mathrm{ref}}$ and $\eta_{T,\mathrm{actual}}$ the viscosities of water at the reference temperature (12.5 °C) and the actual temperature, and c is a constant given by the membrane manufacturer (0.34 for the used membrane). Hydraulic characterization of the different MFS units is presented in the SI. Formation of biofilms on surfaces is initially an exponential process that is rapidly followed by a linear phase due to diffusion limitation of the substrate flux into the biofilm (*10*−*12*). Characklis (*13*) described the progress of biofouling expressed in deposits accumulation, hydrodynamic frictional resistance, or heat transfer resistance with a sigmoid function consisting of an induction and an exponential phase and a plateau phase. The exponential part of this sigmoid function can be explained by the observed exponential relationship between biofilm thickness and PD (SI Figure 1) and the diffusion-limited bacterial growth kinetics of biofilms. Therefore, the NPD increase (NPD_i) in the feed channel of the present study is described as a first order process with

$$\mathrm{NPD}_i = \mathrm{NPD}_t - \mathrm{NPD}_o = e^{R_f t} \text{ and } \ln\frac{\mathrm{NPD}_t}{\mathrm{NPD}_o} = R_f t$$

where NPD_o and NPD_t are the normalized pressure drop at operational time 0 and t (days), R_f is the exponential fouling rate constant (ln $NPD_i \cdot d^{-1}$). The relationship between R_f and S_{ac} was modeled with Figure.P software Inc. (2006).

Total Direct Cell Count (TDC). At the end of a test run the membrane surface and feed spacer of the MFS unit were analyzed for biomass accumulation. The total direct cell count (TDC) was determined as described by Hobbie et al. (*14*). Bacteria were isolated from a membrane sample with product spacer (2/2 cm) in 20 mL sterile tap water by one minute High-energy sonication (HES; Branson Sonifier II W-250) at a constant frequency of 20 kHz with a titanium microtip (5 mm) and power input of 30 W. This treatment was repeated and the TDC value was measured in the combined suspension of 40 mL.

Results

Hydraulic Conditions in the MFS Units. At the start of each experiment the initial NPD (NPD_o) is a characteristic of the hydraulics in the loaded MFS units. The NPD_o values at the different experiments were close to the calculated value of 2.8 kPa, but showed some variation (Table 1). This variation was caused by differences between the used MFS units (see SI), but most likely also by variation in the loading of the MFS. Extrapolation of these values to a spiral-wound NF membrane Trisep 4040-TS80-TSF with a membrane length of 1 m confirmed that the hydraulic conditions in the MFS units were similar to the hydraulics in a spiral-wound membrane as shown previously (*8*).

NPD Increase at Different Acetate Concentrations. At S_{ac} values of 1000 and 500 μg $C \cdot L^{-1}$ an exponential increase of NPD (NPD_i) in the feed channel was observed after a lag-phase (t_{lag}) of approximately 5 days (Figure 1a; Table 1), whereas in the blank no NPD increase occurred. The t_{lag} is defined as the period before the start of the exponential clogging process after an initial linear NPD_i of 0.5−1.0 kPa. Subsequently, S_{ac} values of 25, 10, 5, and 3 μg $C \cdot L^{-1}$ were tested (Figure 1a and b). At S_{ac} of 25 μg $C \cdot L^{-1}$ clogging occurred at a rate comparable to the rate observed at 500 and 1000 μg $C \cdot L^{-1}$. At the S_{ac} values of 10, 5, and 3 μg $C \cdot L^{-1}$ also clogging occurred, but t_{lag} values were clearly higher than observed in the units supplied with 25 μg $C \cdot L^{-1}$ (Figure 1; Table 1). The duplicate at S_{ac} of 10 μg $C \cdot L^{-1}$ showed stagnation in fouling most likely caused by a preferential flow path in the feed channel as concluded from visual inspection. The duplicate at S_{ac} of 5 μg $C \cdot L^{-1}$ was performed in the blank MFS unit (no acetate) after 28 days of operation (blank period). Fouling in this unit started sooner (t_{lag} of 4.5 days) and proceeded more rapidly than in the other unit at 5 μg $C \cdot L^{-1}$ (Figure 1b). It was unclear whether this increase in fouling rate was caused by a difference in temperature (temperature during this experiment was

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TABLE 1. Fouling Characteristics of the Different Acetate Dosing Experiments (Duplicates I and II) Presented in Chronological Sequence of Performance To Illustrate the Differences in Temperature (No Temperature Controlled System)

	I			II		
S_{ac}; temp. (μg C·L^{-1}; °C)	NPD_o(kPa)	t_b; t_{lag}[a] (days)	R_f(ln NPD_i·d^{-1}; 95%CI; r^2)	NPD_o(kPa)	t_{pf}; t_{lag} (days)	R_f (ln NPD_i·d^{-1}; 95%CI; r^2)
1000 (13.5 ± 0.3)	3.4	7.0; 4.0	0.740 (0.506−0.974; 0.97)	2.7	7.0; 5.0	0.844 (0.692−0.996; 0.98)
500 (13.5 ± 0.3)	1.8	7.0; 5.7	0.428 (0.391−0.465; 0.99)	3.7	7.0; 5.0	0.523 (0.326−0.720; 0.90)
1000 (14.3 ± 0.4)	2.8	1.0; 5.0	0.338 (0.134−0.549; 0.90)[b]	3.4	1.0; 4.0	0.511 (0.331−0.691; 0.94)
100 (15.2 ± 0.5)	5.6	8.0; 4.2	0.555 (0.392−0.718; 0.92)			
10 (16.0 ± 0.9)	2.4	8.0; 6.0	0.105 (0.100−0.110; 0.99)	3.3	1.0; 7.9	0.147 (0.141−0.153; 0.99)
5 (16.1 ± 0.9[c] − 19.4 ± 2.0[d])	3.3	1.0; 16.2	0.062 (0.059−0.065; 0.98)[c]	3.9	28.0; 4.5	0.146 (0.136−0.156; 0.96)[d]
25 (16.8 ± 0.5)	5.0	26.0; 1.9	0.463 (0.442−0.484; 0.98)	2.6	26.0; 2.9	0.416 (0384−0.448; 0.99)
3 (14.9 ± 0.6)	4.6	1.0; 7.8	0.057 (0.053−0.061; 0.96)	3.9	54.0; 7.8	0.075 (0.072−0.078; 0.99)
1 (15.5 ± 0.8)	2.1	54.0; 13.8	0.013 (0.012−0.014; 0.95)	3.4	1.0; 27.9	0.011 (0.010−0.012; 0.91)

[a] t_b = time of blank period (no acetate supply) and t_{lag} time before start of the clogging process (NPD increase of 0.5−1.0 kPa). [b] $P = 0.013$. [c] Temperature during test with MFS I. [d] tTemperature during test with MFS II.

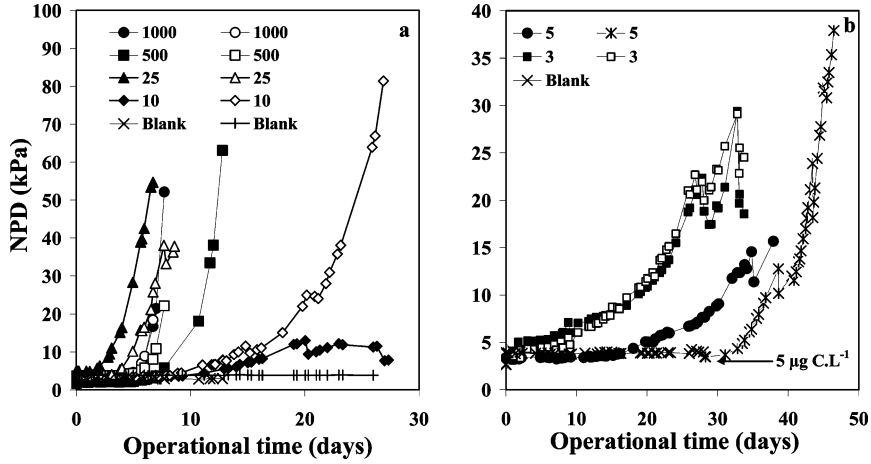

FIGURE 1. Normalized pressure drop in the MFS units supplied with tap water (blank) and with different acetate concentrations (S_{ac}).

about 3 °C higher; Table 1) or by the extended blank period. The fouling rate at S_{ac} of 3 μg C·L^{-1} in both units was in the same order of magnitude as observed at 5 μg C·.L^{-1} (Figure 1b). At S_{ac} of 1 μg C·L^{-1}, however, the fouling rate was much lower (Figure 2a). The NPD values of the units supplied with these low S_{ac} values reached a plateau. For S_{ac} of 3 μg C·L^{-1} a plateau NPD of 20−25 kPa (NPD_i of 300−500%) was reached after approximately 29 days (Figure 1b). The NPD plateau value at 1 μg C·L^{-1} was reached after 50 days at a NPD_i of about 4−6 kPa (100%; Figure 2a).

Effect of Variable Acetate Concentrations. In the MFS units supplied with 1 μg acetate-C·L^{-1} NPD decreased immediately after termination of the acetate supply and the reduction in a period of 9 days was 11−17% of the last measured NPD. Resumption of the dosing caused a second NPD increase period in one of the two units until a final NPD of 8 kPa was reached (Figure 2a). The effect of variation in the acetate supply on the NPD was also investigated at S_{ac} values of 100 and 1000 μg C·L^{-1}. The acetate supply was terminated at 34 and 23 kPa, respectively (Figure 2b) which caused an immediate and exponential NPD decrease to stable values of 25 and 15 kPa, respectively. The NPD decrease was 27 and 36% and was reached after a period of 9 and 6 days, respectively. The residual NPD values after starvation were about 340% of the NPD_o values of the virgin membrane. Resumption of the acetate supply at high (1000), but also at much lower doses of 10 and 5 μg C·L^{-1} resulted in a fast NPD increase starting within a few hours (Figure 2b). At 10 μg C·L^{-1} the fouling rate was approximately the same as observed at 1000 μg C·L^{-1}. During the second starvation period the NPD decreased with 48% within 9 days. The remaining NPD at the end of this starvation period was about two times higher than after the first starvation period.

Fouling Rate. The fouling rate in the MFS units is described with the exponential fouling rate constant R_f (ln NPD_i·d^{-1}; Table 1). The calculations showed that the monitored NPD curves fitted well on the exponential fouling model. All equations but one (S_{ac} of 1000) were highly significant ($p \leq 0.001$) and the regression coefficients (R^2) were ≥0.90. The highest values (0.740−0.844 d^{-1}) were observed at S_{ac} of 1000 μg C·L^{-1} (Table 1). At all other S_{ac} values in the range of 25−1000 μg C·L^{-1} R_f ranged from 0.338 to 0.523 d^{-1} with no clear correlation with the applied S_{ac}. Furthermore, the statistics of the calculations showed a higher level of uncertainty of the R_f values at these S_{ac} values than at S_{ac} values of ≤25 μg C·L^{-1} (cf. Table 1 and Figure 3a). The relationship between R_f and S_{ac} could be described with the exponential growth kinetics model ($P < 0.001$; $R^2 = 0.95$), a compromise between the Blackman and Monod models (*15*), yielding a maximum R_f of 0.555 d^{-1} and a half saturation constant k_f of 15.4 μg acetate-C·L^{-1}. At S_{ac} concentrations of ≤25 μg C·L^{-1} a significant ($P < 0.001$; $R^2 = 0.95$) linear correlation between R_f and S_{ac} was observed (Figure 3a). The fouling rates for the experiments after starvation (Figure 2) were also calculated. Only after the second starvation period at S_{ac} of 10 μg C·L^{-1} the fouling rate of 0.428 d^{-1} (95% CI; 0.326−0.531) was significantly higher than the initial fouling rate of the same concentration (0.105 and 0.147 d^{-1}; Table 1). The fouling rates in the other tests after starvation were similar or slightly higher than the initial fouling rates at the same concentrations (data not presented).

The results at S_{ac} of 5 μg C·L^{-1} indicated that biofilm formation during a blank period with no acetate supply might

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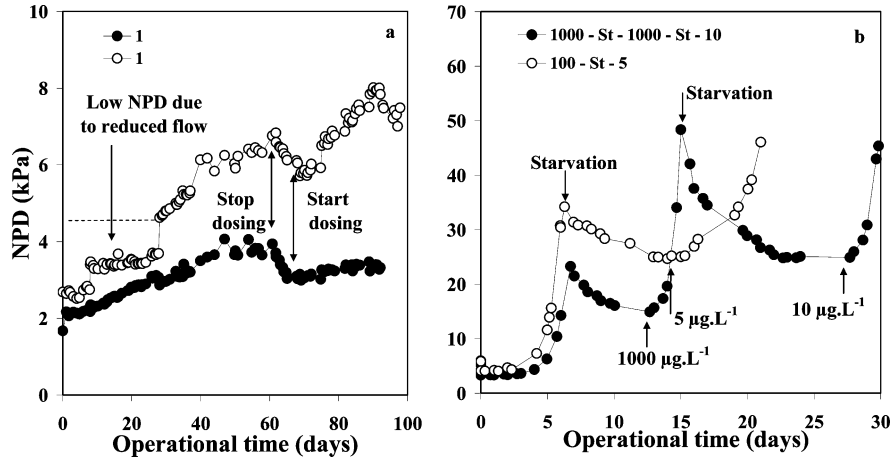

FIGURE 2. Normalized pressure drop in the MFS units supplied with S_{ac} of 1 μg L^{-1} (a; during the first 25 days a unintended reduced flow occurred resulting in too low NPD values) and (b) in the MFS units in multiple dosing periods at different acetate concentrations followed by starvation periods.

enhance the clogging process. The low t_{lag} and high R_f observed at S_{ac} of 5 μg C·L^{-1} with a prolonged blank period, however, was observed at an elevated temperature (Table 1). An enhancing effect of a blank period before starting the acetate supply was demonstrated at S_{ac} of 1 and 3 μg C·L^{-1}. A faster clogging was observed in the units with an extended blank period of 54 days.

Biomass in the MFS Units. The role of biological growth in the feed channel in the clogging process was demonstrated by the effect of a variable regime of the acetate supply on the NPD development. Direct evidence came from the microscopic cell count in the feed channel. TDC numbers in the MFS unit without acetate supply (blank) ranged from 1.5×10^6–2.1×10^6 cells.cm^{-2}. At S_{ac} of 1 μg C·L^{-1} the cell count was more variable and higher (3.7×10^6–2.9×10^7 cells·cm^{-2}). The cell numbers were highest in the MFS at S_{ac} of 25 and 1000 μg C·L^{-1} (7.9×10^7–2.6×10^8 cells·cm^{-2}).

Discussion

Biological Clogging of Membranes at Variable Concentrations of Easily Assimilable Compounds. The results of this study clearly show that biofouling of the feed channel of spiral-wound membranes occurs at low concentrations of an easily assimilable carbon compound added to the feedwater. No biofouling occurred in the feed channel of the MFS under the applied experimental conditions using 1 μm filtered tap water. Therefore this tap water was used to assess the relationship between acetate as a model substrate and biofouling in the MFS monitored with pressure drop. The fouling process expressed as NPD increase could be described with an exponential function based on theoretical considerations (see SI). The derived fouling rate constant R_f showed a linear relationship with the acetate concentration in the range of 1–25 μg C·L^{-1} (acetate-C loads of 0.00014–0.0035 g·m^{-2}·s^{-1}) and a saturation function reaching a maximum fouling rate at concentrations ≥25–1000 μg C·L^{-1}. A similar relationship has been observed for the friction factor in a tubular reactor at glucose-C loads of 0.72–36.8 g·m^{-2}·s^{-1} (*13*) and also for the clogging rate of experimental sand filters supplied with water at variable acetate concentrations (loads of 3.3×10^{-6}–3.3×10^{-4} acetate-C g·m^{-2}·s^{-1} (*16*)). The causative role of microbial growth in the pressure drop increase in the feed channel was demonstrated by a reduction in NPD at the stop of the acetate supply and by the immediate response to resumption of the acetate supply. The high numbers of bacteria on the membrane surface confirmed the role of attached growth in the pressure drop increase. TDC values in the current study (Figure 3b) which were related to NPD increase in the feed channel were close to the "pain level" of 10^8 cells·cm^{-2} derived by Flemming et al. (*17*) for 25% MTC decline. Most of the experiments were performed in "virgin" membrane samples and the results with variable acetate concentrations clearly demonstrated that spiral-wound membranes with extended operational periods may exhibit more biofouling problems.

Threshold Concentration and Growth Kinetics of Bacteria. The results of this study show that the threshold concentration of easily assimilable organic compounds such as acetate for biological clogging of the feed channel of spiral-wound membranes is about 1 μg C·L^{-1}. A similar conclusion was obtained for experimental sand filters supplied with variable acetate concentrations (*16*). In the MFS units at the lowest applied acetate concentration of 1 μg C·L^{-1} (1.4×10^{-4} g C·m^{-2}·s^{-1}) pressure drop reached a plateau value of 2–3 kPa after an operational time of approximately 90 days. In an experimental sand filter operated at a flow rate (0.0003 m·s^{-1}) and an acetate-C concentration of 10 μg L^{-1} (load of 3.3×10^{-6} g C·m^{-2}·s^{-1}) the pressure drop increased with 20 kPa in 200 days (*16*). This higher level of pressure drop increase under lower acetate-load conditions was caused by the lower porosity of the sand (30–40%) compared to the porosity of the feed channel (92%) in the membrane.

The threshold concentration of 1 μg acetate-C·L^{-1} for biofouling demonstrates that the attached bacteria are well adapted to these low substrate concentrations. In growth studies with pure cultures of *Polaromonas* related bacteria, *Pseudomonas* and *Aeromonas* spp. in drinking water at 15 °C, k_s values of 0.4–11.1 μg C·L^{-1} and maximum growth rates μ_{max} of 0.09–0.18 h^{-1} (doubling in 5–10 h) have been observed for growth on acetate (*6, 7*). The k_s values of a *Flavobacterium* spp. on carbohydrates ranged from 5.7 to 8.4 μg C·L^{-1} (*18*). Furthermore, most of these bacteria are capable of utilizing a number of easily assimilable compounds (carboxylic acids, carbohydrates, amino acids, aromatic acids) added as mixtures in drinking water at a concentration of 1 μg C·L^{-1} of individual compounds (*6, 7*). Thus, the threshold acetate concentration for biofouling is consistent with the ability of Aqualic bacteria to multiply in water at concentrations of a few μg of C·L^{-1} of low molecular weight compounds.

The fouling rate in the MFS slowed down at S_{ac} values below 25 μg C·L^{-1} (Figure 3a), which indicates a decrease of the bacterial growth rate in the biofilm at these bulk concentrations. The calculated half saturation concentration (k_f) of 15.4 μg acetate-C·L^{-1} in the feedwater calculated from these data (Figure 3a) is clearly higher than the k_s concentrations for suspended growth in drinking water at the same temperature. Furthermore, the maximum clogging rate is much lower than the maximum growth rates presented

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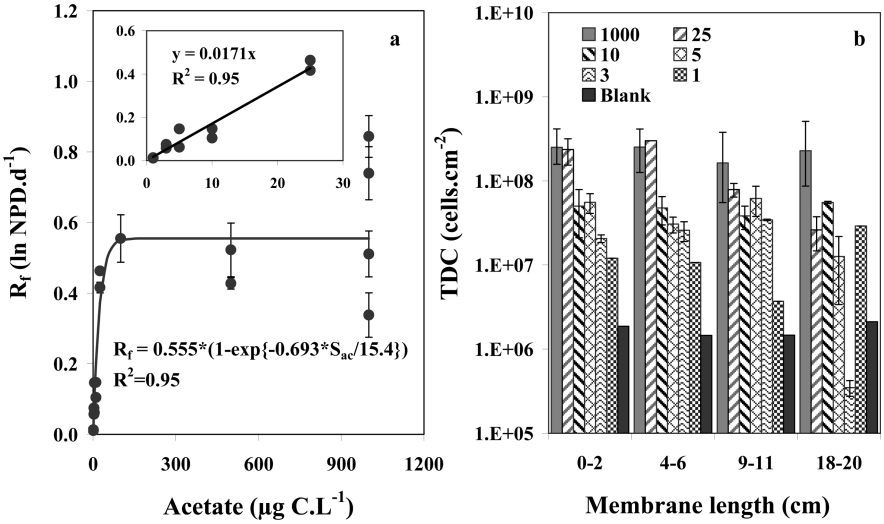

FIGURE 3. Relationship between the acetate-C concentration in the water and the fouling rate constant R_f (a; error bar = SD) and (b) the total direct cell count (TDC) on the membrane as a function of the length the MFS unit (error bar = range of vales) determined at the end of the operational time of the units supplied with different acetate concentrations.

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above. These differences with the growth kinetics of suspended bacteria are attributed to diffusion limited growth of the attached bacteria. The observed plateau in NPD increase at low S_{ac} demonstrates that at these concentrations equilibrium in bacterial growth, decay and sloughing was reached.

Biofouling Potential of the Feedwater. The role of attached microbial growth in biofouling of spiral-wound membranes as documented in literature (*2, 3*) implies that feed waters contain growth-promoting compounds at levels above the derived threshold concentration. Feed water of spiral-wound membranes is usually surface water with limited pretreatment focused on removal of particulates. Optimization of the feedwater quality by biofiltration is a successful antifouling strategy (*19*). Concentrations of easily assimilable organic compounds are reduced in rapid granular filters within a few minutes of contact time (*20*−*24*), even in filters with sintered glass (*25*). However, chemicals added to the feedwater such as antiscalants are potential sources of easily biodegradable compounds (*26*−*28*). Consequently these additives should be of a high quality.

A variety of methods is available for characterization of the biofouling potential of feedwater. Griebe and Flemming (*19*) used the BDOC (biodegradable dissolved organic carbon (*29*)) method and proposed a BDOC value of 0.1 mg C·L^{-1} for the feedwater of membranes to minimize biofilm growth (*22*). The results of the present study, however, show that the level of easily biodegradable organic compounds in the feedwater should be reduced to a much lower level. The AOC method (*30*) and also the BDOC method which has been developed to assess the growth potential of drinking water, are batch tests with exposure times of more than one week enabling utilization of easily and less easily assimilable compounds. Contact times of attached biofilms with the feedwater in membrane elements, however, are in the order of seconds and only small fractions of AOC in water are rapidly available for biofilm formation (*31*). This explains that no clogging occurred at an AOC level of 3−5 μg ac·-C eq·L^{-1} in the unspiked feedwater. In a pilot plant with spiral-wound membranes no biofouling was observed for one year at AOC concentration in the feedwater of 4−6 μg ac·-C eq·L^{-1} (*32*). For determining the biofilm formation rate of water, a continuous flow system has been developed (*33*) in which the concentration of attached growth on glass rings is monitored using ATP analysis (BFR; pg ATP·cm^{2}·d^{-1}). The threshold acetate-C concentration of 1 μg acetate-C·L^{-1} correspondents with a BFR value of 35 pg ATP·cm^{-2}·d^{-1} (*31*). Observations in the feedwater of NF/RO membrane installations showed low NPD increase at BFR values of the feedwater of <1 − 20 pg ATP·cm^{-2}·d^{-1} (*32, 34*), but a rapid pressure drop increase with additional MTC decrease was observed at an AOC of 27 μg ac·-C eq·L^{-1} and BFR of 310 pg ATP·cm^{-2}·d^{-1} caused by the use of a contaminated antiscalant (*25, 26*). In conclusion these field observations in spiral-wound membrane installations with the AOC and BFR methods are consistent with the results of the current study. Moreover, it demonstrates that the definition of the threshold value for biofouling prevention depends on the applied test method. Further data collection on biofouling of NF/RO membranes and feedwater quality assessed with these bioassays is needed to establish guidelines for feedwater quality to prevent or minimize biofouling.

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Supporting Information Available

Theoretical calculations on the relationship between biofilm thickness, pressure drop increase, and porosity decline; Figure of the experimental set up; hydraulic characterization of the MFS. This material is available free of charge via the Internet at http://pubs.acs.org.

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ORIGINAL ARTICLE

Polaromonas and *Hydrogenophaga* species are the predominant bacteria cultured from granular activated carbon filters in water treatment

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Keywords
biofilm, drinking water, GAC filtration, *Polaromonas*.

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Abstract

Aim: Identification of the predominating cultivable bacteria in granular activated carbon (GAC) filters used in a variety of water treatment plants for selecting representative strains to study the role of bacteria in the removal of dissolved organic matter.

Methods and Results: Bacterial isolates were collected from 21 GAC filters in nine water treatment plants treating either ground water or surface water with or without oxidative pretreatment. Enrichment of samples in dilute liquid medium improved culturability of the bacteria by approximately log unit, to 9% up to 70% of the total cell counts. Genomic fingerprinting and 16S rDNA sequence analysis revealed that most (68%) of the isolates belonged to the *Betaproteobacteria* and 25% were identified as *Alphaproteobacteria*. The number of different genera within the *Betaproteobacteria* was higher in the GAC filters treating ozonated water than in the filters treating nonozonated water. *Polaromonas* was observed in nearly all of the GAC filters (86%), and the genera *Hydrogenophaga*, *Sphingomonas* and *Afipia* were observed in 43%, 33% and 29% of the filter beds, respectively. AFLP analysis revealed that the predominating genus *Polaromonas* included a total of 23 different genotypes.

Conclusions: This study is the first to demonstrate that *Polaromonas*, which has mainly been observed in ultraoligotrophic freshwater environments, is a common component of the microbial community in GAC filters used in water treatment.

Significance and Impact of the Study: The predominance of ultraoligotrophic bacteria in the GAC filters indicates that very low concentrations of substrates are available for microbial growth. *Polaromonas* species are suited for further studies on the nutritional versatility and growth kinetics enabling the modelling of biodegradation processes in GAC filters.

Introduction

Granular activated carbon (GAC) filters are used in water treatment for the removal of natural organic matter (NOM) and organic micropollutants. The main removal mechanism is adsorption, but part of the dissolved organic compounds is also removed by biological activity in these filters. Biodegradation processes improve the biological stability of the water by removing biodegradable nonadsorbable organic compounds that may cause bacterial regrowth and biofilm formation in drinking water distribution systems (LeChevallier *et al.* 1987; Van der Kooij *et al.* 1989; Servais *et al.* 2004). Furthermore, biodegradation of adsorbable organic compounds delays the saturation of adsorption sites, thereby increasing the quantity of organic matter that can be removed by a GAC filter in a certain period of time (Wilcox *et al.* 1983). The concentration of micropollutants in filtered

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water most often determines the operational period of a GAC filter and postponed breakthrough of these compounds therefore reduces the costs of water treatment (Orlandini *et al.* 1997).

The efficiency of the biodegradation processes depends on the concentration and activity of the microbial biomass and the nutritional properties of the microbial community. The properties of the microbial community are in turn determined by the composition and concentration of NOM and by the operational parameters (filtration rate, contact time and GAC properties) and temperature. Several methods are available for determining the concentration (or activity) of biomass in GAC filters, e.g. oxygen consumption, tetrazolium reduction, phospholipids and adenosine triphosphate (ATP) (Camper *et al.* 1985; Urfer and Huck 2000; Fonseca *et al.* 2001; Magic-Knezev and Van der Kooij 2004; Velten *et al.* 2007). Based on the data about ATP concentrations observed in the GAC filters, it can be estimated that at least 6–125 kg of dry weight of microbial biomass carbon is present in a filter bed containing 100 m^3 of GAC (Magic-Knezev and Van der Kooij 2004).

Information about the nutritional properties of the predominating bacteria is needed to elucidate the biodegradation capacity of GAC filters. Bacteria isolated from GAC filters have been identified in most cases as members of the genera *Pseudomonas*, *Acinetobacter*, *Caulobacter*, *Alcaligens*, *Flavobacterium* and *Bacillus* (McElhaney and McKeon 1978; Wilcox *et al.* 1983; Stewart *et al.* 1990). However, these bacteria may not represent the microbial community because substrate-rich solid media was used with an incubation time of a few days, yielding only small fractions of the bacteria present in oligotrophic environments (Reasoner and Geldreich 1985). Furthermore, many aquatic isolates remained unidentified because of restricted reactions with substrates used at high concentrations in the classic tests for the identification of bacteria. To date, molecular analytical methods enable effective identification of isolated bacteria. Furthermore, culture-independent techniques revealed the existence of many unknown and uncultured bacteria and stimulated researchers to adapt the cultivation techniques for bacteria from environmental samples (Button *et al.* 1993; Bussmann and Schink 2001; Page *et al.* 2004). An advantage of using cultivation methods is that organisms are obtained for further studies.

The objectives of our study were: (i) identification of cultivable bacteria predominating in GAC filters used in water treatment and (ii) selection of representatives of these bacteria for further study. In this study, the number of cultivable bacteria was determined in GAC filters at a variety of full-scale water treatment plants in relation to the total microscopic count using enrichment media with low nutrient concentration to favour growth of aquatic bacteria. Subsequently, isolates were collected and identified based on 16S rDNA sequences, and fingerprinting techniques were applied to determine the diversity of predominant isolates.

Materials and methods

Isolation of predominant bacteria

Bacteria were isolated from 21 different GAC filters, the sand of six dual media (anthracite/sand) filters and one slow sand filter (SSF), respectively, at nine full-scale water treatment plants in The Netherlands. The GAC filters were operated at different conditions. The empty-bed contact time varied between 10 and 45 min and the filtration rate was in the range of 3–10 m h^{-1}. The concentration of dissolved organic carbon varied between 1·5 and 5·5 mg l^{-1} and the water temperature ranged from 5 to 21°C. Filter material was collected, stored and treated with ultrasound to obtain suspended biomass as described previously (Magic-Knezev and Van der Kooij 2004). The bacteria in the obtained suspensions were cultivated on solid medium. Appropriate decimal dilutions of the obtained bacterial suspensions were spread in triplicate over the surface of R2A agar (Oxoid) plates and incubated during 10 days at 25°C (Reasoner and Geldreich 1985). The colonies were counted and from these counts, the number of colony-forming units (CFU) cm^{-3} was calculated. From each sample, three colonies of the three most abundant colony types and colonies with a distinctive morphological feature were collected for further analysis.

The most probable number (MPN) method was used for enumeration of the concentration of the bacteria in samples of nine GAC filters. The liquid medium used for this purpose was prepared from the autoclaved influent of the GAC filter enriched with 5 mg l^{-1} of yeast extract (YE). The obtained biomass suspensions were decimally diluted in autoclaved tap water and inoculated in fivefold in 9-ml volume of the YE medium. Growth was monitored with ATP analysis during 14 days of incubation at 22°C. Subsequently, the concentration of bacteria was determined according to MPN method (Clesceri *et al.* 1996). Volumes of 0·1 ml from the highest dilution with growth were plated on R2A medium to obtain pure cultures. A total of 186 isolates were collected from the R2A plates and 25 isolates were collected from the liquid medium.

Identification of isolated bacteria

Collected isolates were screened with the repetitive extragenic palindrome *rep*-PCR to identify colonies with

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similar appearance and select the representatives for the sequencing of 16S rDNA. Full sequences were used to determine the phylogenetic position of an isolate and partial sequences were used to confirm the identity of isolates with *rep*-PCR pattern similarity >90%. AFLP fingerprints were used to evaluate the diversity within predominant genus.

Rep-PCR analysis

Freshly grown colonies on R2A medium were suspended in 5^{-1} μl of DNA-free water and DNA was extracted using the Bio-Rad DNA Extraction Kit, following the procedure prescribed by the manufacturer. Subsequently, a genomic fingerprint was generated using specific primers for the *REP* regions of DNA as described previously (Versalovic *et al.* 1991). The PCR products were separated using agarose gel electrophoresis (2·5% fine NuSieve 3:1; BMA, Rockland, ME, USA) at 60 V for 16 h. Gels were stained for 30 min with Cyber gold and digitized with Bio-Rad Gel Documentation System (Bio-Rad). *REP*-patterns were compared by the BIONUMERICS software ver. 3.2 (Applied Maths NV) after the normalization to the 100-bp standard reference lanes (Invitrogen 100-bp DNA ladder, Cat no. 15628019), applying the unweighed pair group method. *Rep*-PCR fingerprints were generated from DNA of *Aeromonas hydrophila* (strain M800), *Ps. fluorescens* (strain P17) and *Spirillum* sp. (strain NOX) in six to nine series to determine the variability of the procedure. These strains were grown and maintained on Lab-Lemco Agar (Oxoid).

AFLP analysis

The AFLP technique for DNA fingerprinting adapted for analysis of *Campylobacter* strains was used (Duim *et al.* 1999). The final products were diluted 1 : 1 together with an internal lane standard (PE Applied Biosystems) and analysed on a short capillary/POP four polymer by using a model ABI 310 automated DNA sequencer. The obtained chromatograms were normalized with internal lane standard and compared using BIONUMERICS software ver. 3.2.

Sequencing and phylogenetic analysis

The 16S rDNA was amplified after DNA isolation with PCR using Taq DNA polymerase with primers for conserved domains (Devereux and Willis 1995). Full 16S rDNA sequences were analysed using two internal primers (530R16 and 907F16) and two terminal primers (8R16 and 1510R16) and partial 16S rDNA sequences were analysed using one internal primer (907F16). The 16S rDNA sequences were assembled with the Croma Tool and the DNASTAR software package (DNAStar Inc., WI). Automatically aligned and subsequently manually corrected sequences were added to the ARB SSU rDNA database tree (Technische Universität München, Freising, Germany) using the quick parsimony tool without filters. The identity of isolates on the genus level was derived from their position in the ARB SSU rDNA database tree (Ludwig *et al.* 2004). Sequences were also compared with the sequences from the GenBank database using the Blast tool (http://www.ncbi.nlm.nih.gov/blast) to identify the closest relatives.

Statistical analysis

The differences between the proportions of different bacterial species or groups originating from the filters treating different water types were tested using the *Z*-test (http://onlinestatbook.com/rvls/) on difference between proportions at a confidence level of 95%.

Nucleotide sequence accession numbers

All partial or full-length sequences from 16S rRNA gene sequences determined in this study have been deposited in the GenBank database under accession numbers EU130949–EU131006 and EU180508–EU180567.

Results

Isolation of predominant cultivable bacteria

The colony counts of bacteria on R2A medium ranged from 1×10^7 to 5×10^9 CFU cm^{-3} and represented 0·2–9% of the total direct cell count (TDC) values that ranged from 1×10^9 to 4×10^{10} cell per cm^3 GAC (Magic-Knezev and Van der Kooij 2004). The predominating colony types represented 20–50% of the colony counts and the second and third most abundant colony types represented 10–20% of these counts. A total of 186 isolates originating from different filter materials were collected from R2A plates. The number of isolates obtained from the GAC filters treating surface water (SW) exceeded the number of isolates from the GAC filters treating ground water (GW) as a result of the larger variety of colony types on R2A plates inoculated with biomass obtained from filters treating SW. The first, second and third dominant colony types yielded a total of 86, 50 and 33 isolates, respectively. The remaining 17 isolates represented less abundant colony types with a distinctive morphologic feature, not found in other isolates, e.g. colour, shape or roughness of the colony.

The concentration of cultivable bacteria, estimated as MPN in liquid medium, ranged from $1{\cdot}1 \times 10^8$ to $4{\cdot}3 \times 10^{10}$ cells per cm^3 GAC. These concentrations were 0·4–2·2 log units (median 0·98) higher than the corresponding HPC values (Table 1). A total of 25 isolates were obtained as pure culture from the highest dilution with growth in liquid medium.

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Table 1 Concentration of bacteria from nine GAC samples cultivated on solid (HPC) and in liquid medium (MPN)

Sample	HPC CFU ml^{-1} (SD)	MPN N ml^{-1} (lower–upper*)	Log (MPN/HPC)
A	$1{\cdot}2 \times 10^7$ ($1{\cdot}3 \times 10^6$)	$1{\cdot}2 \times 10^8$ ($3{\cdot}7 \times 10^7$–$4{\cdot}2 \times 10^8$)	1·0
B	$4{\cdot}0 \times 10^7$ ($5{\cdot}4 \times 10^6$)	$1{\cdot}1 \times 10^8$ ($3{\cdot}6 \times 10^7$–$3{\cdot}8 \times 10^8$)	0·4
C	$5{\cdot}9 \times 10^7$	$1{\cdot}4 \times 10^8$ ($3{\cdot}6 \times 10^7$–$4{\cdot}2 \times 10^8$)	0·4
D	$1{\cdot}9 \times 10^8$ ($1{\cdot}3 \times 10^7$)	$9{\cdot}3 \times 10^8$ ($1{\cdot}8 \times 10^8$–$4{\cdot}2 \times 10^8$)	0·7
E	$2{\cdot}0 \times 10^8$ ($2{\cdot}3 \times 10^7$)	$1{\cdot}1 \times 10^9$ ($4{\cdot}0 \times 10^8$–$2{\cdot}9 \times 10^9$)	0·7
F	$2{\cdot}4 \times 10^8$ ($6{\cdot}1 \times 10^7$)	$2{\cdot}3 \times 10^9$ ($4{\cdot}6 \times 10^8$–$9{\cdot}4 \times 10^9$)	1·0
G	$3{\cdot}6 \times 10^8$ ($6{\cdot}9 \times 10^7$)	$4{\cdot}3 \times 10^{10}$ ($9{\cdot}0 \times 10^9$–$1{\cdot}8 \times 10^{10}$)	2·1
E	$4{\cdot}9 \times 10^8$ ($8{\cdot}5 \times 10^7$)	$2{\cdot}0 \times 10^{10}$ ($4{\cdot}5 \times 10^9$–$4{\cdot}2 \times 10^{10}$)	1·6
H	$7{\cdot}1 \times 10^8$ ($1{\cdot}8 \times 10^8$)	$7{\cdot}5 \times 10^9$ ($1{\cdot}7 \times 10^9$–$2{\cdot}0 \times 10^9$)	1·0

*95% confidence limits.

Selection and identification of cultured bacteria

The pattern similarity of the *rep*-PCR genomic fingerprint determined for three control strains in multiple experiments varied between 84% and 98% for *Aer. hydrophila* strain M800 ($n = 6$) and *Ps. fluorescens* strain P17 ($n = 8$) and from 89% to 99% for *Spirillum* sp. strain NOX ($n = 9$). The *rep*-patterns within a species show more variation than between strains and a *rep*-PCR pattern similarity >90% was used for clustering of the isolates. *Rep*-PCR fingerprints were generated for all isolates collected from the solid medium. A total of 130 (70%) of the isolates were divided into 39 clusters, each with 2–12 isolates, with >90% similarity. Furthermore, 56 isolates (30%) occurred individually in the *rep*-PCR dendrogram. For representatives of each cluster and all individually occurring isolates, the sequence of 16S rDNA gene was determined. Full sequences were used to determine the phylogenetic position of an isolate and partial sequences were used to confirm the identity of isolates with *rep*-PCR pattern similarity >90%. Isolates within clusters with *rep*-PCR pattern similarity >90% showed >97% 16S rDNA sequence similarity confirming the validity of the applied selection criterion for *rep*-PCR pattern similarity (>90%). A total of 112 selected isolates obtained from R2A plates and 25 isolates grown in the MPN tests were identified based on the complete or partial 16S rDNA sequence.

The phylum of the *Proteobacteria* represented about 94% of all isolates (Table 2). The proportions of *Alpha*- and *Betaproteobacteria* differed for the two culturing methods. *Alphaproteobacteria* represented 22% of the isolates obtained from the solid medium and 44% of the isolates obtained from the liquid medium.

A total of 25% of the identified isolates were assigned to the class *Alphaproteobacteria* of which *Sphingomonas* (7·1%) and *Afipia* (4·3%) were most frequently represented. The isolates identified as members of the genus *Sphingomonas* were found in 33% of the filter beds (7/21) and had >97% sequence similarities with five different *Sphingomonas* sp. strains (AB033945, AF395031, AF428806, AY038702 and AY509378). The isolates identified as members of the genus *Afipia*, which were found in 29% of the filter beds (6/21), had >97% sequence similarity with *Afipia* genosp. 11 (AGU87782 and AGU87779) or *Afipia massiliensis* (AY568510).

The *Betaproteobacteria* represented 68% (143/211) of all isolates, of which the Comamonadaceae represented 76% (108/143) and included 51% (108/211) of all collected isolates. The most frequently represented genus of this group was *Polaromonas* (26·5%), which represented 50% of the predominating colony types and 43% of the second predominating colony types. *Polaromonas*-related bacteria were isolated from 86% (18/21) of the filter beds (Table 2). Their concentration estimated from the colony counts ranged from 5×10^6 to 3×10^9 CFU cm^{-3} GAC and represented 1–8% of the TDC values. The concentration of *Polaromonas*-related bacteria in GAC filters estimated from the MPN values ranged from 5×10^7 to 3×10^{10} cells per cm^3 GAC, i.e. 5–75% of the TDC values. The genus *Hydrogenophaga* comprised 13·7% of all isolates and 20% of the *Betaproteobacteria*. *Hydrogenophaga*-related bacteria were isolated from 43% (9/21) of the filter beds investigated (Table 2) and were dominant in two filter beds. These bacteria had >97% sequence similarity with five sequences in the NCBI database and represented mainly *Hydrogenophaga palleronii* (AF445679, AF523030, AF523047 and AF523069) or *Hydrogenophaga taenospiralis* (AF078768). Isolates identified as *Hydrogenophaga* were 21% of the second predominant colony types and 26% of the third predominant colony types. The concentration of *Hydrogenophaga* in GAC filters was estimated at 2×10^6–1×10^9 CFU cm^3 GAC, i.e. 0·2–3% of the TDC values. The concentration of *Hydrogenophaga*-related bacteria in GAC filters obtained from the MPN tests was estimated at 2×10^7–1×10^{10} cells per cm^3 GAC, i.e. 2–25% of the TDC values. A total of 7·6% of all isolates were related to a methyl *tert*-butyl ether (MTBE), degrading bacterium (AF176594) of the

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Table 2 Identity and number (proportion) of the isolated bacteria, the number (proportion) of GAC filter beds in which a certain group is detected and the number (proportion) of filter beds in which a group was predominant

Identity	Number of isolates (%)	Presence in filter beds (%)	Predominant in filter beds (%)
Betaproteobacteria			
Polaromonas	56 (26·5)	18 (86)	11 (52)
Hydrogenophaga	29 (13·7)	9 (43)	2 (10)
Methylibium	13 (6·2)	4 (19)	3 (14)
Ultramicrobacterium	9 (4·3)	2 (10)	1 (5)
Aquaspirillum	8 (3·8)	4 (19)	0
Ideonella	6 (2·8)	2 (10)	0
Acidovorax	4 (1·9)	1 (5)	0
Comamonadaceae, unclassified	3 (1·4)	1 (5)	0
Variovorax	3 (1·4)	1 (5)	0
Alcaligenes	2 (0·90	1 (5)	0
Burkholderia	2 (0·9)	1 (5)	0
Pseudomonas	2 (0·9)	1 (5)	0
Rubrivivax	2 (0·9)	1 (5)	0
Xylophilus	2 (0·9)	1 (5)	0
Aquamonas	1 (0·5)	1 (5)	0
Unclassified	1 (0·5)	1 (5)	0
Alphaproteobacteria			
Sphingomonas	15 (7·1)	7 (33)	2 (10)
Afipia	9 (4·3)	6 (29)	1 (5)
Unclassified	6 (2·8)	4 (19)	1 (5)
Bradyrhizobium	5 (2·4)	2 (10)	0
Brevundimonas	4 (1·9)	2 (10)	0
Rhodobacter	4 (1·9)	2 (10)	0
Methylomonas	3 (1·4)	1 (5)	0
Roseomonas	2 (0·9)	1 (5)	0
Acetobacter	1 (0·5)	1 (5)	0
Caulobacter	1 (0·5)	1 (5)	0
Deviosa	1 (0·5)	1 (5)	0
Rhizobium	1 (0·5)	1 (5)	0
Actinobacteria	9 (4·3)	3 (14)	0
Bacteriodetes	4 (1·9)	3 (14)	0
Gammaproteobacteria	1 (0·5)	1 (5)	0
Firmicutes	2 (0·90)	2 (10)	0

unclassified Burkholderiales. These isolates had >97% sequence similarity with four sequences (AY622242, AY662010, AY212718 and AF176594).

Cultivation in the liquid medium did not yield different genera when compared with the isolates from the solid medium.

Diversity of *Polaromonas*

Most (66%) of the *Polaromonas* isolates had 99% similarity with an uncultured bacterium clone C-15 (AF523046) of the Comamonadaceae, obtained from bottled mineral water (Loy *et al.* 2005). The remaining isolates of the *Polaromonas* group had >97% sequence similarity with five other sequences (AY250094, AY752100, AF523013, AF407400 and AF523046). The complete or partial 16S rDNA sequences of these isolates showed >97% similarity with the sequences of *Polaromonas vacuolata* (Irgens *et al.* 1996), *Polaromonas naphthalenivorans* (Jeon *et al.* 2004) and *Polaromonas aquatica* (Kampfer *et al.* 2006; Fig. 1). AFLP analysis revealed that the isolates could be divided into 23 genotypes with >90% internal similarity (Fig. 2)

Identity of the predominating cultured bacteria in relation to water origin and pretreatment

The GAC filters studied were supplied with different water types. The GAC filters treating either GW or SW showed similar relative abundances of the *Betaproteobacteria* (data not shown). The genus *Polaromonas* represented a significantly ($P < 0·01$) larger proportion (33%) of the isolates cultured from GAC filters supplied with ozonated water (OW) than from filters without oxidative pre-treatment (16%). Isolates of the genus *Variovorax* were only observed in filters treating nonoxidized groundwater. The genus *Sphingomonas* represented the predominating *Alphaproteobacteria* in GAC filters treating SW and the genus *Afipia* represented the predominant

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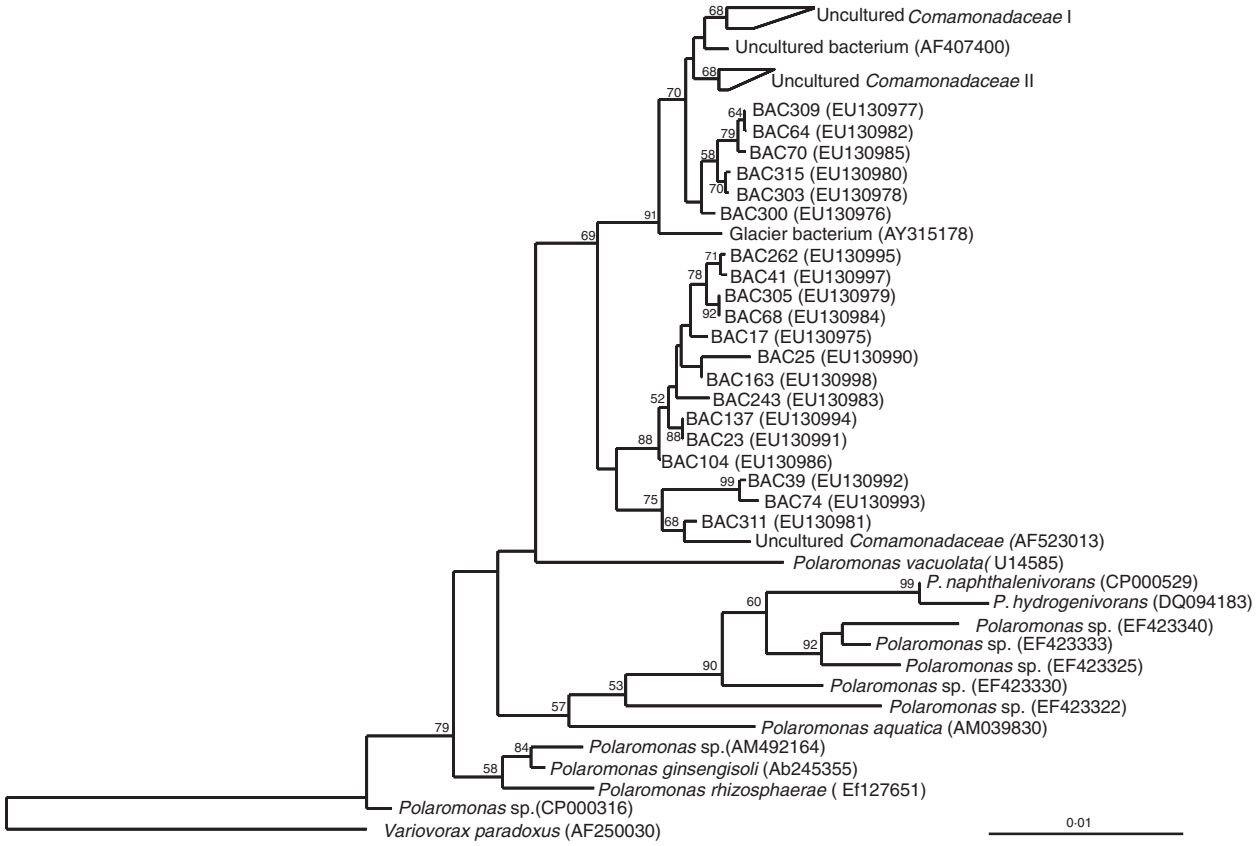

Figure 1 Phylogenetic tree showing the diversity and the relatedness of isolates from GAC filters classified as members of genus *Polaromonas*. The tree is constructed from full-length 16S rRNA sequences using the neighbour-joining method with Felstenstein correction and termini filter using 1000 bootstrap replicates. Bootstraps values above 50% are shown. The scale bar represents 1% evolutionary distance. *Variovorax paradoxus* is used as the out-group.

Alphaproteobacteria in the filters treating GW. The genera *Caulobacter*, *Devosia*, *Methylobacterium* and *Rhodobacter* were only obtained from filters treating nonoxidized ground water, whereas members of the family Acetobacteraceae and the genera *Brevundimonas*, *Rhizobium* and *Roseomonas* were obtained only from filters treating OW. The latter genera and *Rhodobacter* were specific for filters treating SW and members of the genera *Caulobacter*, *Devosia* and *Methylobacterium* were specific for filters treating GW.

The isolates collected from sand and anthracite layers of the rapid sand filtration (RSF) or slow sand filtration (SSF) were identified as members of the same genera that were observed in GAC filters. Also in these filters, members of the Comamonadaceae family were predominant (53%).

Discussion

Abundance and diversity

The range of HPC values in GAC samples observed in this investigation correspond well with values reported earlier for GAC filters in water treatment (Camper *et al.* 1985; Stewart *et al.* 1990). These values represent a minor proportion (0·2–9%) of the TDC values and similar fractions of cultivable bacteria have been reported for oligotrophic aquatic environments and soil (Reasoner and Geldreich 1985; Bruns *et al.* 2001; Page *et al.* 2004; Janssen 2006). Adjustment of the culturing technique improved the cultivability of bacteria from GAC filters by approximately 1 log unit (median value; Table 2). This observation is consistent with results of other studies, viz. an increase of the average cultivability of lake bacteria with 1·25 log units when the substrate and oxygen concentration were adjusted and for marine bacteria, a cultivability of 2–60% was reported with the dilution-culture technique (Button *et al.* 1993; Bussmann and Schink 2001). However, we did not observe differences in the identity of isolates harvested with the two culturing techniques. Hence, the dilution-culture technique yields isolates representing a significant proportion of the total bacterial community, which therefore can be used for further studies.

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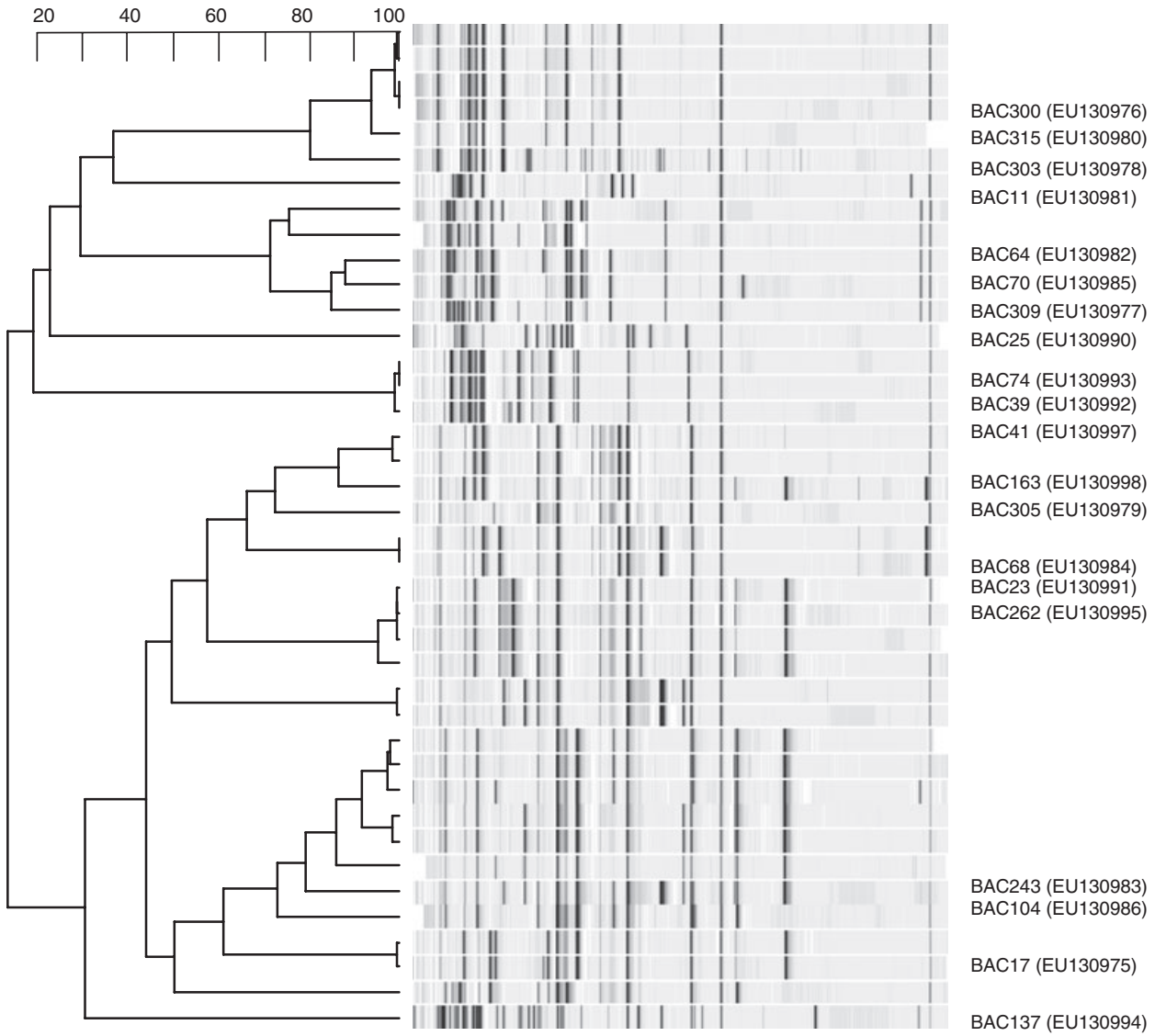

Figure 2 Pearson correlation dendogram from AFLP patterns of *Polaromonas*-related isolates from GAC filters. The accession numbers were shown only for the isolates that were used to build the phylogenetic tree based on 16S rRNA sequences in Fig. 1.

In previous studies, a limited number of rapidly growing bacteria, which were able to metabolize substances at high concentrations, and bacteria with specific features, e.g. formation of spores, were isolated from GAC filters, viz. members of the genera *Pseudomonas*, *Alcaligenes*, *Acinetobacter*, *Flavobacterium*, *Caulobacter* and *Bacillus* (Parsons *et al.* 1980; Stewart *et al.* 1990; White *et al.* 1996). The identity of the isolates collected in this study differed clearly from the identity of bacteria isolated in earlier studies on GAC filters. The differences may be attributed to: (i) optimized removal of bacteria from GAC surface, (ii) adjustment of culturing techniques and (iii) use of molecular methods for identification of isolated bacteria. Furthermore, the nomenclature of many previously isolated bacteria has been adapted since the introduction of phylogenetic analysis based on 16S rDNA sequences. The species *Alcaligenes*, *Ps. acidovorans* and *Ps. testosteroni* have been reclassified to the *Comamonadaceae* as *Variovorax paradoxus*, *Comamonas acidovorans* and *Comamonas testosteroni*, respectively (Willems *et al.* 1991). Bacteria previously identified as *Pseudomonas* are presently classified as *Sphingomonas* (White *et al.* 1996). In this study, *Polaromonas* was identified as predominant genus in GAC filters and these bacteria represent a high proportion of the TDC values (up to 70%). To our knowledge, representatives of the genus *Polaromonas* have not been reported in earlier studies on GAC. Only a few species of this genus have yet been described (Irgens *et al.*

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1996; Jeon *et al.* 2004; Kampfer *et al.* 2006). The genomic diversity of the isolates identified in this study as *Polaromonas* spp. (Figs 1 and 2) suggests that further classification into additional species may be expected in the future.

Comparison with the bacterial communities of freshwater habitats, including SW, treated water and bottled water and of soil, yield a number of similarities and differences, but this comparison is biased by the application of cultivation in this study. The predomination of the *Betaproteobacteria* (61–80%) in GAC filters in water treatment is consistent with the position of this bacterial class in communities of biofilms and planktonic bacteria in natural freshwater environments (Zwart *et al.* 2002; Brummer *et al.* 2003; Bruns *et al.* 2003; Eiler and Bertilsson 2004; Page *et al.* 2004; Gich *et al.* 2005) and in drinking water distribution systems (Kalmbach *et al.* 1997). The smaller proportion of *Alphaproteobacteria* is consistent with studies on natural fresh water, but these bacteria represented more than 90% of isolates from a distribution system simulator with a monochloramine residual and from a chlorinated drinking water distribution system (Williams *et al.* 2003). *Alphaproteobacteria* also are major constituents of the microbial communities in soil (Bodour *et al.* 2003; Janssen 2006), and in particle-associated and free-living bacterial communities in water treatment systems and river water (Crump *et al.* 1999; Macdonald and Brozel 1999). The small fractions of representatives of the *Gammaproteobacteria* (<1%), the Actinobacteria (4·3%) and the Firmicutes (<1%) are consistent with studies on natural freshwater communities, bottled natural mineral water and soil (Eiler and Bertilsson 2004; Loy *et al.* 2005). However, the Bacteriodetes (1·9%) were a significant fraction (>25%) of the bacterioplankton community in Swedish lakes (Eiler *et al.* 2004) and the Actinobacteria equalled the presence of the *Alpha-* and *Betaproteobacteria* in a number of lakes and rivers (Zwart *et al.* 2002). These variations may be due to both differences in water composition and comparing clones with isolates. The relatively high proportion of *Alphaproteobacteria* among isolates cultivated in liquid medium (44%) suggests that the abundance of *Alphaproteobacteria* in GAC filters may be underestimated by cultivation on R2A medium.

Relationship between identity of predominating bacteria and environmental conditions in GAC filters

The presence of bacteria in GAC filters depends on the interactions between environmental conditions and their physiological properties. The significant contribution of a few bacterial types found in the GAC filters treating different water types indicates that these bacteria are typical constituents of the bacterial community in GAC filters used in water treatment (Table 2). On the contrary, bacterial types represented by only a single isolate (singletons) also constituted a relatively large proportion (30%) of the isolated bacteria. The bacteria isolated from the GAC filters may have different origins, viz. (i) the feed water, (ii) the biomass accumulating between the GAC particles and (iii) the bacteria attached to GAC surface. Previous observations showed that the main proportion of bacteria in GAC filters is present on the surface of GAC particles (Magic-Knezev and Van der Kooij 2004). Therefore, the predominating bacterial types represent mainly attached bacteria and/or bacteria present as accumulated biomass. The predominating types of bacteria cultured from the GAC filters belonged to a few genera, of which *Polaromonas* was most frequently observed. Some genera were only incidentally observed but such observations could not be attributed to differences in environmental conditions.

The concentration of biodegradable compounds in the feed water of the GAC filters usually is relatively low. Oxidative pretreatment of the water increases the concentration and diversity of biodegradable compounds leading to the increased bacterial abundance in these filters (Gagnon *et al.* 1997). Concentrations of easily assimilable organic carbon (AOC), mainly including carboxylic acids and aldehydes, may attain a level of 40–150 μg of C/l after ozonation. Without such oxidation, the AOC concentration remains <10 μg C/l in GW and <35 $\mu g\ l^{-1}$ in SW (Van der Kooij *et al.* 1989; Van der Kooij 1992). Descriptions of the nutritional versatility of the predominating genera confirm that *Polaromonas*, *Hydrogenophaga* and *Afipia* prefer carboxylic acids and/or amino acids as growth substrates but are limited in the utilization of carbohydrates and generally do not hydrolyse proteins, carbohydrates or fats, (Garrity *et al.* 2005; Magic-Knezev and Van der Kooij 2006). *Polaromonas* spp. generally are not observed in natural freshwater environments (Zwart *et al.* 2002; Eiler and Bertilsson 2004), but the organism predominated in an ultraoligotrophic Crater lake, in contaminated GW and in bottled natural mineral water (Jeon *et al.* 2004; Page *et al.* 2004; Loy *et al.* 2005). Representatives of the genera *Pseudomonas*, *Aeromonas*, *Aquaspirillum* and *Flavobacterium* can multiply at concentrations of a few $\mu g\ l^{-1}$ of low molecular weight compounds (Van der Kooij et al. 1982; Van der Kooij and Hijnen 1984, 1985), but were not detected in GAC filters or are present at a very low concentration. Obviously, *Polaromonas* spp. multiply even more efficiently at low concentrations of biodegradable compounds and/or attach more efficiently onto the GAC surface than these organisms. Indeed, the batch tests with *Polaromonas* isolates collected from the GAC filters revealed that these bacteria are able to

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multiply at very low concentrations of carboxylic acids and aromatic acids (Magic-Knezev and Van der Kooij 2006). Elucidation of the predominant position of *Polaromonas* requires further investigations, e.g. survival at a low flux of nutrients, attachment properties and utilization of NOM present in SW and GW. Furthermore, the contribution of these bacteria to the microbial community in GAC filters should be determined with culture-independent methods

Microbiological contaminant removal

Adsorption processes play a major role in the removal of organic contaminants with GAC filtration in water treatment (Orlandini 1999). *Hydrogenophaga* spp. can degrade MTBE (Hatzinger *et al.* 2001; Kane *et al.* 2007). *Polaromonas*-related bacteria can utilize cis-dichloroethane or naphthalene as sole carbon source at a concentration of a few mg l^{-1} (Coleman *et al.* 2002; Jeon *et al.* 2004). Furthermore, *Sphingomonas* spp. are capable degrading a wide variety of xenobiotic compounds, including pesticides (White *et al.* 1996). The concentrations of MTBE and pesticides in SW used for water treatment usually is low (<1 μg l^{-1}; Achten *et al.* 2002) and it is uncertain if biodegradation of these compounds occurs in GAC filter beds in the presence of clearly higher concentrations of biodegradable NOM. In addition, a contribution of these bacteria to the removal of xenobiotic organic compounds at suddenly elevated concentrations is uncertain, because adaptation seems to be slow (Coleman *et al.* 2002).

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ORIGINAL ARTICLE

Utilization of oligo- and polysaccharides at microgram-per-litre levels in freshwater by *Flavobacterium johnsoniae*

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Abstract

Aims: To obtain a bacterial strain that can be used to quantify biodegradable polysaccharides at concentrations of a few micrograms per litre in freshwater.
Methods and Results: *Flavobacterium johnsoniae* strain A3 was isolated from tap water supplemented with laminarin, pectin or amylopectin at 100 μg C l^{-1} and river Rhine water. The organism utilized 14 of 23 oligo- and polysaccharides, and 1 of 9 monosaccharides, but none of the sugar acids, sugar alcohols, carboxylic acids or aromatic acids tested at 10 μg C l^{-1}. Amino acids promoted growth of strain A3, but not in coculture with assimilable organic carbon (AOC) test strain *Pseudomonas fluorescens* P17, which utilized these compounds more rapidly than strain A3. Compounds released by strain P17 and AOC test strain *Spirillum* sp. NOX grown on acetate promoted the growth of strain A3 at N_{max} values of $\geq 2 \times 10^5$ CFU ml^{-1} of strain P17 and $\geq 5 \times 10^5$ CFU ml^{-1} of strain NOX. Significant growth of strain A3 was observed in surface water and in tap water in the presence of strain P17 (N_{max} P17 $< 2 \times 10^5$ CFU ml^{-1}).
Conclusions: Strain A3 utilizes oligo- and polysaccharides at microgram-per-litre levels. In surface water and in tap water, the organism was able to utilize compounds that were not utilized by strain P17. These compounds may include oligo- and/or polysaccharides.
Significance and Impact of the Study: Phytoplanktonic and bacterial polysaccharides can constitute an important biodegradable fraction of natural organic matter in water and may promote growth of heterotrophic bacteria during water treatment and drinking water distribution. Strain A3 can be used to quantify a group of compounds that includes oligo- and polysaccharides at microgram-per-litre levels in freshwater.

Introduction

Surface water and groundwater used to produce drinking water usually contain a complex mixture of natural organic compounds, collectively called natural organic matter (NOM). Humic substances typically represent the largest NOM fraction in natural waters, but polysaccharides, proteins and low molecular weight compounds (e.g. amino acids, carboxylic acids and monosaccharides) can significantly contribute to NOM as well (Croué *et al.* 1999).

Phytoplankton can be responsible for significant input of NOM in surface water because of the excretion of extracellular compounds during growth (Myklestad 2000). In addition, intracellular compounds may be released into the surface water upon lysis of phytoplankton cells that are under prolonged stress or degraded by other micro-organisms (Myklestad 2000). Extracellular compounds produced by phytoplankton consist mainly of complex polysaccharides, whereas the main intracellular compounds of phytoplankton are polysaccharides, proteins, lipids and low molecular weight intermediates (Myklestad 1995, 2000; Børsheim *et al.* 2005). Extracellular polysaccharides (EPS), storage and cell wall polysaccharides produced by freshwater phytoplankton can dominate the polysaccharide fraction in surface water that

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is used to produce drinking water (Croué *et al.* 1999). In addition, heterotrophic bacteria present in surface water, in water treatment processes and in drinking water distribution systems have the ability to produce biodegradable EPS (Lazarova and Manem 1995; Zhang and Bishop 2003). These phytoplanktonic and bacterial polysaccharides represent one of the biodegradable NOM fractions that may promote growth of heterotrophic bacteria during water treatment and drinking water distribution. However, information on the concentrations of (biodegradable) polysaccharides in natural and treated water is lacking, presumably, because no chemical analytical methods seem to be available to quantify these compounds at microgram-per-litre levels (Croué *et al.* 1999).

In the Netherlands, *c.* 30% of the drinking water is produced from surface water. Microbial regrowth in drinking water distribution systems in the Netherlands is controlled by producing biologically stable drinking water instead of maintaining a disinfectant residual during distribution (Van der Kooij *et al.* 1999). Biologically stable drinking water has a low concentration of biodegradable organic compounds (Rittmann and Snoeyink 1984). A variety of tests has been developed to assess the biological stability of treated water, including the biodegradable dissolved organic carbon (BDOC) and the assimilable organic carbon (AOC) tests. The BDOC test determines the decrease in DOC concentration of the water sample because of the growth of indigenous bacteria or incubation with sand from a biological filter (Joret and Levy 1986; Servais *et al.* 1987). The original AOC test determines the concentration of easily assimilable organic compounds based on the growth of *Pseudomonas fluorescens* strain P17 and *Spirillum* sp. strain NOX in a pasteurized water sample (Van der Kooij 1992). Strain P17 utilizes almost all amino acids and some carboxylic acids, whereas strain NOX is specialized in the utilization of carboxylic acids, including those that strain P17 cannot utilize (Van der Kooij *et al.* 1982; Van der Kooij and Hijnen 1984).

The bacterial strains of the AOC test allow quantification of biodegradable organic compounds at concentrations of a few micrograms per litre. In addition, the bacterial strains of the AOC test enable characterization of two biodegradable NOM fractions. However, polysaccharides are not quantified in the AOC test. The objective of our study was to obtain a bacterial strain that is able to utilize polysaccharides at microgram-per-litre levels in freshwater.

Materials and methods

Isolation and identification of bacterial strains

Tap water prepared from anaerobic groundwater by aeration and rapid sand filtration was collected in borosilicate glass Erlenmeyer flasks (600 ml per flask), which had been cleaned as previously described (Van der Kooij 1992). This tap water contained 1·9 mg of dissolved organic carbon l^{-1} and was supplemented with laminarin, pectin, amylopectin or glucose at 100 μg C l^{-1}. Subsequently, NO_3^- and PO_4^{3-} were added at a concentration of 125 μg N l^{-1} and 50 μg P l^{-1}, respectively. River Rhine water was filtered through a sterile 1·2-μm cellulose nitrate filter (Sartorius AG, Germany), and 0·2% (v/v) river Rhine filtrate was added to each water sample. Water samples were prepared in duplicate flasks and incubated at 15°C until maximum heterotrophic plate counts were reached. Heterotrophic plate counts were determined periodically using R2A agar streak plates (Reasoner and Geldreich 1985) which were incubated for 10 days at 25°C. Total direct cell (TDC) counts were determined using acridine orange (Hobbie *et al.* 1977) and epifluorescence microscopy (Leica DMRXA, The Netherlands).

Eight bacterial strains that formed predominant colony types on the R2A plates were isolated and identified by 16S rRNA gene sequencing. Primers 8f and 1392r (Table 1) were used to amplify the 16S rRNA gene of each bacterial isolate. PCR mixtures (50 μl) contained 25 μl of PCR Master Mix (Promega, USA), 10 pmol of each primer and 5 μl of 100 times diluted template DNA. PCR amplification was performed in a GeneAmp® PCR System 9700 (Applied Biosystems, USA), according to the following programme: 5 min at 95°C; 35 cycles of: 45 s at 95°C, 60 s at 57°C, 2 min at 72°C; and 7 min at 72°C. The size of the PCR product was confirmed on a 2% (w/v) agarose gel stained with SYBR® Gold. The PCR product was cleaned using a DNA Clean & Concentrator™-5 Kit, according to the manufacturer's instructions (Zymo Research, USA). Approximately 700 base pairs of the 16S rRNA gene of each bacterial isolate were sequenced using primer 8f (Baseclear, The Netherlands). Close relatives of each 16S rRNA gene were obtained from the GenBank database by BLAST homology searches. The 16S rRNA gene of one bacterial isolate (strain A3) was completely sequenced using primers 8f, 518f, 530r,

Table 1 Sequences of primers used for PCR amplification and sequence analysis

Primer	Sequence
8f	5′ AGA GTT TGA TCM TGG CTC AG 3′
518f	5′ CCA GCA GCC GCG GCG GTA AT 3′
530r	5′ GTA TTA CCG CGG CTG CTG 3′
907f	5′ AAA CTY AAA KGA ATT GAC GG 3′
907r	5′ CCG TCA ATT CMT TTG AGT TT 3′
1392r	5′ ACG GGC GGT GTG TAC A 3′
1510r	5′ TAC GGY TAC CTT GTT ACG ACG ACT T 3′

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907f, 907r and 1510r (Table 1). The 16S rRNA gene sequences of bacterial isolates A1 through A8 have been deposited in GenBank under accession numbers EU860079 through EU860086.

Assessment of the nutritional versatility of strain A3 at low substrate concentrations

The nutritional versatility of one selected bacterial isolate (strain A3) was tested with mixtures of organic compounds and with individual organic compounds at low concentrations in tap water. Tap water (600 ml) was collected in Erlenmeyer flasks, pasteurized for 30 min at 60°C and supplemented with NO_3^- (125 N μg N l^{-1}) and PO_4^{3-} (50 μg P l^{-1}). Solutions of mixtures of organic compounds were prepared in tap water and heated for 15 min at 121°C, whereas solutions of individual organic compounds were heated for 30 min at 60°C. Mixtures of organic compounds were added at a concentration of 10 μg C l^{-1} per compound. The mixtures were (i) a mixture of 20 amino acids, (ii) a mixture of 11 aromatic acids, (iii) a mixture of 18 carboxylic acids and (iv) a mixture of 19 carbohydrates, sugar acids and sugar alcohols (Table 2). In addition, 39 carbohydrates, sugar acids and sugar alcohols were tested as individual growth substrate for strain A3 at 10 μg C l^{-1} (Table 2). Fructose, galactose, glucose, xylose and galacturonic acid were tested at 100 μg C l^{-1} as well.

Strain A3 was precultured in a mineral salts medium supplemented with 100 μg of amylopectin-C l^{-1} and 100 μg of NO_3^- N l^{-1}. The mineral salts medium contained the following per litre of demineralized water: KH_2PO_4 2·7 mg; K_2HPO_4 4·0 mg; Na_2HPO_4 3·2 mg; $CaCl_2$ 38 mg; $CoCl_2$ 0·03 mg; H_3BO_3 0·1 mg; $MgSO_4$ 24 mg; $CaSO_4$ 0·06 mg; $MnSO_4$ 2·7 mg; $ZnSO_4$ 0·06 mg; $FeSO_4$ 1·6 mg. The preculture of strain A3 was incubated at 15°C and stored at 4°C when maximum colony counts (N_{max}) had been reached. Each tap water sample was inoculated with 50–200 CFU ml^{-1} of precultured strain A3 and incubated at 15°C. Water samples were prepared in duplicate flasks, unless otherwise stated. Colony counts of strain A3 were determined using Lab-Lemco (LLA) agar streak plates (Oxoid), which were incubated for 3 days at 25°C. The growth rate ν (h^{-1}) of strain A3 during the exponential growth phase was calculated using the equation: $\nu = \log N_2 - \log N_1/[\log 2 \times (t_2 - t_1)]$, where N is the colony count and t_2 and t_1 are time points. TDC counts were determined as described earlier. N_{max} values and TDC counts were compared by an unpaired, two-sided t-test.

Growth of strain A3 with *P. fluorescens* strain P17

Growth of strain A3 with *P. fluorescens* strain P17 was tested in pasteurized tap water (600 ml) supplemented with a amino acid mixture (Table 2), a carbohydrate

Table 2 Mixtures of and individual organic compounds tested as growth substrates for strain A3 in tap water at 15°C

Amino acids mixture	Aromatic acids mixture	Carboxylic acids mixture	Carbohydrates, sugar acids and sugar alcohols	
			Mixture & individual*	Individual†
L-Alanine	Anthranilate	Acetate	L-Arabinose	L-Fucose
L-Arginine	Benzoate	Adipate	D-Fructose	D-Glucuronate
L-Asparagine	Ferulate	Citrate	D-Galatactose	D-Galacturonate
L-Aspartate	Gallate	Formate	D-Glucose	Galactobiose
L-Cysteine	*p*-Hydroxybenzoate	Fumarate	D-Mannose	Laminaribiose
L-Glutamine	*p*-Hydroxyphenylacetate	Glyoxylate	L-Rhamnose	Melezitose
L-Glutamate	Mandelate	Glycolate	D-Ribose	Melibiose
Glycine	Nicotinate	β-Hydroxybutyrate	D-Xylose	Trehalose
L-Hystidine	Phthalate	α-Ketoglutarate	D-Cellobiose	Xylobiose
L-Isoleucine	Salicylate	DL-Lactate	Lactose	Alginate
L-Leucine	Vanillate	DL-Malate	Maltose	Amylopectine
DL-Lysine		Maleate	Raffinose	Fucoidan
L-Methionine		Malonate	Saccharose	Galactan
DL-Phenylalanine		Propionate	Stachyose	Gellan gum
L-Proline		Pyruvate	d-Gluconate	Laminarin
DL-Serine		Oxalate	Glycerol	Pectin
L-Threonine		L-Tartrate	Myo-inositol	Rhamnogalacturonan
L-Tryptophan		Valerate	D-Mannitol	Xanthan gum
L-Tyrosine			D-Sorbitol	Xylan
L-Valine				Xyloglucan

*Organic compounds of mixture were also tested as individual substrate for growth of strain A3.
†Additional organic compounds tested as individual substrate for growth of strain A3.

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mixture (fructose, cellobiose, maltose, saccharose, raffinose and stachyose) or with both mixtures. The amino acid mixture was prepared as described earlier; the carbohydrate mixture was prepared in tap water and pasteurized for 30 min at 60°C. Each mixture was added to tap water at a concentration of 10 μg C l^{-1} per compound. When only one mixture was added, NO_3^- and PO_4^{3-} were added as described earlier, but when both mixtures were added, NO_3^- and PO_4^{3-} were added at a concentration of 250 μg N l^{-1} and 100 μg P l^{-1}, respectively. Tap water samples were prepared in duplicate flasks and inoculated with 50–200 CFU ml^{-1} of strain A3, strain P17 or both strains. For this purpose, strain A3 was precultured as described earlier. Strain P17 was precultured in a similar manner, using 1 mg of acetate-C and 0·2 mg of NH_4^+ N per litre of mineral salts medium. After inoculation, the water samples were incubated at 15°C. Colony counts of strain A3 and strain P17 were determined using LLA medium, except for the colony count of strain A3 in coculture with strain P17 in the water samples with amino acids or amino acids and carbohydrates. The colony count of strain A3 in these samples was determined using LLA-carbohydrate medium which contained the following per litre of demineralized water: Lab-Lemco powder (Oxoid) 0·3 g; starch 2·5 g; sucrose 1·0 g; agar technical (Oxoid) 15·0 g. All plates were incubated for 3 days at 25°C.

Growth of strain A3 was also tested at different N_{max} values of either strain P17 or *Spirillum* sp. strain NOX. For this purpose, pasteurized tap water (600 ml) was supplemented with acetate at concentrations ranging from 5 to 500 μg C l^{-1} plus 250 μg N l^{-1} and 100 μg P l^{-1}, inoculated with either strain P17 or strain NOX, and subsequently incubated at 15°C. The colony count of strain P17 or strain NOX was determined on LLA medium as described earlier. Strain A3 was added when strain P17 or strain NOX had reached the N_{max} level. Colony counts of strain A3 were determined using LLA-carbohydrate medium as described earlier. Individual growth of strain A3 was tested in tap water supplemented with 25 or 500 μg acetate-C l^{-1}, 250 μg N l^{-1} and 100 μg P l^{-1}.

Growth of strain A3 in surface water

Surface water was collected from a freshwater canal in the Netherlands in May. The chlorophyll-a content of the surface water was determined according to the ISO 10260:1992 method. Subsequently, the surface water was filtered through a 1·2-μm cellulose nitrate filter (Sartorius AG, Germany) which had been rinsed with Milli-Q® Ultrapure Water (Millipore, USA) before use. The surface water filtrate was collected in Erlenmeyer flasks (600 ml water per flask), pasteurized for 30 min at 60°C, supplemented with NO_3^{-1} and PO_4^{3-} at a concentration of 0·8 mg N l^{-1} and 0·3 mg P l^{-1} and inoculated with strain P17, strain A3 or both strains. Surface water filtrate supplemented with 30 μg amylopectin-C l^{-1} was inoculated with strain P17 and strain A3 as well. Surface water samples were prepared in duplicate flasks and incubated at 15°C. Colony counts of strain P17 and strain A3 were determined using LLA plates that were incubated for 3 days at 25°C.

Results

Isolation and identification of bacterial strains utilizing polysaccharides

Laminarin, pectin, amylopectin and glucose were tested individually at 100 μg C l^{-1} and promoted bacterial growth in tap water inoculated with 0·2% (v/v) river Rhine water. Maximum heterotrophic plate counts (N_{max}) were reached within 7 days of incubation and ranged from 2·2 (± 0·2) × 10^5 to 1·4 (± 0·2) × 10^6 CFU ml^{-1}, whereas an N_{max} value of 3·8 (± 0·2) ×10^4 CFU ml^{-1} was reached in the blank. Plating efficiency was 79 (± 12) % as determined by comparison of N_{max} values with TDC values (data not shown). Eight bacterial strains (strain A1 through A8) were isolated from tap water samples with laminarin, pectin or amylopectin based on differences in colony morphology on R2A medium. Bacterial strains A1 through A5 and A7 were identified as representatives of *Flavobacterium* species by 16S rRNA gene sequencing, whereas strain A6 and strain A8 belonged to *Rhodoferax* and *Curvibacter*, respectively (Table 3). Strain A3 was the only bacterial strain isolated from each of the tap water samples with laminarin, pectin or amylopectin, respectively (Table 3). The other bacterial strains (A1, A2 and A4 through A8) were obtained with one or two of these carbohydrates. The complete 16S rRNA gene of strain A3 showed 98% similarity with *F. johnsoniae* strain DSM 425.

Nutritional versatility of *F. johnsoniae* strain A3

The nutritional versatility of strain A3 was studied in more detail using mixtures of organic compounds and individual organic compounds. Only the carbohydrate mixture and the amino acid mixture promoted the growth of strain A3, which also showed some growth in the blank (Fig. 1). The similarity of the growth curves in duplicate flasks and the low standard deviation of the N_{max} values of strain A3 in duplicate flasks demonstrate the high reproducibility of the experimental set-up. Furthermore, statistical analysis showed no difference between the N_{max} values of strain A3 and the TDC values

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Table 3 Identity of the bacterial strains isolated from tap water samples supplemented with laminarin, pectin or amylopectin at 100 μg C l^{-1} and 0·2% (v/v) River Rhine water and incubated at 15°C

Strain no.	Substrate added to water sample	Description of 16S rRNA gene sequence with most significant alignment (GenBank accession no.)	Similarity (%)
A1	Laminarin	*Flavobacterium* sp. CL1·152 (AM934685)	99
		Flavobacterium succinicans DSM 4002 (AM230492)	97
A2	Laminarin	*Flavobacterium* sp. WB-1·1·56 (AM177392)	99
		Flavobacterium frigidimaris (AB183888)	98
A3	Laminarin, pectin, or amylopectin	*Flavobacterium* sp. WB1·2-3 (AM934629)	99
		Flavobacterium johnsoniae DSM 425 (AM230488)	98
A4	Laminarin or amylopectin	*Flavobacterium* sp. WB 2·1-80 (AM167560)	98
		Flavobacterium succinicans DSM 4003 (AM230493)	97
A5	Laminarin or amylopectin	*Flavobacterium* sp. GOBB3-209 (AF321038)	99
		Flavobacterium succinicans DSM 4002 (AM230492)	98
A6	Laminarin or amylopectin	β-proteobacterium LI2-55 (AJ964892)	98
		Curvibacter delicates (AF078756)*	96
		'Aquamonas fontana' AQ11 (AB120965)†	96
A7	Amylopectin or glucose	*Flavobacterium* sp. PR30-2 (EU057847)	98
		Flavobacterium granuli (AB180738)	97
A8	Laminarin	β-proteobacterium LH90 (DQ535028)	98
		Rhodoferax ferrireducens (AF435948)	97

**Aquaspirillum delicatum* has been reclassified as *Curvibacter delicatus*.
†Name '*Aquamonas fontana*' has been suggested as reclassification of the genus *Aquaspirillum*, but has not been validly published.

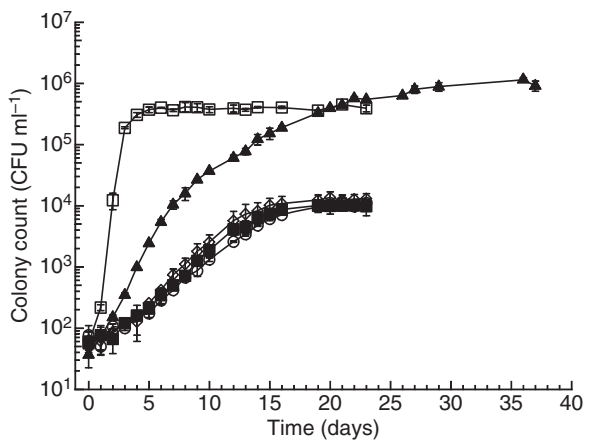

Figure 1 Growth of *Flavobacterium johnsoniae* strain A3 in tap water supplemented with mixtures of organic compounds. Symbols: (○) blank, no organic compounds added; (▲) mixture of 20 amino acids added; (◇) mixture of 11 aromatic acids added; (□) mixture of 19 carbohydrates added; (■) mixture of 18 carboxylic acids added. Individual compound concentration was 10 μg C l^{-1}. Error bars indicate standard deviation of colony count in duplicate flasks.

in the samples ($P = 0{\cdot}98$). The growth rate of strain A3 with the mixture of 19 carbohydrates ($0{\cdot}20 \pm 0{\cdot}002$ h^{-1}) was higher than with the mixture of 20 amino acids ($0{\cdot}05 \pm 0{\cdot}002$ h^{-1}), but the N_{max} value with the carbohydrate mixture [$4{\cdot}3$ ($\pm 0{\cdot}5$) $\times 10^5$ CFU ml^{-1}] was lower than with the amino acid mixture [$1{\cdot}1$ ($\pm 0{\cdot}1$) $\times 10^6$ CFU ml^{-1}] (Fig. 1).

Of the 19 carbohydrates included in the mixture, only fructose, cellobiose, maltose, saccharose, raffinose and stachyose promoted the growth of strain A3 at 10 μg C l^{-1}, which could explain the relatively low N_{max} value of strain A3 on the carbohydrate mixture. In addition, growth of strain A3 was tested on 20 other carbohydrates at 10 μg C l^{-1}, of which laminaribiose, amylopectin, galactan, gellan gum, laminarin, pectin, rhamno-galacturonan, xylan and xyloglucan promoted the growth of strain A3 (Table 4). N_{max} values of strain A3 were compared with TDC values for three selected carbohydrates, viz. amylopectin, laminarin and pectin, and no statistical difference between these parameters was observed ($P = 0{\cdot}83$). The average N_{max} value of strain A3 on the growth-promoting carbohydrates at 10 μg C l^{-1} was $1{\cdot}6$ ($\pm 0{\cdot}3$) $\times 10^5$ CFU ml^{-1}.

Fructose was the only monomer that promoted the growth of strain A3 at 10 μg C l^{-1}. Tests with fructose, glucose, galactose, xylose and galacturonic acid at 100 μg C l^{-1} also demonstrated that only fructose was utilized. Thus, strain A3 is able to grow on a large number of

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Table 4 Maximum colony counts of *Flavobacterium johnsoniae* strain A3 in tap water supplemented with individual carbohydrates at 10 μg C l^{-1}*

Carbohydrate added (10 μg C l^{-1})	N_{max} (CFU ml^{-1})	Carbohydrate added (10 μg C l^{-1})	N_{max} (CFU ml^{-1})
Monosaccharides		Sugar acids & alcohols	
L-Arabinose	n.g.†		
L-Fucose	n.g.	D-Gluconate	n.g.
D-Fructose‡	$1{\cdot}7 (\pm 0{\cdot}09) \times 10^5$	D-Glucuronate	n.g.
D-Galactose‡	n.g.	D-Galacturonate‡	n.g.
D-Glucose‡	n.g.	Glycerol	n.g.
D-Mannose	n.g.	Myo-inositol	n.g.
L-Rhamnose	n.g.	D-Mannitol	n.g.
D-Ribose	n.g.	D-Sorbitol	n.g.
D-Xylose‡	n.g.		
Oligosaccharides		Polysaccharides	
D-Cellobiose	$1{\cdot}8 \times 10^5$	Alginate	n.g.
Galactobiose	n.g.	Amylopectine‡	$1{\cdot}0 (\pm 0{\cdot}2) \times 10^5$
Lactose	n.g.	Fucoidan	n.g.
Laminaribiose	$1{\cdot}4 \times 10^5$	Galactan‡	$1{\cdot}4 (\pm 0{\cdot}07) \times 10^5$
Maltose	$2{\cdot}0 \times 10^5$	Gellan gum	$0{\cdot}2 \times 10^5$
Melezitose	n.g.	Laminarin‡	$1{\cdot}2 (\pm 0{\cdot}1) \times 10^5$
Melibiose	n.g.	Pectin‡	$0{\cdot}8 (\pm 0{\cdot}05) \times 10^5$
Raffinose	$2{\cdot}0 \times 10^5$	Rhamnogalacturonan	$0{\cdot}5 \times 10^5$
Saccharose	$1{\cdot}7 \times 10^5$	Xanthan gum	n.g.
Stachyose	$1{\cdot}5 \times 10^5$	Xylan	$1{\cdot}3 \times 10^5$
Trehalose	n.g.	Xyloglucan	$1{\cdot}6 \times 10^5$
Xylobiose	n.g.		

*N_{max} value in the blank was $7{\cdot}8 (\pm 0{\cdot}3) \times 10^3$ CFU ml^{-1}.
†n.g., growth not different from growth in blank after 23 days of incubation.
‡Compound tested in duplicate flasks; unmarked compounds tested in single flask.

oligo- and polysaccharides, whereas monosaccharides, sugars acids and sugar alcohols are not degraded at ≤ 100 μg C l^{-1}, with the exception of fructose. Hence, strain A3 appears to be specialized in the utilization of oligo- and polysaccharides (Table 4), although it can utilize amino acids as well.

Growth of *F. johnsoniae* strain A3 with *P. fluorescens* strain P17 and mixtures of organic compounds

Growth measurements with strain A3 and strain P17 were conducted in tap water supplemented with a mixture of 20 amino acids, a mixture of 6 carbohydrates (i.e. fructose, cellobiose, maltose, saccharose, raffinose and stachyose) or with both mixtures to determine if strain A3 and strain P17 would influence each other in their growth. Strain P17 utilized only the amino acid mixture and reached the same N_{max} value in tap water with the amino acid mixture as in tap water with both mixtures (Table 5). The similarity of the N_{max} values of strain P17 in tap water with and without strain A3 shows that strain A3 did not influence the growth of strain P17 (Table 5). The N_{max} of strain A3 was the same in tap water with and without strain P17, as was the N_{max} of strain A3 on the carbohydrate mixture in tap water with and without strain P17 (Table 5). In contrast, the N_{max} value of strain A3 on the amino acid mixture was clearly higher in tap water without strain P17 than with strain P17, demonstrating that strain P17 influenced the growth of strain A3.

In the absence of strain P17, the N_{max} value of strain A3 in tap water with both mixtures was equal to the sum of its N_{max} value in tap water with the amino acid mixture and its N_{max} value in tap water with the carbohydrate mixture (Table 5), indicating that strain A3 utilized amino acids and carbohydrates. In addition, strain A3 required the same number of days to reach N_{max} in tap water with both mixtures as in tap water with only the carbohydrate mixture. These observations show that strain A3 utilized the amino acids simultaneously with the carbohydrates. In the presence of strain P17, however, strain A3 reached the same N_{max} value in tap water with both mixtures as in tap water with only the carbohydrate mixture, demonstrating that strain A3 utilized carbohydrates and strain P17 amino acids. (For growth curves see Figs S1–S3.)

Nevertheless, in tap water with both bacterial strains and the amino acid mixture, strain A3 reached a higher N_{max} value than in the blank, whereas the N_{max} value of

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Table 5 Growth of *Flavobacterium johnsoniae* strain A3 and *Pseudomonas fluorescens* strain P17 in tap water supplemented with mixtures of organic compounds

	Strain P17		Strain A3	
Substrates and strains added*	N_{max} (± std) (CFU ml^{-1})	Days (± std) needed to reach N_{max}	N_{max} (± std) of (CFU ml^{-1})	Days (± std) needed to reach N_{max}
Blank + strain P17	0·9 (± 0·2) × 10^4	27 (± 0)	–	–
Blank + strain A3	–	–	0·6 (± 0·3) × 10^4	27 (± 5)
Blank + strain P17 + strain A3	1·0 (± 0·2) × 10^4	34 (± 0)	0·5 (± 0·0) × 10^4	40 (± 2)
AA + strain P17	1·6 (± 0·1) × 10^6	4 (± 0)	–	–
AA + strain A3	–	–	1·1 (± 0·1) × 10^6	36 (± 3)
AA + strain P17 + strain A3	1·5 (± 0·1) × 10^6	4 (± 1)	9·6 (± 0·3) × 10^4	45 (± 0)
CH + strain P17	1·3 (± 0·5) × 10^4	34 (± 0)	–	–
CH + strain A3	–	–	7·6 (± 0·3) × 10^5	7 (± 1)
CH + strain P17 + strain A3	1·6 (± 0·1) × 10^4	39 (± 7)	7·8 (± 0·9) × 10^5	9 (± 0)
AA + CH + strain P17	1·6 (± 0·1) × 10^6	5 (± 1)	–	–
AA + CH + strain A3	–	–	1·7 (± 0·1) × 10^6	7 (± 0)
AA + CH + strain P17 + strain A3	1·4 (± 0·1) × 10^6	3 (± 0)	9·2 (± 0·1) × 10^5	8 (± 1)

*Abbreviations: (blank) no organic compouds added; (AA) mixture of 20 amino acids added; (CH) mixture of 6 carbohydrates added; (strain P17) *P. fluorescens* strain P17 added; (strain A3) *F. johnsoniae* strain A3 added. Individual compound concentration was 10 μg C l^{-1}.

strain P17 was the same as in tap water without strain A3 (Table 5). These findings suggest that strain A3 utilized either remaining amino acids or organic compounds produced by strain P17 during growth. In tap water in which strain P17 had grown to different N_{max} values, growth of strain A3 was promoted at ≥ 2·1 (± 0·05) × 10^5 CFU ml^{-1} of strain P17 reached on ≥ 50 μg acetate-C l^{-1}. The N_{max} of strain A3 was directly proportional to the N_{max} of strain P17, when ≥ 3 × 10^5 CFU ml^{-1} (Fig. 2). In addition, growth of strain A3 was promoted at N_{max} values of strain NOX ≥ 5·0 (±0·6) × 10^5 CFU ml^{-1} grown on ≥ 25 μg acetate-C l^{-1} (Fig. 2). Also here, a directly proportional relationship was found between the N_{max} of strain A3 and the N_{max} of strain NOX, when ≥ 7 × 10^5 CFU ml^{-1} (Fig. 2). The yield factor on acetate-C was 4·1 (± 0·06) × 10^6 CFU μg^{-1} for strain P17 and 1·3 (± 0·01) × 10^7 CFU μg^{-1} for strain NOX. The N_{max} value of strain A3 at 25 and 500 μg acetate-C l^{-1} was 1·1 (± 0·2) × 10^4 CFU ml^{-1} and 1·5 (± 0·01) × 10^4 CFU ml^{-1} compared to 0·8 (± 0·3) × 10^4 in the blank. Thus, strain A3 utilized a negligible amount of acetate, demonstrating that growth of the organism in the tap water with strain P17 or strain NOX was caused by compounds originating from these two strains. From the proportional relationships and the average N_{max} value of strain A3 on 10 μg carbohydrate-C l^{-1} [1·6 (± 0·3) × 10^5 CFU ml^{-1}], it was estimated that the amount of excreted compounds utilized by strain A3 was about 7 μg carbohydrate-C equivalents per 100 μg acetate-C utilized by either strain P17 or strain NOX.

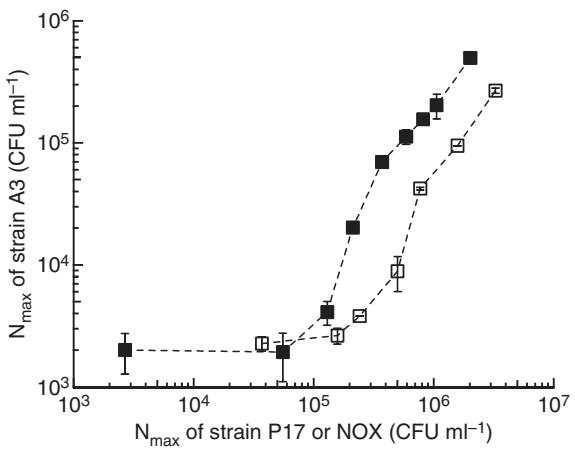

Figure 2 Growth of *Flavobacterium johnsoniae* strain A3 in tap water with *Pseudomonas fluorescens* strain P17 or *Spirillum* sp. strain NOX grown at different acetate concentrations. Symbols: (■) strain A3 grown with strain P17; (□) strain A3 grown with strain NOX. Error bars indicate standard deviation of N_{max} in duplicate flasks.

Growth of *F. johnsoniae* strain A3 in surface water

Growth of strain A3 with strain P17 was tested in surface water that contained 15 μg chlorophyll-a l^{-1} and was collected from a freshwater canal to determine to what extent strain A3 utilized compounds that were not utilized by strain P17. Growth of strain P17 and strain A3 in the surface water was readily promoted, and N_{max} values were reached within 7 days of incubation. Strain

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Table 6 Growth of *Flavobacterium johnsoniae* strain A3 and *Pseudomonas fluorescens* strain P17 in surface water

Substrate and strains added	N_{max} (± std) of strain P17 (CFU ml^{-1})	N_{max} (± std) of strain A3 (CFU ml^{-1})
Blank + strain A3	–	1·4 (± 0·01) × 10^6
Blank + strain P17	1·6 (± 0·02) × 10^5	–
Blank + strain P17 + strain A3	1·6 (± 0·09) × 10^5	1·2 (± 0·1) × 10^6
30 μg amylopectin –C l^{-1} + strain P17 + strain A3	1·5 (± 0·03) × 10^5	1·5 (± 0·1) × 10^6

P17 reached the same N_{max} value in the surface water with and without strain A3, whereas strain A3 reached the same N_{max} value in the surface water with and without strain P17 (Table 6). The N_{max} value of strain A3 in coculture with strain P17 was higher with 30 μg C l^{-1} of added amylopectin than without. From the N_{max} value of strain P17, it was calculated that the amount of AOC available for strain P17 in the surface water was 39 μg acetate-C equivalents l^{-1}, whereas the N_{max} value of strain A3 grown in the surface water with strain P17 corresponded with *c.* 75 μg carbohydrate-C equivalents l^{-1}. Hence, strain A3 utilized a significant amount of AOC in the surface water tested that strain P17 was unable to utilize.

Discussion

Involvement of *Flavobacterium* species in the degradation of polysaccharides

In our study, six of the eight bacterial isolates were identified as representatives of the genus *Flavobacterium*. *Flavobacterium* species cultured from natural waters are known to degrade a variety of polysaccharides at concentrations of a few grams per litre of growth medium (Humphry *et al.* 2001; Bernadet and Bowman 2006). The ecological significance of tests for growth at high concentrations of polysaccharides is limited, because NOM concentrations in aquatic environments typically range between 0·5 and 100 mg C l^{-1} (Frimmel 1998), and polysaccharides represent only a fraction of the total NOM concentration (Croué *et al.* 1999). Studies have shown that bacteria of the *Cytophaga-Flavobacteria* group can account for ≥ 30% of all heterotrophic bacteria present during periods of phytoplankton growth (e.g. seasonal blooms) in natural waters (Riemann *et al.* 2000; Eiler and Bertilsson 2004, 2007). During these phytoplankton blooms, relatively large amounts of high-molecular extracellular and intracellular compounds, mainly polysaccharides, are released (Myklestad 2000; Børsheim *et al.* 2005). Several studies have shown that bacteria of the *Cytophaga-Flavobacteria* group contribute to the degradation of these phytoplanktonic polysaccharides (Cottrell and Kirchman 2000; Elifantz *et al.* 2005). *Flavobacterium* species commonly occur in treated water (Norton and LeChevallier 2000), and *Flavobacterium* species that are able to utilize low concentrations of polysaccharides (≤ 100 μg l^{-1}) have been isolated from tap water before (Van der Kooij and Hijnen 1981, 1983). The isolation of the *Flavobacterium* strains in our study confirms that Flavobacteria are involved in the degradation of polysaccharides at microgram-per-litre levels in freshwater.

Growth of *F. johnsoniae* strain A3 at low concentrations of oligo- and polysaccharides

All mono- and oligosaccharides, sugar acids and sugar alcohols tested in our study as growth substrates for strain A3 can diffuse through the porins of the outer membrane, which have a size exclusion limit of about 700 Da (Nikaido 2003). However, strain A3 utilized only fructose, and 6 of the 12 oligosaccharides tested, suggesting that substrate-specific transport systems are required to transport these compounds across the cytoplasmic membrane.

Strain A3 also utilized a variety of polysaccharides, and phenotypic characterization tests have shown that *F. johnsoniae* produces extracellular enzymes to degrade various polysaccharides and proteins (Bernadet and Bowman 2006). Our results imply that oligosaccharides are the main products of polysaccharide degradation by strain A3. The organism reached an average N_{max} value of 1·3 × 10^5 CFU ml^{-1} with amylopectin, galactan, laminarin, xylan and xyloglucan at 10 μg C l^{-1}, indicating that strain A3 may have high-affinity transport systems for the oligosaccharides that are released upon degradation of these polysaccharides. The lower N_{max} values observed with gellan gum, pectin and rhamnogalacturonan suggest that strain A3 either was unable to break certain linkages in these polysaccharides or could not transport certain degradation products across its cytoplasmic membrane. Investigations on the molecular level could elucidate the processes involved in the utilization of polysaccharides by strain A3, but were beyond the scope of our study.

High molecular organic compounds in aquatic environments are usually considered to be more resistant to bacterial degradation than low molecular weight compounds

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(Sundh 1992). The ability to specifically utilize high molecular compounds in oligotrophic environments could enable strain A3 to successfully compete with bacteria that can only utilize low molecular compounds. Growth of strain A3 in the surface water tested did not promote the growth of strain P17, but it remains unclear whether the degradation of high-molecular compounds by strain A3 may also be beneficial to other bacteria.

Utilization of phytoplanktonic and bacterial polysaccharides by *F. johnsoniae* strain A3

The various polysaccharides tested in our study as growth substrates for strain A3 were selected to reflect the structural diversity of natural polysaccharides that could be present in natural and treated water. Extracellular, storage and cell wall polysaccharides produced by phytoplankton are assumed to be the most important natural polysaccharides in surface water used for drinking water production (Croué *et al.* 1999). Many of the EPS produced by phytoplankton are species-specific heteropolymers with a complex chemical structure including multiple types of monosaccharides and glycosidic linkages (Myklestad 1995; Paulsen *et al.* 1998; Giroldo and Vieira 2005). Furthermore, a wide range of storage and cell wall polysaccharides can be found among phytoplankton as well (Painter 1983; Biersmith and Benner 1998; Sigee 2005). Hence, surface water may contain a large variety of phytoplanktonic polysaccharides, especially during seasonal phytoplanktonic blooms. The polysaccharide fraction will partially be removed during water treatment, but treated water may still contain polysaccharides of phytoplanktonic origin. Furthermore, bacteria can release EPS in treated water particularly from biofilms formed in water treatment processes and distribution systems. Like phytoplanktonic EPS, bacterial EPS display considerable structural heterogeneity (Kumar *et al.* 2007).

Based on the structure of the polysaccharides tested as substrate for growth of strain A3, it appears that the organism can utilize homopolymers of glucose, xylose or galactose and heteropolymers with varying proportions of glucose, xylose, galactose, rhamnose, arabinose, galacturonic acid and glucuronic acid. The main linkages between the monosaccharide constituents in the polysaccharides that strain A3 utilized are α-(1 $\rightarrow$ 2)-, β-(1 $\rightarrow$ 3)-, α/β-(1 $\rightarrow$ 4)- and α/β-(1 $\rightarrow$ 6)-. These monosaccharide constituents and linkage patterns are present in a large fraction of the storage and cell wall polysaccharides produced by phytoplankton and occur in phytoplanktonic and bacterial EPS as well. Hence, strain A3 appears to be able to utilize a significant proportion of these natural polysaccharides at microgram-per-litre levels.

Application of *F. johnsoniae* strain A3 for the quantification of oligo- and polysaccharides

Chemical analytical methods for quantification of natural oligo- and polysaccharides at concentrations $\leq$ 100 μg l^{-1} in freshwater appear to be unavailable (Croué *et al.* 1999). Recently, a test has been developed that determines the AOC concentration in a water sample using a natural bacterial consortium from treated water as inoculum and flow cytometry for enumeration (Hammes and Egli 2005). This test is less time-consuming and more automated than the original AOC test, but does not differentiate between the NOM fractions utilized. The original AOC test differentiates two NOM fractions because of the use of two specialized bacterial strains, strain P17 and strain NOX, which are particularly proficient in the utilization of amino acids and carboxylic acids, respectively (Van der Kooij *et al.* 1982; Van der Kooij and Hijnen 1984). However, strain P17 and strain NOX cannot utilize oligo- and polysaccharides at low concentrations. Strain A3 is capable of utilizing a variety of oligo- and polysaccharides and may have potential to be used for the quantification of these compounds in freshwater. Strain A3 can also utilize amino acids, but strain P17 utilizes these compounds much more rapidly than strain A3. Consequently, growth of strain A3 on amino acids can be prevented when strain P17 is used simultaneously in a water sample.

Organic compounds produced by strain P17 and strain NOX enhanced the growth of strain A3 in tap water in which strain P17 had grown to N_{max} levels $\geq$ 2·1 ($\pm$ 0·05) $\times$ 10^5 CFU ml^{-1} or strain NOX to $\geq$ 5·0 ($\pm$ 0·6) $\times$ 10^5 CFU ml^{-1} using acetate. The nature of the organic compounds produced by strain P17 and strain NOX has not yet been elucidated. The growth-promoting organic compounds originating from strain P17 may have been EPS, which can be produced by *P. fluorescens* strains (Hung *et al.* 2005; Kives *et al.* 2006). Strain P17 and strain NOX produced the growth-promoting organic compounds at a ratio of *c.* 7 μg carbohydrate-C equivalents per 100 μg acetate-C. Thus, the total biomass production on acetate was significantly increased by growth of strain A3. Cell densities of biofilms and sediments formed during water treatment and distribution typically range from 10^5 cells cm^{-2} to more than 10^7 cells cm^{-2} (Boe-Hansen *et al.* 2003; Van der Kooij *et al.* 2003). Consequently, bacteria in these environments may benefit from the growth of other bacteria by highly efficient utilization of excreted growth-promoting compounds.

N_{max} values of strain P17 and strain NOX in tap water are usually lower than 2 $\times$ 10^4 and 2 $\times$ 10^5 CFU ml^{-1} (Van der Kooij 1992), respectively, and therefore, strain P17 and strain NOX will not promote growth of strain

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A3 in tap water. Furthermore, growth of strain A3 was not promoted by strain P17 at 1·6 ($\pm$ 0·09) $\times$ 10^5 CFU ml^{-1} in the surface water from a freshwater canal (Table 6). A significant amount of AOC was available for strain A3 in the surface water in comparison with the amount of AOC available for strain P17. Similarly, AOC was available for strain A3 in the tap water tested in the presence of strain P17. The metabolic capacities of strain A3 suggest that the compounds promoting the growth of the organism in the surface and tap water may have included oligo- and/or polysaccharides, but strain A3 could (also) have utilized other compounds (e.g. proteins). Determination of the nature of the compounds available for strain A3 in various types of natural and treated water requires further investigation. Nevertheless, growth of strain A3 can be used as an indicator for the presence of biodegradable, natural oligo- and polysaccharides at microgram-per-litre levels in freshwater.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Growth of *Flavobacterium johnsoniae* strain A3 in tap water supplemented with a mixture of 21 amino acids, a mixture of 6 carbohydrates, or both mixtures in the absence and presence of *Pseudomonas fluorescens* strain P17. Symbols: (●) blank, no organic compounds added; (○) carbohydrate mixture added; (▲) amino acid mixture added; (□) both mixtures added; (■) both mixtures and strain P17 added. Individual compound concentration was 10 μg C l^{-1}. Error bars indicate standard deviation of colony count in duplicate flasks.

Figure S2 Growth of *P. fluorescens* strain P17 in tap water supplemented with a mixture of 21 amino acids, a mixture of 6 carbohydrates, or both mixtures in the absence and presence of *F. johnsoniae* strain A3. Symbols: (●) blank, no organic compounds added; (○) carbohydrate mixture added; (Δ) amino acid mixture added; (□) both mixtures added; (■) both mixtures and strain A3 added. Individual compound concentration was 10 μg C l^{-1}. Error bars indicate standard deviation of colony count in duplicate flasks.

Figure S3 Growth of *F. johnsoniae* strain A3 in (●) tap water supplemented with strain P17, and (○) in tap water supplemented with a mixture of 21 amino acids and strain P17. Error bars indicate standard deviation of colony count in duplicate flasks.

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Ammonia-Oxidizing Bacteria and Archaea in Groundwater Treatment and Drinking Water Distribution Systems▽†

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The ammonia-oxidizing prokaryote (AOP) community in three groundwater treatment plants and connected distribution systems was analyzed by quantitative real-time PCR and sequence analysis targeting the *amoA* gene of ammonia-oxidizing bacteria (AOB) and archaea (AOA). Results demonstrated that AOB and AOA numbers increased during biological filtration of ammonia-rich anoxic groundwater, and AOP were responsible for ammonium removal during treatment. In one of the treatment trains at plant C, ammonia removal correlated significantly with AOA numbers but not with AOB numbers. Thus, AOA were responsible for ammonia removal in water treatment at one of the studied plants. Furthermore, an observed negative correlation between the dissolved organic carbon (DOC) concentration in the water and AOA numbers suggests that high DOC levels might reduce growth of AOA. AOP entered the distribution system in numbers ranging from 1.5×10^3 to 6.5×10^4 AOPs ml^{-1}. These numbers did not change during transport in the distribution system despite the absence of a disinfectant residual. Thus, inactive AOP biomass does not seem to be degraded by heterotrophic microorganisms in the distribution system. We conclude from our results that AOA can be commonly present in distribution systems and groundwater treatment, where they can be responsible for the removal of ammonia.

Ammonia can be present in source water used for drinking water production or added to treated water with chlorine to form chloramines as a disinfectant. However, the presence of ammonia in drinking water is undesirable because nitrification might lead to toxic levels of nitrite (29) or adverse effects on water taste and odor (4) and might increase heterotrophic bacteria, including opportunistic pathogens (29). Two-thirds of the drinking water in The Netherlands is produced from groundwater. Most of the groundwater used for drinking water production is anoxic with relatively high concentrations of methane, iron, manganese, dissolved organic carbon (DOC), and ammonia. Treatment of anoxic groundwater aims at achieving biologically stable water, because drinking water in The Netherlands is distributed without a disinfectant residual. As a result, a highly efficient nitrification process during rapid medium filtration is required.

Nitrification is the microbial oxidation of ammonia to nitrate and consists of two processes: the oxidation of ammonia to nitrite by ammonia-oxidizing prokaryotes (AOP) and the oxidation of nitrite to nitrate by nitrite-oxidizing bacteria (NOB). Recently it was shown that in addition to bacteria, archaea also are capable of ammonia oxidation (13). Since then, ammonia-oxidizing archaea (AOA) have been found in many different ecosystems, including wastewater treatment systems (10, 20, 24). However, it is currently unknown if AOA are present in drinking water treatment processes and distribution systems. Recent studies have focused on nitrification in drinking water treatment (16, 28). In those studies, AOB and NOB were enumerated by traditional most-probable-number (MPN) methods using selective liquid media. However, MPN methods are time-consuming and underestimate the numbers of AOP and NOB (3). Quantitative real-time PCR has been used to quantify AOB in drinking water (12) and might be a useful tool for quantifying AOB and AOA in drinking water.

In our study, a real-time PCR method targeting the *amoA* gene of AOB or AOA was developed to quantify numbers of AOP in drinking water. This real-time PCR method was used together with a phylogenetic analysis of the *amoA* gene of AOB and AOA to do the following: (i) determine the treatment steps where AOP dominates in the groundwater treatment train of three drinking water production plants in The Netherlands, (ii) quantify the AOP entering the distribution system and determine the fate of AOP during transport in the distribution system, and (iii) elucidate the role of AOA in nitrification during drinking water treatment and in distribution systems.

MATERIALS AND METHODS

Samples. Water samples were taken from drinking water treatment and the distribution systems of water treatment plants A, B, and C. The oxic groundwater of plant A is treated with limestone filtration. The treatment train of the anoxic groundwater of plant B consists of plate aeration, first rapid sand filtration, tower aeration, water softening, and second rapid sand filtration. Treatment plant C uses anoxic groundwater from a deep and middle-deep aquifer. The treatment train of the middle-deep groundwater consists of tower aeration, first rapid sand filtration, $KMnO_4$ dosing, second rapid sand filtration, granular activated carbon (GAC) filtration, and UV. The treatment train of the deep groundwater consists of tower aeration, water softening, HCl dosing, and rapid multimedium (sand/anthracite) filtration. In general, 100 ml of raw water, 100 ml of water after each treatment step, and 100 ml of finished water were taken at all three treatment plants. The distribution systems of plants A, B, and C were analyzed by sampling 100 ml water at the tap of three different house addresses at the proximal, central, and distal parts of the distribution system (resulting in a total of nine

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samples per distribution system). All water samples were stored at 4°C and processed within 24 h.

DNA isolation. In general, 100 ml of a water sample was filtrated over a 25-mm polycarbonate filter (0.22-μm pore size, type GTTP; Millipore, The Netherlands). The filter and a DNA fragment of an internal control were added to phosphate and MT buffer of the FastDNA Spin kit for soil (Qbiogene) and stored at −20°C. The internal control was used to determine the PCR efficiency. DNA was isolated using the FastDNA Spin kit for soil according to the supplier's protocol.

Real-time PCR for *amoA* genes of AOB and AOA. The *amoA* genes of AOB and AOA were amplified with previously described primers (10, 22). Reaction mixtures of 50 μl contained 25 μl of 2× IQTM SYBR green supermix (Bio-Rad Laboratories BV, The Netherlands), 10 pmol of forward and reverse primer, and 20 μg bovine serum albumin. Amplification, detection, and data analysis were performed in an iCycler IQ real-time detection system (Bio-Rad laboratories BV, The Netherlands). The amplification program used was as follows: 95°C for 3 min; 40 cycles of 20 s at 95°C, 1 min at 60°C, and 1 min at 72°C. The PCR cycle after which the fluorescence signal of the amplified DNA is detected (threshold cycle [C_T]) was used to quantify the concentration of AOB and AOA *amoA* gene copies. Quantification was based on comparison of the sample C_T value with the C_T values of a calibration curve based on known copy numbers of the *amoA* gene of AOB or AOA. The AOB numbers were calculated by assuming two *amoA* gene copy numbers per cell (5) and the AOA numbers by assuming one *amoA* gene copy number per cell (18).

Phylogenetic analyses. Phylogenetic analyses of the *amoA* genes of AOB and AOA were performed on water sampled at the treatment train and distribution system of groundwater treatment plants A, B, and C. DNA was isolated from the water samples, and the *amoA* genes of AOB and AOA were amplified as described above. PCR products were purified using the Qiaquick PCR purification kit (Qiagen, The Netherlands) according to the supplier's protocol. Subsequently, PCR products were cloned into *Escherichia coli* JM109 by using the pGEM-T Easy Vector system (Promega, The Netherlands). Inserts were amplified with pGEM T Easy Vector-specific primers T7 and SP6, using the following PCR conditions: 94°C for 5 min; 30 cycles of 94°C for 20 s, 48°C for 45 s, and 72°C for 45 s; and finally 72°C for 7 min. About 48 PCR products of the *amoA* gene of AOB and 48 PCR products of the *amoA* gene of AOA with the correct insert size from each sample were sequenced using pGEM-T Easy Vector primer T7 (Baseclear, The Netherlands).

The computer program DOTUR was used to determine *amoA* gene sequences with more than 98% sequence similarity (23), at which sequences were considered to belong to the same operational taxonomic unit (OTU) (10). Similarity between samples was determined by comparing OTU presence and abundance using the Morisita index for similarity (27, 30). The *amoA* nucleotide sequences were translated into amino acid sequences, aligned by ClustalX, and manually checked. Sequences were compared with the GenBank data library using protein-protein and nucleotide-nucleotide BLAST to obtain the nearest phylogenetic relatives. Subsequently, evolutionary distances of the AmoA protein sequences were calculated using the Kimura correction model. Neighbor-joining trees with 1,000 bootstrap replicates were constructed using the computer program TreeConW. Chimeras were detected by comparing AmoA protein trees based on the first 100 to 200 bp and the last 100 to 200 bp. These trees gave the same phylogenetic structure, except for one bacterial AmoA protein sequence, which was omitted from the results.

Chemical analyses. The ATP concentration was determined in water samples by measuring the amount of light produced in the luciferin-luciferase assay. A nucleotide-releasing buffer (Celsis) was added to the water sample to release ATP from the cells. The intensity of the emitted light was measured in a Celsis Advance luminometer calibrated with solutions of free ATP (Celsis) in autoclaved tap water according to the supplier's protocol. Orthophosphate was determined colorimetrically using the ascorbic acid method (ISO 6878:2004), ammonium was determined colorimetrically using the Kjeldahl method (according to the Dutch standard method NEN 6576), and DOC was measured using an infrared gas analyzer according to ISO 8245:1999.

Statistical analyses. The data for AOB and AOA numbers in three parts of the distribution system (proximal, central, and distal) were statistically analyzed. Analysis of variance (ANOVA) using general linear models with Bonferonni post hoc testing was used to determine differences between locations in the distribution net. Differences were considered statistically significant at P values of <0.05. All statistical procedures were calculated using the SPSS 16.0 software program.

Nucleotide sequence accession numbers. *amoA* gene sequences from this study are deposited in the GenBank library under accession numbers EU852659 to EU852722.

RESULTS

Real-time PCR. A calibration curve with 10-fold dilutions of known copy numbers of the *amoA* gene of AOB and AOA was constructed to quantify the numbers of AOB and AOA by real-time PCR. Calibration curves for the *amoA* genes of AOB and AOA resulted in log linear relationships (with an r^2 value above 0.99) between copy numbers and the C_T in the range of 10 to 10^6 *amoA* gene copy numbers. The real-time PCR for the *amoA* genes of AOB and AOA showed high PCR efficiencies: 95.5% for the *amoA* gene of AOB and 94.8% for the *amoA* gene of AOA. Consequently, the developed real-time PCR methods for the *amoA* genes of AOB and AOA can be used to quantify AOB and AOA in drinking water samples.

AOP in groundwater treatment plants. The chemical composition of the (an)oxic groundwater extracted by water treatment plants A, B, and C is shown in Table S1 in the supplemental material. Water treatment plant A extracts oxic groundwater with an ammonia concentration below the detection limit of 0.05 mg liter^{-1}. The AOB numbers in the water sampled from the treatment train were low or not detectable (Fig. 1A). In contrast, AOA numbers were relatively high (1,000 times higher than those of AOB) and were not reduced by limestone treatment (Fig. 1A).

Besides 3.4 mg liter^{-1} ammonia, the raw water of treatment plant B contains total organic carbon, Fe, Mn, and CH_4 as well. During treatment, AOP reduce ammonia to concentrations below 0.05 mg liter^{-1}. AOB were present in raw water at low numbers, and numbers increased in water sampled after plate aeration, the first rapid sand filter, tower aeration, and softening (Fig. 1A). In water sampled after the second rapid sand filter and in finished water, AOB numbers were lower, although numbers remained relatively high. AOA were not detected in water sampled after the first two treatment steps. In water sampled after tower aeration, low AOA numbers were present, and these numbers decreased in water sampled after softening and the second sand filter (Fig. 1A).

Groundwater treatment plant C extracts water from middle-deep and deep aquifer layers. Groundwater from these two depths differs in its geochemistry, with middle-deep groundwater having higher total organic carbon, Fe, and Mn concentrations. Both middle-deep and deep groundwater has an ammonia concentration of ~1.0 mg liter^{-1}, and this concentration is reduced to below 0.05 mg liter^{-1} in finished water. Raw water from the deep and middle-deep aquifer is anoxic, and consequently, AOB and AOA numbers were low or below the detection limit in raw water. In the treatment train of middle-deep extracted groundwater, the AOA numbers remained relatively stable until the UV treatment. Directly after UV treatment, AOA were no longer detected in the water (Fig. 1B). AOB numbers increased until the second sand filter but decreased during the last two treatment steps. For deep groundwater treatment, the number of AOB increased after tower aeration but remained relatively constant through the rest of the treatment train (Fig. 1B). In contrast, AOA increased until the multimedium filtration step. The finished water (a mix of treated middle-deep and deep groundwater) contained high AOB and AOA numbers.

The ammonia concentration removed at each treatment step in plant C was analyzed as well. The statistical characteristics of

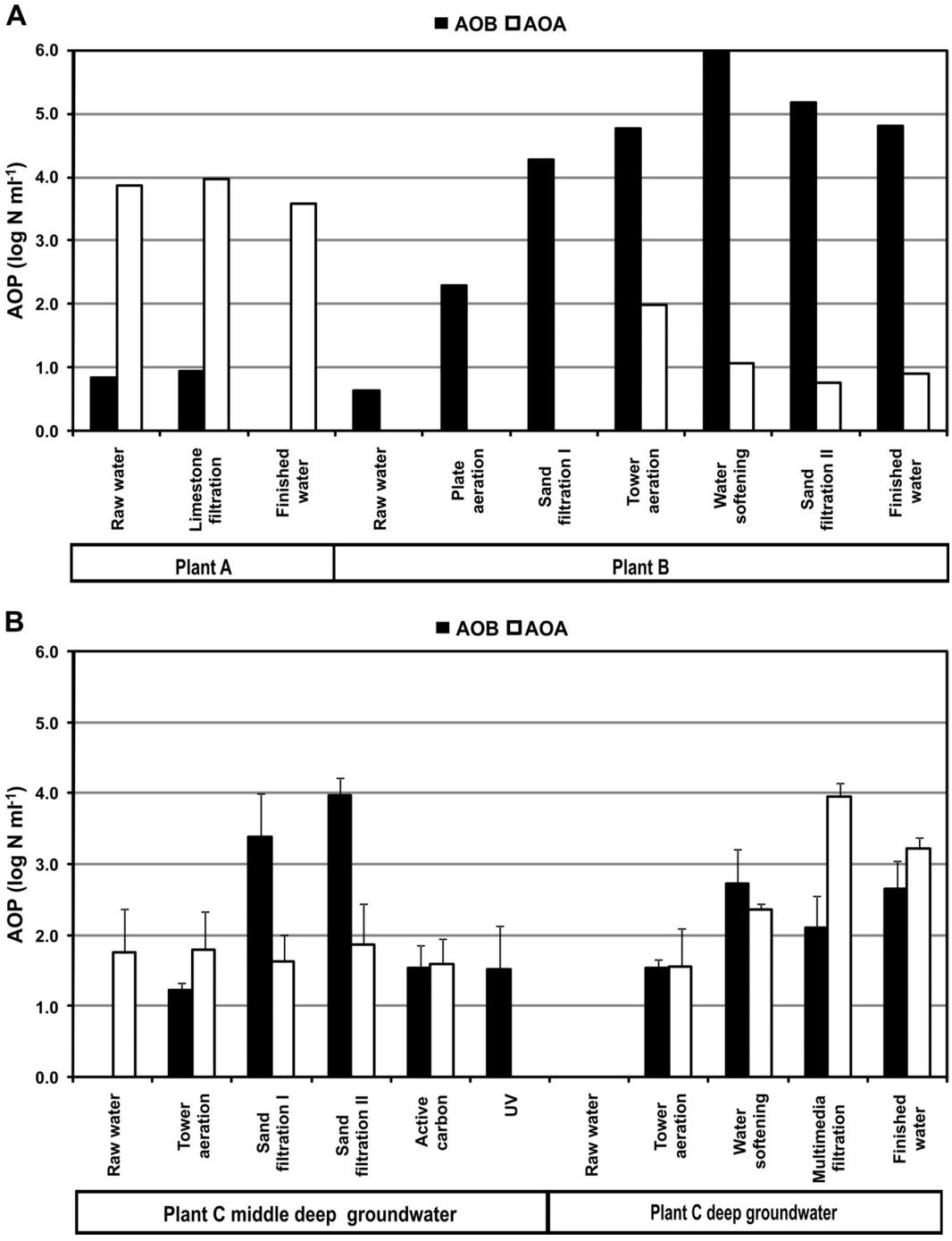

FIG. 1. The number of AOB and AOA in water sampled after different treatment stages at water treatment plants A and B (A) or at the middle-deep and deep extracted groundwater of water treatment plant C (B). Values for plants A and B were based on duplicate samples taken on a single day. Values for plant C were average numbers for duplicate samples taken on three different days during 2008.

the correlations between ammonia removal at a specific treatment step and the number of AOP, AOB, or AOA in the water sampled directly after that treatment step are shown in Table 1. The ammonia concentration removed at each treatment step showed a significant and strong correlation with the log number of AOP in the water sampled after the treatment step. Thus, the AOP determined by the real-time PCR analysis were responsible for ammonia degradation in the treatment trains of plant C. For AOB, a strong, significant correlation was obtained with ammonia removal during treatment processes in the middle-deep extracted groundwater train (Table 1). No significant correlation was observed between AOB and ammonia removal in the treatment of deep extracted groundwater. In contrast, ammonia removal during treatment of the deep extracted groundwater correlated strongly with log numbers of AOA (Table 1). We conclude from these results that AOB are

TABLE 1. Statistical characteristics of the correlations between the amount of ammonia removed at a treatment step and the log number of AOP, AOB, or AOA in water sampled directly after the treatment step at groundwater treatment plant C

Sample type	Parameter 1	Parameter 2	Correlation between parameters	
			P value	r^2 value
Middle-deep and deep extracted groundwater	Ammonia removed (mg liter^{-1})	AOP (log organisms ml^{-1})	<0.01	0.73
		AOB (log organisms ml^{-1})	0.03	0.22
		AOA (log organisms ml^{-1})	<0.01	0.45
Middle-deep extracted groundwater	Ammonia removed (mg liter^{-1})	AOP (log organisms ml^{-1})	<0.01	0.79
		AOB (log organisms ml^{-1})	<0.01	0.78
		AOA (log organisms ml^{-1})	>0.1	
Deep extracted groundwater	Ammonia removed (mg liter^{-1})	AOP (log organisms ml^{-1})	<0.01	0.77
		AOB (log organisms ml^{-1})	>0.1	
		AOA (log organisms ml^{-1})	<0.01	0.86

responsible for the removal of ammonia during treatment of the middle-deep groundwater, whereas AOA degrade ammonia in the treatment train of deep extracted groundwater.

To elucidate possible factors that determine whether AOB or AOA become dominant in the treatment trains of plant C, relationships between DOC, ATP, and orthophosphate and AOB, AOA, and AOP were studied. The DOC concentration in the water that feeds a treatment process where ammonia is removed had a significant positive correlation with AOB numbers in the water sampled directly behind the treatment process ($P < 0.01$; $r^2 = 0.57$), whereas AOA numbers were significantly negatively correlated with the DOC concentration ($P < 0.01$; $r^2 = 0.56$). ATP and orthophosphate concentrations in the water did not show any significant correlation with the number of AOB or AOA.

AOP in distribution systems. AOP were observed in all water samples from the distribution systems of the three water treatment plants (Fig. 2). The AOB numbers were relatively high in distribution water sampled from groundwater treatment plants B and C (Fig. 2B and C). AOB were not observed in water sampled from the distribution system of plant A. In contrast, the AOA numbers were relatively high in water sampled from the distribution system of plant A (Fig. 2A). AOA numbers in the distribution system of plants B and C were in general 10 to 100 times lower than those in the distribution system of plant A, but some sample locations in the distribution system of plant B had exceptions: water samples with low numbers of AOB (<1.2 or 1.6 log *n* ml^{-1}) had relatively high numbers of AOA (4.2 or 3.0 log *n* ml^{-1}). As a result, relatively high standard deviations were observed for AOB and AOA in water sampled from the distribution system of plant B.

The AOB and AOA numbers were not significantly different between proximal, central, and distal parts of the distribution system of plants B and C ($P > 0.05$). In the distribution system of plant B, AOB numbers decreased with increasing distance from the treatment plant, whereas AOA numbers slightly increased (Fig. 2B), but these differences were not statistically significant. The AOA numbers increased from proximal to distal parts of the distribution system of plant A (Fig. 2A), and AOA numbers at the distal part of the distribution system were significantly higher than at the proximal part ($P < 0.05$).

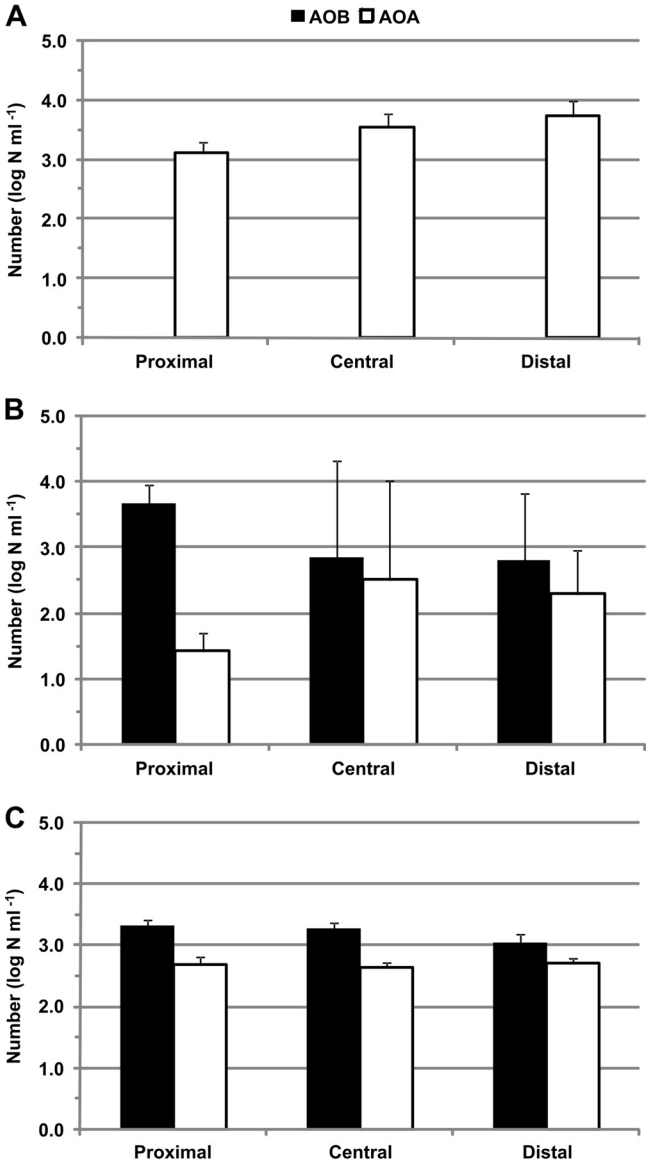

FIG. 2. The average numbers of AOB and AOA in the proximal, central, and distal parts of the distribution system of water treatment plants A, B, and C.

TABLE 2. *amoA* gene sequences, OTU numbers, and Shannon index of diversity[a]

Location	Analysis of:					
	AOB			AOA		
	No. of seq	No. of OTUs	Shannon index	No. of seq	No. of OTUs	Shannon index
Treatment plant A						
Limestone	0	ND	ND	48	10	1.93
Distribution	0	ND	ND	47	8	1.61
Treatment plant B						
Rapid sand filter I	47	7	1.16	ND	ND	ND
Tower aeration	47	1	0	47	1	0
Rapid sand filter II	48	2	0.10	48	4	1.09
Distribution system	47	1	0	48	5	1.33
Treatment plant C						
Rapid sand filter I	ND	ND	ND	48	5	1.13
GAC filter	12	2	0.64	34	4	1.08
Rapid multimedia filter	48	1	0	48	4	0.57
Distribution system	48	1	0	48	3	0.84
Biofilm	47	2	0.10	48	3	0.77

[a] seq, sequences; ND, not detected.

Phylogenetic analyses. The *amoA* gene sequences of AOB revealed a low number of OTUs in samples taken from the treatment train and the distribution system of treatment plants B and C (Table 2). Consequently, the Shannon diversity index was between 0 and 0.64 for most samples (Table 2). The OTU numbers based on the *amoA* gene sequences of AOA were generally higher than those for for AOB. Water sampled after limestone treatment and drinking water in the distribution system at plant A had a relatively high Shannon index, whereas the other samples had an index between 0 and 1.33 (Table 2).

The Morisita similarity index between the AOB communities of most of the analyzed water and biofilm samples was around 1, indicating that these AOB communities had the same composition, irrespective of the water treatment plant (Fig. 3A). The Morisita index between the AOB community of the water sampled after the first rapid sand filter at plant B and the communities of the other samples was slightly lower, whereas the index between the AOB community in water sampled after GAC filtration at plant C and the AOB communities in the other samples was low (Fig. 3A). The Morisita index between the AOA community of water and biofilm sampled from the distribution system of plant C was high (Fig. 3B). Remarkably, the AOA community in water sampled after the rapid multimedium filter at plant C was highly similar to the AOA population in water sampled after tower aeration at plant B. The Morisita similarity index between the AOA communities of samples from plant A was high as well and clustered together in the unweighted pair group method with arithmetic mean analysis. In contrast, the index between AOA *amoA* gene sequences obtained from water sampled at the distribution system of plant B, water sampled after the first rapid sand filtration, and water sampled after GAC filtration of plant C were very low (Fig. 3B).

The dominant AOB *amoA* sequence in most water samples was related to a noncultured *Nitrosomonas* species (Fig. 4A). Apparently, this AOB species is capable of colonizing different

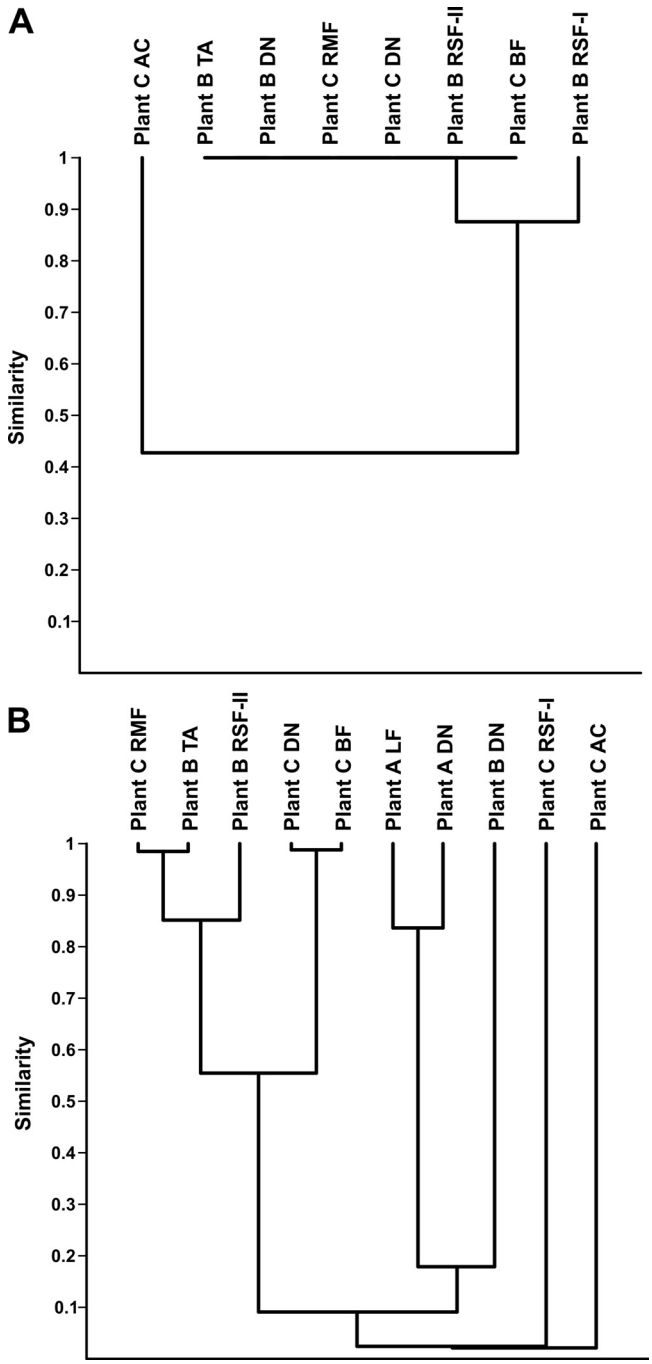

FIG. 3. Unweighted pair group method with arithmetic mean cluster analysis based on the Morisita similarity index between the OTUs of the *amoA* gene sequences of ammonia-oxidizing bacteria (A) or ammonia-oxidizing archaea (B). Abbreviations: AC, GAC filtration; BF, biofilm; DN, distribution system; LF, limestone filtration; RMF, rapid multimedia filter; RSF-I, first rapid sand filter; RSF-II, second rapid sand filter; TA, tower aeration.

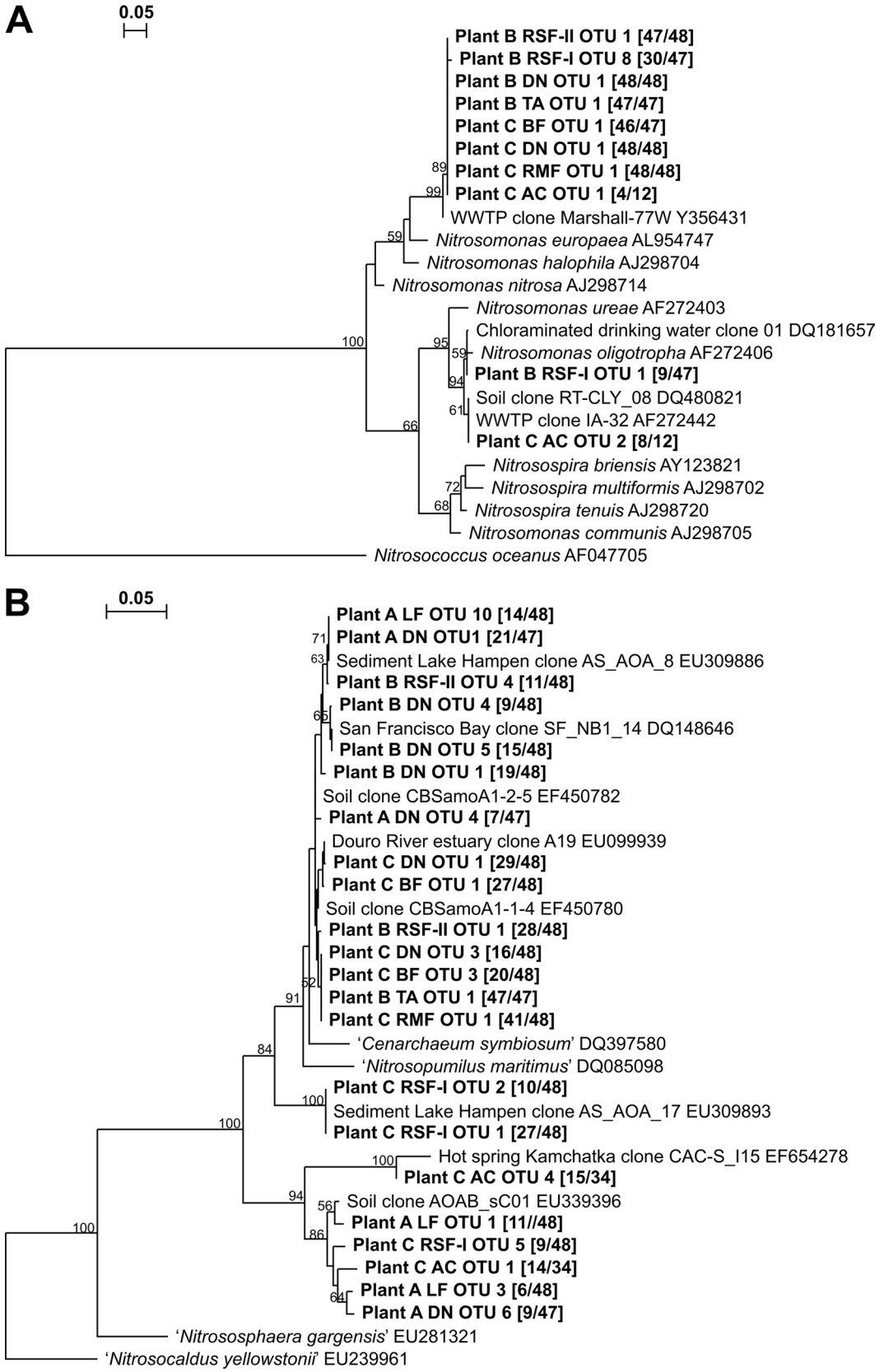

FIG. 4. Phylogenetic tree based on deduced amino acid sequences of the *amoA* gene sequences of ammonia-oxidizing bacteria (A) or archaea (B). Only OTUs that had more than five sequences in a sample are shown. Trees were constructed with the neighbor-joining method using 1,000 bootstrap replicates. Abbreviations are as in Fig. 3. Between brackets: sequence number belonging to that OTU/total sequence number. The bar represents a 5% evolutionary distance, and bootstrap values above 50% are shown. *amoA* gene sequences from this study are deposited in the GenBank library under accession numbers EU852706 to EU852722. "Nitrosopumilus maritimus," "Nitrososphaera gargensis," and "Nitrosocaldus yellowstonii" are "*Candidatus*" names.

water treatment systems and remains dominant in the distribution system (in both water and biofilm) as well. The dominant AOB *amoA* sequence in the water sampled after GAC filtration at plant C was related to sequences obtained from clone libraries constructed from wastewater, soil, and river sediment. Eight *amoA* sequences obtained from water sampled after the first rapid sand filter at plant B belonged to *Nitrosomonas oligotropha* (Fig. 4A). Most of the *amoA* sequences of AOA, obtained from water sampled in the treatment train of middle-deep groundwater at plant C and from water sampled after limestone treatment and in the distribution system of plant A, were related to sequences obtained from clone libraries constructed from soil and a hot spring (Fig. 4B). The remaining *amoA* sequences of AOA were related to sequences obtained from clone libraries constructed from soil, rhizosphere, and sediments from rivers, lakes, and estuaries (Fig. 4B).

DISCUSSION

Quantitative PCR. Recent studies of nitrification in drinking water treatment or distribution systems used culturing methods to determine AOP numbers (16, 28) or fixed nitrifying biomass (14). The AOP numbers were determined with an MPN method using liquid culture medium for nitrifying bacteria. The fixed nitrifying biomass was determined by measuring the appearance rate of nitrite and nitrate produced by prokaryotes in a nitrifier medium. These methods underestimate the AOP number and biomass, because many environmental AOB and AOA are unable to grow in artificial growth medium (3). In addition, the MPN method takes approximately 4 to 9 weeks before MPN tubes can be scored positive or negative for growth (17). Some of the studies recognized these shortcomings, but the lack of an alternative method resulted in the use of the MPN method (6, 16). Moreover, Cunliffe (6) noted the pressing need for a fast quantification method for AOP in chlorinated distribution systems, because rapid diagnosis of increased AOP populations in the distribution system enables process controllers to react quickly and avoid use of excessive free chlorine in the system.

Real-time PCR methods have been used to quantify AOB numbers in drinking water (12). In our study, we developed two real-time PCR methods that quantified *amoA* copies of AOB and AOA in drinking water. The high correlation between *amoA* copy numbers and C_T values and the high PCR efficiencies observed show that the real-time PCR methods are reliable tools for quantifying AOB and AOA in drinking water. Real-time PCR methods are fast and can detect both culturable and nonculturable AOP, which makes the method advantageous compared to culturing methods. Still, PCR methods detect DNA from both live and dead microorganisms, which might constrain viability analyses.

AOP in drinking water treatment and distribution system. In The Netherlands, drinking water is distributed without a disinfectant. Raw water used for drinking water might contain ammonia, which is removed during biological filtration in the treatment plant. This nitrification process occurs at the surface of sand/anthracite or GAC filters (28). Consequently, it is generally assumed that nitrification occurs mainly in those treatment processes that involve biologically active filters (8). In contrast to this assumption, we observed relatively high AOP numbers in water sampled after water softening at plant C and in water sampled after tower aeration and water softening at plant B. The pivotal function of tower aeration is not nitrification but physical removal of carbon dioxide and/or methane from water (8). The higher number of AOP in water sampled after tower aeration and pellet softening than in water from the first rapid sand filter at plant B implies that biological nitrification is limited in the first rapid sand filter. The raw water of plant B contains high concentrations of DOC, methane, iron, and manganese, which might limit nitrification during biological filtration (2, 9).

Most striking was the observation that AOB dominated the AOP fraction in the biological treatment processes in the treatment train of the middle-deep extracted groundwater at plant C, whereas AOA dominated these processes during treatment of the deep extracted groundwater. Correlation studies confirmed that each dominant AOP fraction was responsible for ammonia removal during the biological filtration processes in the respective treatment train. Moreover, the number of AOB was positively correlated with the DOC concentration in the water, whereas AOA numbers correlated negatively with DOC concentrations. These results indicate that a high DOC content in the water might limit the growth of AOA. Treatment plant B, which extracts groundwater with a high DOC concentration as well, also showed low AOA numbers in the treatment train, confirming the correlation results between DOC and AOA at plant C. However, it might be that the correlation between DOC and AOA or AOB numbers is spurious. For instance, not only is the DOC concentration higher in the middle-deep extracted groundwater at plant C and the groundwater extracted at plant B, but iron and manganese concentrations are considerably higher in these water types as well. Furthermore, AOA dominated AOB in soils with relatively low and high water-extractable carbon (15), which demonstrates that the correlation between AOB or AOA does not occur in all environments. Therefore, additional in vitro experiments have to be conducted to elucidate the role of DOC in the establishment of an AOP population in water treatment, but such a study goes beyond the scope of our paper.

Previous studies have shown that MPN counts of AOP increase toward the distal part of the distribution system (16, 17) due to an increased amount of ammonia in chloraminated drinking water (6). Because drinking water is distributed without a disinfection residual in The Netherlands, ammonia concentrations are below the detection limit in the distribution system and growth of AOP will be absent or very low. Indeed, we observed that the numbers of AOB and AOA did not increase along the distribution system of treatment plants B and C. In contrast, the number of AOA in water samples taken from the distal part of the distribution system of plant A was significantly higher than that in water samples taken at the proximal part of the distribution system. Furthermore, the cluster analysis of the AOA OTUs showed that AOA populations in the distribution system were different from AOA populations in the last treatment stages. These observations imply that certain AOA species grew in the distribution system. This was especially unexpected for plant A, because ammonia was not detected in the extracted raw water and the concentration of organic carbon in the finished water was very low, resulting in a high level of biological stability (25). It has not been

extensively tested if the cultivated AOAs described thus far are able to grow on substrates other than ammonia (7, 11, 13). The cultivated AOA "*Candidatus* Nitrosopumilus maritimus" is related to marine group I crenarchaea (13), and other authors have suggested that some crenarchaeal species belonging to marine group I grow heterotrophically (1, 19). Perhaps some of the AOA species present in drinking water can grow on both organic carbon and ammonia, but activity studies have to be performed to prove this hypothesis.

No *Nitrosospira amoA*-like sequences were obtained in our study, demonstrating that the conditions during water treatment favor growth of *Nitrosomonas* species. The same *Nitrosomonas* phylotype dominated most water samples at plants B and C. Apparently, the geographic distance between the two water treatment plants (135 km) and the difference in raw water quality between the two plants did not influence the population structure of AOB, and the conditions at each water treatment plant resulted in the same dominant phylotype. Studies of AOB phylogenetics in distribution systems in the United States and Finland have found a low diversity of AOB as well (16, 21). The obtained dominant AOB genotypes belonged to the *Nitrosomonas oligotropha* cluster (United States) or the *Nitrosomonas marina* cluster (Finland) but not to the *Nitrosomonas europaea* cluster that harbored the dominant phylotype obtained in our study. However, in contrast to our study, the other studies used chloraminated drinking water, which is likely to select for AOB populations that are more resistant to chloramine.

Inactive nitrifying biomass that enters the distribution system might be degraded by heterotrophic bacteria, which could result in biofilm formation and growth of undesired microorganisms (e.g., *Aeromonas* or *Legionella*) (26). Although we showed that AOP biomass enters the distribution system, AOA and AOB numbers were not significantly lower at the distal part of the distribution system. The nitrification in the treatment train of plant B and C seems to be sufficient to prevent growth of nitrifying prokaryotes on ammonia in the distribution system. Moreover, the inactive nitrifying biomass entering the distribution system is not instantly removed by settling or consumption by protozoa.

AOA in drinking water. Only recently it was demonstrated that AOA can be responsible for the oxidation of ammonia (13). Moreover, in several soil samples, AOA were numerically dominant over AOB (15), stressing their importance in some environments. The results from our study demonstrate that AOA can also be present in (drinking) water sampled from the treatment train and distribution system of water treatment plants in The Netherlands. The highest number of AOA was observed at water treatment plant A, and AOA dominated the ammonia-oxidizing population. However, the raw water of plant A has an ammonia concentration below the detection limit (<0.05 mg NH_4 liter^{-1}), and numbers of AOA remain stable during water treatment because nitrification does not occur in the treatment train. As a result, the AOA population in raw and finished water at plant A probably reflects the composition of the ammonia-oxidizing populations in the saturated sandy soil surrounding the extraction well. This explanation is supported by the following: (i) our observation that some of the *amoA* gene sequences of AOA clustered with sequences obtained from clone libraries of soil DNA and (ii) observations that the number of AOA in sandy soils can exceed the number of AOB by a factor of 1,200 (15). Treatment plant C contained approximately equal amounts of AOA and AOB, and numbers increased simultaneously during water treatment, suggesting that these organisms contribute equally to the biological nitrification of ammonia in the treatment trains. Overall, we conclude that AOA can be significant contributors to nitrification in drinking water treatment and can be present in the distribution system.

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36 Nutritional versatility of two *Polaromonas* related bacteria isolated from biological granular activated carbon filters

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Abstract Physiological properties of two bacterial strains isolated from GAC filters and identified as members of the genus *Polaromonas* were investigated in order to characterize the biodegradation processes in GAC filters used in water treatment. The results of batch culture growth experiments with mixed and individual substrates at concentrations typical for drinking water indicate that the two examined *Polaromonas* strains have similar physiological properties. Favourite substrates for growth are carboxylic acids and aromatic acids, while only a few amino acids and carbohydrates are utilized for growth. The maximum growth rate (0.09–0.11 h^{-1}) was reached at low substrate concentrations (10 μg C/l) of acetate. The saturation constant for growth of strain P-315 on acetate is 0.015μM and 0.039 μM for the growth on benzoate indicating a higher affinity of this strain for acetate. These results indicate that biodegradation of low molecular weight organic compounds in GAC filters is possible at very low concentration of substrate. The obtained results can be used to model the biodegradation in GAC filters for examining the effects of operational parameters on the efficiency of the removal of organic compounds by the combined adsorption and biodegradation in GAC filters.

Keywords Water treatment, GAC, biodegradation, *Polaromonas,* saturation constant, growth rate

Introduction

Biodegradation processes in granular activated carbon (GAC) filters are beneficial for the removal of organic compounds in the process of water treatment. The extent of biodegradation in GAC filters is determined by the concentration of active biomass and by the metabolic properties of this biomass for available substrates. For the optimal exploitation of biodegradation in GAC filtration detailed information is needed about quantitative and qualitative properties of biomass in the GAC filters. Several methods are available for the

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assessment of biomass concentration associated with GAC (Magic-Knezev and Van der Kooij 2004, onas strains isolated from biological granular activated carbon filters rioriter-ing van DZH meneenen. Urfer and Huck 2000, Wang *et al.* 1995) but information about metabolic properties of bacteria from GAC is scarce. According to the ATP-based method (Magic-Knezev and Van der Kooij, 2004), 6–125 kg of active biomass can be present in a filter bed of 100 m^3 GAC. Further characterisation and optimisation of biodegradation in GAC filters requires information about the identity and physiological properties of predominant bacteria in GAC filters. In a study that was based on cultivation-dependant techniques, dominant cultivable bacteria in GAC filters used in drinking water treatment were identified as members of the genus *Polaromonas* of the *Comamonadaceae* family (Magic-Knezev *et al* 2003). Very little information about the physiological properties of species belonging to this genus is available. Therefore, the aim of this research was to determine growth characteristics of two strains belonging to the genus *Polaromonas* that were isolated from GAC filters. Experiments were designed to obtain the information about:

1. nature of favourite substrates at concentrations typical for water treatment and
2. kinetic parameters of growth on selected substrates at concentrations typical for water treatment.

The information about the nature of favourite substrates and kinetic parameters of growth for these two strains, together with the information about the concentration of biomass in a GAC filter contributes to the understanding of the removal of organic matter by the biodegradation processes in a GAC filter.

Methods

Bacterial strains

Strain P-315 was isolated from a GAC filter treating surface water that was pre-treated by coagulation and sedimentation followed by rapid sand filtration, softening and ozonation. Strain P-305 was isolated from a GAC filter treating ground water pre-treated with rapid sand filtration and softening.

According to 16S rDNA sequence analysis both strains were identified as members of the genus *Polaromonas*. Closest cultured relative with both strains is *Polaromonas vacuolata* with a sequence similarity >97% (NCBI accession number UAU14585). Although a high level of similarity was observed for 16S rDNA sequence, analysis of genomic DNA with AFPLP fingerprinting (not published) indicated that these two strains belonged to different genotypes.

Inoculum cultures to be used in the growth experiments were prepared by inoculating a 100-ml infusion flask containing basal medium supplemented with 1mg acetate-C /l with a suspension of a fresh culture grown on an R2A agar. Flasks were incubated at 15°C until the maximum colony counts were obtained (4–8 10^6 cfu/ml). These cultures are kept at 4°C.

Growth on mixed substrates

The growth experiments were performed in 1 l glass-stoppered Erlenmeyer flask treated with 550°C for 4 hours to eliminate traces of carbon. The pipettes were cleaned with a 10%

solution of $K_2Cr_2O_7$ in concentrated H_2SO_4, followed by 4 hour rinsing with tap water and heating overnight at 250°C. A total of four different types or organic compounds were used as a substrate for growth: I, 20 amino acids; II, 11 aromatic compounds; III, 17 carboxylic acids and IV, 20 carbohydrates. Solutions were prepared in 600 ml of dune-infiltrated river water treated by powdered activated carbon, followed by softening, rapid and slow sand filtration (assimilable carbon concentration (AOC) <5 μg acetate C/l). Indigenous bacteria were eliminated by pasteurisation of water at 60°C for 30 minutes. Mixtures of substrates were prepared by adding individual substrates from stock solutions, which had been prepared as described previously (Van der Kooij and Hijnen, 1985). The final concentration of each individual substrate in the mixture was 1 μg C/l. Phosphorus (Na_2HPO_4) and nitrogen were added (NH_4Cl) to avoid growth limitation other than by the carbon source. Flasks were inoculated with the pre-cultured suspension of the strains to reach a start concentration of 100–500 CFU/ml. The Erlenmeyer flasks were incubated without shaking in the dark at 15 ± 1°C and growth was monitored by periodic determination of viable cells counts on R2A medium (Oxoid) in triplicate after incubation at 25°C during 5–7 days. Parallel to the determination of viable cell counts, concentrations of ATP were measured. Each mixture or concentrations was tested in duplicate flasks.

Growth on individual substrates

For the preparation of 1 mg C/l solutions autoclaved tap water was supplemented with nitrogen, phosphorous and individual carbon source from the stock solution and inoculated with the 100–1000 CFU of the pre-cultured strain. Growth was carried out in 9-ml test tubes at 15 ± 1°C. The growth was monitored by measurement of the ATP concentration on days 7 and 14 after inoculation. Solutions containing 10 μg C/l of individual substrates were prepared in the same way as described for the solutions of mixed substrate except only one substrate was added per Erlenmeyer flask. Flasks were inoculated with the pre-cultured suspension of strain to reach a start concentration of 100 to 500 CFU/ml and monitoring of growth was identical as in the experiments with mixed substrates. For each substrate duplicate test was performed. The experiments conducted to determine kinetic parameters of growth (growth yield coefficient (Y), maximum growth rate ($1/G_{min}$) and substrate saturation constant K_s) were carried out in treated aerobic ground water containing a very low concentration of assimilable organic carbon. The test water was supplemented with nitrogen, phosphorous and the selected carbon source (acetate or benzoate) at concentrations ranging from 1 to 500 μg C/l. The flasks were incubated at 15 ± 1°C and growth was monitored by periodic determination of viable cell counts on R2A medium (Oxoid) in triplicate after incubation at 25°C during 5–7 days. Parallel to the determination of viable cell counts ATP concentration was measured.

ATP analysis

The measurement of ATP is based on the production of light in the luciferine-luciferase assay. ATP was released from suspended cells with nucleotide-releasing buffer (NRB, Celsis). The intensity of the emitted light was measured in a luminometer (Celsis Advance^{TM}) calibrated with solutions of free ATP (Celsis) in autoclaved tap water following the procedure as given by the manufacturer. The detection limit was 1 ng ATP L^{-1} of sample.

Growth kinetics

The growth yield Y was calculated from the maximum colony counts ($N_{max,}$CFU/ml) obtained for the applied substrate concentration (dS).

$$Y = dN_{max}/dS \qquad \text{(eq. 1)}$$

The doubling time (G in hours) of the cultures was calculated with the equation:

$$G = \log 2(t' - t)/(\log N_{t'} - \log N_t) \qquad \text{(eq. 2)}$$

where $t' - t$ is the incubation time in number of viable cells (N) increased from N_t to $N_{t'}$.

The saturation constant K_s and minimum generation time G_{min} was calculated with Lineweaver-Burk equation:

$$G = G_{min} + G_{min} \cdot K_s/S \qquad \text{(eq. 3)}$$

Where S is the concentration of the added substrate (dS) and the natural substrate concentration (S_n) which was derived from the N_{max} values observed in water without substrate addition. (Van der Kooij and Hijnen, 1985).

Results and discussion

Growth on mixed substrates

The growth on mixed substrate at the concentration of individual substrate of 1 μg C/l is performed for four major groups of organic compounds, vz 20 AA, 11 AR; 17 CA and 11 CH. The growth response is compared to the growth in water without added substrate. The strains responded similarly to substrate mixtures. Both strains showed significant growth in the mixtures of the AA, AR and CA compounds and a low response was observed with CH (Table 1). Best response was observed for the AR mixture indicating a high affinity of the strains for these types of organic compounds.

Growth on individual substrates

The growth of strains on individual carbon sources was tested for two concentrations: 1 mg C/l and 10 μg C/l. The results of the growth experiments on a wide range of individual compounds at a concentration of 1 mg C/l are presented in Table 2.

Table 1 Growth response as the ratio of the maximum concentration of two strains (C_m) in the suspensions of mixed substrates and in blank water(C_b) (R_{CFU} or $R_{ATP} = C_m/C_b$). AA = amino acids; CA = carboxylic acids; AR = aromatic compounds; CH = carbohydrates .

	Strain P-305		Strain P-315	
Mixture of substrates	R_{CFU}	R_{ATP}	R_{CFU}	R_{ATP}
Amino acids (AA)	8.1	6.6	6.8	9.1
Carboxylic acids (CA)	3.8	4.2	6.4	5.7
Aromatic acids (AR)	21.3	7.0	13.9	6.8
Carbohydrates (CH)	1.5	1.9	2.3	2.0

Table 2 Utilisation of individual compounds in drinking water by two *Polaromonas* strains at a concentration of 1 mg/l.

		Number (%) of compounds utilized	
Substrate type	***Number of compounds***	**Strain P-315**	**Strain P-305**
Amino acids (AA)	20	6 (30)	3 (15)
Aromatic compounds (AR)	11	6 (55)	6 (55)
Carboxylic acid (CA)	17	17 (100)	15 (88)
Carbohydrates (CH)	21	3 (14)	5 (24)

The strains showed some differences in response to the substrates from four major groups but both strains grew well on the majority of tested carboxylic acids and aromatic compounds. The utilisation of amino acids and especially grow on carbohydrates was limited to a number of compounds from this two groups. The strain isolated from the filter treating ground water (P-305) had a slightly lower response in terms of yield to individual substrates than the strain P-315 that was isolated from the filter treating surface water. Furthermore, strain P305 grew slower in the water without substrates added.

A number of compounds was selected for further growth experiments at the concentration of 10 μg C/l. Best response was observed for acetic acid, tartaric acid and benzoic acid (data not shown).

Growth kinetics of the *Polaromonas* strains for acetic and benzoic acid

Acetic acid and benzoic acid were selected as substrates for the determination of the kinetic parameters of growth. Acetic acid is a product of ozonation and forms an important part of assimilable organic carbon in drinking water. Benzoic acid was selected to obtain information about the degradation of aromatic compounds which are present in drinking water as a part of natural organic matter (humic and fulvic acid), or as a xenobiotic compounds. Furthermore, kinetic parameters of growth on acetate for *Spirillum sp.* (strain NOX) and *Pseudomonas fluorescens* (strain P17) which are widely applied for the analysis of AOC concentration in treated water, were determined earlier (Van der Kooij et al 1990).

The kinetic parameters of growth were determined for the concentration range 1–500 μg C/l. The growth curves observed in water supplied with substrates demonstrated that N_{max} values and the growth rate depend on the substrate concentration. Both substrates promoted the growth rate and increased the colony counts et al concentrations tested (Figure 1.).

The growth yield coefficients were calculated with maximum values of the ATP concentration and the plate count (CFU) for the applied substrate concentrations. These yield coefficient values differ slightly for the examined substrates and were used to calculate the natural indigenous substrate concentration (S_n) in the blank water (Table 3). The S_n concentration calculated from the yield coefficient derived from the ATP measurement were slightly higher than the concentrations calculated with the yield coefficient derived from the plate counts. The average value of the derived indigenous substrate concentration was used for the correction of S (S_n + dS) in the calculation of G_{min} and K_s with the Lineweaver-Burk equation (eq. 3). A plot of the generation time (G) of strain P-315 versus 1/S gave linear

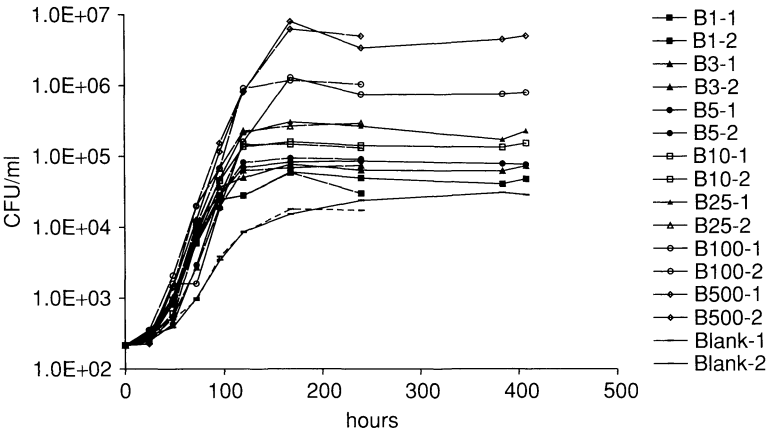

Figure 1 Growth of strain P-315 with different concentrations of benzoate at 15°C.

function for the growth on acetate and a non-linear function for the growth on benzoate (Figure 2).

These results reveal that S_n and added acetate were utilized simultaneously, while added benzoate is utilized after the depletion of S_n. Due to the preferential utilisation of S_n in the solutions with added benzoate, kinetic parameters for growth on benzoate could be determined for the concentrations above 10 μg C/l. The minimum generation time for both substrates was for strain P-315 between 9 and 11 h (0.09-0.11 h^{-1}) but the K_s value for acetate was lower than the K_s value for benzoate (table 4) indicating higher affinity of strain P-315 for acetate than for benzoate.

The observed K_s values are low and similar values have been reported for the growth of *Aeromonas hydrophyla* strain M800 on oleate (Van der Kooij and Hijnen 1988) and for the growth of *Flavobacterium sp.* strain S12 on oligosaccharides (Van der Kooij and Hijnen 1985). Furthermore, a similar saturation constant is reported for growth of a *Mycobacterium sp.* on phenantrene (table 4). Information about the physiological properties of bacteria belonging to the genus *Polaromonas* is scarce. There are only few species described in this genus and one of the species is isolated from a GAC filter treating polluted ground water (Coleman *et al.* 2002). The results of our research indicate that *Polaromonas* strains

Table 3 Growth yield on acetate (Y_{ac}) and benzoate (Y_{ar}) acid and the concentration of indigenous substrate (S_n) derived from the growth yield and the maximum growth in the blank.

Strain	Substrate	Y_{CFU} (CFU/μg C)	$S_{n(CFU)}$ (μg C/l)	Y_{ATP} (ng ATP/μg C)	$S_{n(ATP)}$ (μg C/l)
P- 305	Acetate	2.1 10^7	1.9	0.8	2.1
	Benzoate	1.7 10^7	2.4	0.8	2.1
P- 315	Acetate	1.4 10^7	1.7	0.8	2.4
	Benzoate	1.4 10^7	1.7	1.0	1.9

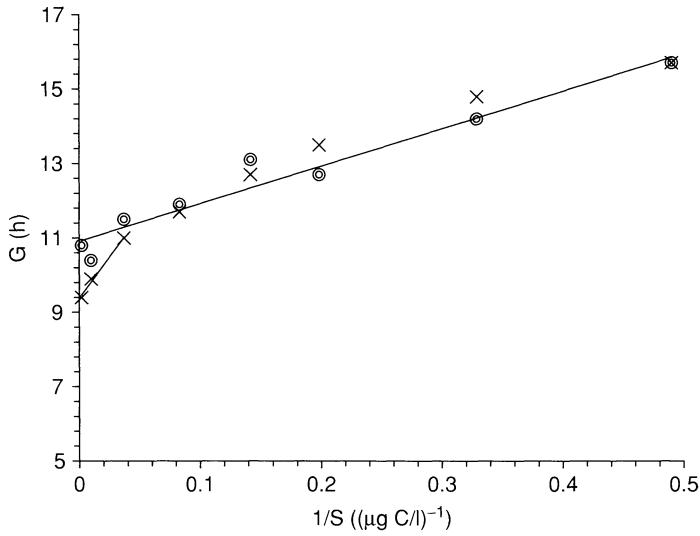

Figure 2 Lineweaver-Burk plot of strain P-315 for growth on acetate (○) and benzoate (×).

isolated from GAC filters are able to grow on very low concentration of carboxylic acids and aromatic compounds (1 µg C/l).

Carboxylic acids, which are the favourite substrates of *Polaromonas* strains, are formed by the oxidation of natural organic matter and are common constituents of organic matter at the end of the treatment train. Their presence in drinking water may contribute significantly to the biofilm formation in distribution systems. Due to their polar nature, carboxylic acids can not be removed by the adsorption on GAC, and their removal by biodegradation results in the improvement of biological stability of drinking water.

Table 4 Saturation constants for growth of some aquatic bacteria on different substrates.

strain	Substrate	K_s (µM)	G_{min} (h)	Reference
Polaromonas P-315	Acetate	0.015	10.9	This research
	Benzoate	0.039	9.4	
Polaromonas	cis-dichloroethene	1.6		Coleman *et al.* (2002)
Mycobacterium sp.	Phenantrene	0.026		Miyata *et al.* (2004)
Aeromonas hydrophyla	Oleate	0.01	4.3	Van der Kooij
Strain M800	Acetate	0.46	6.7	and Hijnen (1988)
Flavobacterium sp.	Maltotetraose	0.019	2.3	Van der Kooij
	Maltotetraose	0.016	2.2	and Hijnen (1985)
	Maltotetraose	0.015	2.2	
Spirillum sp.	Oxalate	0.14	4.1	Van der Kooij (1990)
Pseudomonas fluorescens	Acetate	0.17	5.5	
Pseudomonas spp. GF1TP	Toluene	2.1	2.6	Massol-Deya *et al.* (1997)
Pseudomonas spp. GF161	Toluene	2.7	3.0	
Ralstonia taiwanensis	Phenol	5.5		Chen *et al.* (2004)

The removal of organic compounds in GAC filters is a result of adsorption and biodegradation processes. The removal by the adsorption is determined by the adsorption capacity of GAC and the adsorption kinetic of organic compounds in the water, while the removal by the biodegradation is determined by the concentration of biomass and its metabolic potential for the organic compounds that are available as a substrate. The adsorption capacity of GAC for aromatic compounds can differ significantly, but aromatic compounds are more adsorbable and will therefore occupy the adsorption sites on GAC. When these compounds are biodegraded, adsorption capacity of GAC will be used more optimally for the target compounds that are non-biodegradable and adsorbable resulting in improved performance of a filter and improved water quality. The information about the biodegradation kinetics for carboxylic acids by bacteria from GAC will be used for modeling the removal of these compounds in GAC filters, aiming at optimizing the use of this treatment process.

Conclusions

Two strains of the genus *Polaromonas*, isolated as predominant cultivable bacteria from GAC filters used in water treatment, have a similar metabolic potential and the following physiological properties of these strains were observed:

1. Preferable substrates for growth are carboxylic acids and aromatic compounds while both strains grow only on a limited number of amino acids and carbohydrates;
2. Growth on benzoate and acetate occurs at very low concentration of these substrates (1 μg/l) with relatively low growth rate ($0.09\,h^{-1}$);
3. Higher Ks value for growth on benzoate (0.039 μM) than for acetate (0.015) indicate higher affinity of strain P-315 for acetate than for benzoate;
4. The ATP concentration is suited parameter for determination of bacterial growth yield and for determination of kinetic growth parameters at concentrations >10 μg C/l.

Hence, bacteria in GAC filters used in water treatment are able to utilise biodegradable low molecular weight compounds at very low concentrations, thus contributing to the production of biologically stable drinking water.

Acknowledgements

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Optimisation and significance of ATP analysis for measuring active biomass in granular activated carbon filters used in water treatment

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Abstract

A method for determining the concentration of active microbial biomass in granular activated carbon (GAC) filters used in water treatment was developed to facilitate studies on the interactions between adsorption processes and biological activity in such filters. High-energy sonication at a power input of 40 W was applied to GAC samples for the detachment of biomass which was measured as adenosine triphosphate (ATP). Modelling of biomass removal indicated that a series of six to eight sonication treatments of 2 min each yielded more than 90% of the attached active biomass. The ATP concentrations in 30 different GAC filters at nine treatment plants in The Netherlands ranged from 25 to 5000 ng ATP cm^{-3} GAC, with the highest concentrations at long filter run times and pretreatment with ozone. A similar concentration range was observed in nine rapid sand (RS) filters. ATP concentrations correlated significantly ($p<0.05$) with total direct bacterial cell counts in each of these filter types, but the median value of the ATP content per cell in GAC filters (2.1×10^{-8} ng ATP/cell) was much lower than in the RS filters (3.6×10^{-7} ng ATP/cell). Average biofilm concentrations ranging from 500 to 10^5 pg ATP cm^{-2} were calculated assuming spherical shapes for the GAC particles but values were about 20 times lower when the surface of pores >1 μm diameter is included in these calculations. The quantitative biomass analysis with ATP enables direct comparisons with biofilm concentrations reported for spiral wound membranes used in water treatment, for distribution system pipes and other aquatic environments.
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Keywords: Granular activated carbon; Adenosine triphosphate; Biological filtration; Biofilms; Ultrasound

1. Introduction

Granular activated carbon (GAC) filtration is used in water treatment for the removal of organic compounds that cause undesirable colour, odour or taste, and for the removal of pesticides and other xenobiotics. Adsorption and biodegradation occur simultaneously during GAC filtration. A significant part of the costs of GAC filtration in water treatment is caused by the required thermal regeneration. Ozonation increases the biodegradability and reduces the adsorbability of natural organic matter (NOM) dissolved in water (Sontheimer et al., 1978; Van Leeuwen et al., 1985;

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Nomenclature

ATP = adenosine triphosphate
CFU = colony-forming units
DOC = dissolved organic carbon
GAC = granular activated carbon
HES = high-energy sonication
HPC = heterotrophic plate count
LES = low-energy sonication
NOM = natural dissolved organic matter
NOX = *Spirillum* sp. strain NOX
P17 = Pseudomonas fluorescens strain P17
TDC = total direct cell count
UST = ultrasonic treatment

Carlson et al., 1996). Consequently, preozonation enhances the microbial activity in GAC filters and delays the occupation of adsorption sites by NOM, resulting in postponed regeneration of GAC for the removal of persistent xenobiotics (Orlandini, 1999). More detailed knowledge about the interactions between treatment conditions and biodegradation in GAC filters may lead to a more efficient use of GAC filters in water treatment. A method for determining the concentration of active biomass on the surface of GAC was developed as a first step toward elucidation of these interactions.

A variety of techniques has been used to assess the activity or the concentrations of bacteria present in GAC filters, e.g. oxygen consumption (Klotz et al., 1975; Van der Kooij, 1983; Urfer and Huck, 2000), heterotrophic plate counts (Klotz, 1979; Camper et al., 1985), total direct cell counts (DiGiano et al., 1992), scanning electron microscopy (Weber et al., 1978; Cairo et al., 1979), phospholipids (Wang et al., 1995; Carlson et al., 1996), uptake of labelled substrates (White et al., 1977, Servais et al., 1994) and reduction of 2-(p-iodo-phenyl)-3(p-nitrophenyl)-3 phenyl tetrazolium chloride (INT) (Fonseca et al. 2001), respectively. Factors hampering the general application of one of these methods include the laborious nature of the analytical procedure and difficulties with the interpretation of the data: e.g. oxygen uptake by activated carbon itself, the large percentage of viable but uncultivable microorganisms and no differentiation between viable and non-active bacteria, respectively. This paper describes an ATP-based method for determining the concentration of the active biomass in GAC filters. ATP was selected because of the speed and accuracy of the analysis and its general use in aquatic microbiology (Karl, 1980; Webster et al., 1985; Van der Kooij et al., 1995). The microorganisms were removed from GAC surface with ultrasound, a technique that is often used for the detachment of particle-associated microorganisms and biofilms (Mathieu et al. 1993; Van der Kooij et al., 1995). However, sonication can inactivate microorganisms (Hua and Thompson, 2000) and therefore the effects of ultrasonic treatment on the ATP concentration and cultivability of pure cultures and detached biofilm bacteria were investigated in this study.

The main objectives of this investigation were:

1. To optimise ultrasonic treatment for the removal of bacteria from GAC in combination with the use of ATP analysis for biomass quantification;
2. To determine the relationship between ATP concentrations and selected biomass parameters (HPC, TDC) in GAC filter beds and obtain information about effects of process conditions on the biomass concentration in these filters. For this purpose a limited survey on biomass concentrations in GAC filters, rapid sand (RS) filters and slow sand (SS) filters as applied in water treatment was conducted.

2. Materials and methods

2.1. Method development

Method development was conducted in four phases: (i) determination of the effects of ultrasound on the ATP concentration and cultivability with selected pure cultures of aquatic bacteria (*Spirillum* sp., strain NOX and *Pseudomonas fluorescens*, strain P17) and suspensions of mixed bacterial communities detached from GAC samples. The suspensions were treated (10 times) with ultrasound and after each treatment ATP and cultivability were determined. Untreated suspensions served as a control; (ii) determination of the effect of the adsorptive properties of GAC and the release of fines on the concentration of free ATP. Free ATP was added to a suspension of virgin GAC or GAC with developed biomass and incubated for 2 h. A solution of free ATP in autoclaved tap water was used as control; (iii) testing of the efficiency of the sonication treatment to remove attached bacteria with bacterial biofilms on plasticised polyvinylchloride (PVCp), GAC and sand. The materials were subsequently treated up to 20 times with ultrasound at different power inputs and after each treatment biomass removal was measured as the ATP concentration. The ATP yield was plotted against the number of treatments; (iv) from the results of these experiments an optimal method for the removal and

measurement of active biomass was defined and applied to a series of samples from different filter beds.

2.2. Samples from filter beds

Samples of GAC, sand and anthracite were obtained from nine water treatment plants in The Netherlands. Filter material (10–100 g) was collected from below the filter bed surface (2–10 cm) with a properly cleaned multisampler (Eijkelkamp) and stored in 100 cm^3 screw-capped borosilicate flasks in the filtrate of the sampled unit at 4 °C for a maximum period of 36 h before the analysis. The specific density of the filter material was calculated from the weight of 50 cm^3 of GAC, sand or anthracite that had been dried for four hours at 105 °C. The volume is determined with a measuring cylinder (100 cm^3). Prior to its use in experiments, virgin carbon (Norit 0.8 ROW Supra) was kept in demineralised water for 48 h to allow saturation with oxygen. Subsequently, acid (1 M HCl) was added to obtain pH 8.

2.3. Calculation of external surface of GAC

Data for the external surface of the GAC particles assuming spherical shapes were derived from the literature (Sontheimer et al., 1985). The estimation of the surface of the pores with a diameter $>1\,\mu m$ is based on data provided by the manufacturer about GAC porosity as based on penetration of butan at increasing pressure (Capelle and Vooys, 1983) and the assumption that pores had a cylindrical shape.

2.4. Test strains, mixed GAC community and stationary biofilm

Spirillum sp. strain NOX and *Pseudomonas fluorescens* strain P17 were obtained from the stationary growth phase of AOC tests in treated water as described earlier (Van der Kooij, 1992). A mixed microbial community as present on GAC was obtained with a 2 min low-energy sonication (LES) treatment of a GAC sample (2 g wet weight) in 50 cm^3 autoclaved tap water. Subsequently, the suspended bacteria were separated from the GAC particles and kept in autoclaved tap water for 4 h at 20 °C to allow adaptation to the new environment. A biofilm was grown on pieces (20 cm^2) of plasticized PVC (PVC-P) incubated for 10 days at 15 °C in 600 cm^3 of tap water inoculated with 1 cm^3 of filtered river water (1.2 μm membrane filter) and enriched with nitrogen and phosphorus to prevent growth limitation (Van der Kooij and Veenendaal, 2001).

2.5. Ultrasonic treatment

A total of two to five gram of wet filter material was added to 50 cm^3 of autoclaved tap water (pH 8.4 ± 0.2) in a 100 cm^3 screw-capped borosilicate flask. Sonication was applied for 2 min. A volume of 5 cm^3 of the obtained suspension was collected and kept on ice for examination. The surplus liquid was decanted, 50 cm^3 autoclaved tap water was added and sonication was repeated. This procedure was repeated several times depending on the objective of the experiment. LES was applied with a Branson sonication unit 5050 at a constant frequency of 43 kHz and 180 W power output. Samples contained in 50 cm^3 of autoclaved tap water were placed in the sonication chamber with 10 L of demineralised water. High-energy sonication (HES) was applied with a Sonifier II W-250 at a constant frequency of 20 kHz and an adjustable power output. Ultrasound was applied to the sample via a titanium microtip ($D = 5$ mm), with power inputs ranging from 10 to 40 W.

2.6. ATP measurement

The measurement of ATP is based on the production of light in the luciferine-luciferase assay. ATP was released from suspended cells with nucleotide-releasing buffer (NRB, Celsis). The intensity of the emitted light was measured in a luminometer (Celsis Advance™) calibrated with solutions of free ATP (Celsis) in autoclaved tap water following the procedure as given by the manufacturer. The detection limit was 1 ng ATP L^{-1} of sample. Standard additions of free ATP dissolved in autoclaved tap water were used for recovery experiments.

The influence of the adsorptive properties of GAC on the ATP measurement was tested by addition of free ATP to suspensions of virgin and preloaded GAC in autoclaved tap water (0.04–0.1 g GAC cm^{-3}, dry weight). The concentrations of added ATP were of 2, 100, 600 and 3000 ng L^{-1}, respectively. Autoclaved tap water with similar concentrations of free ATP was used as control. Recovery of added ATP was calculated from the measured ATP concentrations in the controls and in samples, which were corrected for the concentration of autochthonous ATP. The influence of carbon fines on the efficiency of light measurement was determined with GAC sampled from a filter bed and suspended in autoclaved tap water at concentrations of 0.01–0.3 g GAC cm^{-3} (dry weight) and subsequently sonicated. The liquid phase with GAC fines was separated from the GAC particles by decantation. After the measurement of autochthonous ATP, free ATP was added and the recovery was calculated from the measured values in the samples and the control solutions, respectively.

2.7. Total direct cell count TDC

A defined volume of the microbial suspension as obtained by sonication of GAC samples was filtered

through a 0.22 μm polycarbonate filter, stained with acridine orange as described by Hobbie et al. (1977) and observed with epifluorescence microscopy (1000 x, Leica DM RXA). A total of ten random fields, each containing 20–200 cells, were analysed.

2.8. Heterotrophic plate counts HPC

Volumes of 0.05 cm^3 of appropriate decimal dilutions of microbial suspensions obtained by sonication of GAC, sand or anthracite in autoclaved tap water were spread in triplicate over the surface of R_2A agar (Oxoid Ltd.) plates, which were incubated during 10 days at 25 °C.

2.9. Statistic analysis

The statistical significance of the effects of ultrasonic treatment on microbial ATP and cultivability was determined by a *t*-test after correction of the data for the values measured in the controls.

3. Results

3.1. Effects of sonication on microbial ATP and cultivability

The concentration of ATP in a suspension of NOX and P17 grown in treated water and in suspensions of the microbial community obtained from GAC samples ($n = 3$) remained unaffected (102±2%) after ten LES treatments of 2 min each. This treatment also had no effect on the HPC values in these samples (105±15%). Ten HES treatments of 2 min each at power inputs of 20 and 40 W also did not significantly affect the ATP concentration in microbial suspensions of NOX and P17 and mixed microbial communities obtained from GAC (recovery > 90%; $n = 4$). HPC values increased after the first few treatments at both power inputs but further sonication obviously damaged the bacteria. This damage occurred after four to six treatments in the samples treated with 20 W and after two to four treatments in the samples that were treated with 40 W. Typical examples of this effect are shown in Fig. 1. The decrease of the HPC values following HES treatment fitted to an exponential function:

$$B_T = B_0 \cdot e^{-k \times t}, \quad (1)$$

where B_0 is the HPC value in the untreated sample, and k (min^{-1}) is the coefficient of exponential decrease. Values of k for strain NOX, strain P17 and a mixed microbial community obtained from a GAC filter were calculated from the effects of ten subsequent HES treatments at power inputs of 20 and 40 W (Table 1). From these values it can be derived that the HPC values

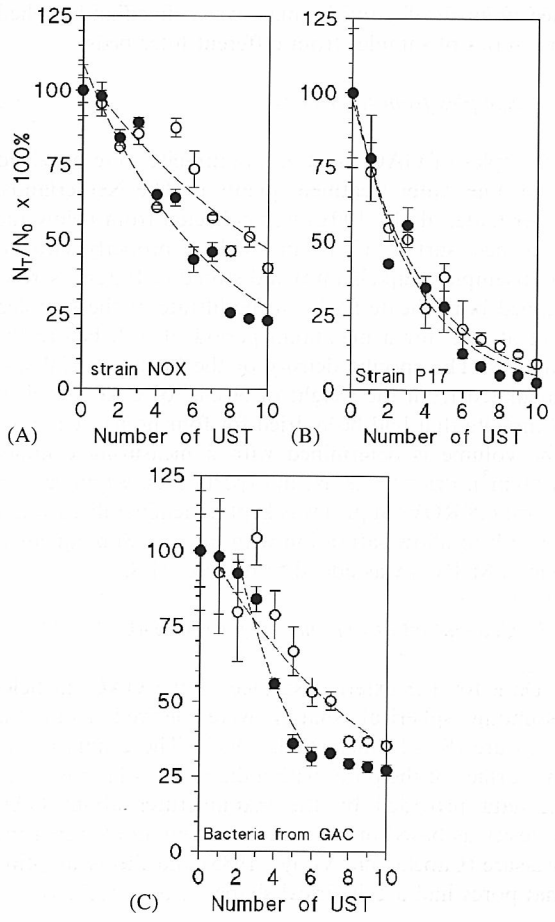

Fig. 1. Effect of HES treatment on the HPC values of strain NOX and strain P17 grown in tap water and a mixed microbial community obtained from a GAC filter. Abbreviations: N_0, concentration at start (time zero, untreated sample); N_T, concentration at time T. Symbols: ○, 20 W; ●, 40 W.

of strain NOX and the mixed microbial community decreased with 1–6% for each minute of HES treatment in most cases but with strain P17 also decreases of 15–20% were observed (Fig. 1b). HES treatment affected strain P17 significantly ($p < 0.05$) more than strain NOX and the mixed microbial community from GAC. Furthermore, treatment at 40 W gave significantly larger k values than treatment at 20 W ($p < 0.05$).

3.2. Stability of ATP in the presence of GAC

The effect of the adsorptive properties of GAC on the concentration of free ATP was tested with the internal standard addition method. The average recovery of free ATP was 104±7% ($n = 8$) 5 min after the addition to tap water suspensions of virgin GAC and GAC from a filter bed. Two hours after the addition, the recovery of free ATP was 74±13% ($n = 4$) for virgin GAC and

Table 1
Coefficients of exponential decrease (k, min^{-1}) of the cultivability of strain NOX, strain P17 and a mixed microbial community obtained from a GAC filter, after ten subsequent HES treatments at two power inputs.

Organism	High energy sonication (HES)	
	20 W	40 W
Spirillum sp. strain NOX	0.018 ± 0.006 ($r^2 = 0.59$)	0.064 ± 0.006 ($r^2 = 0.95$)
	0.0287 ± 0.002 ($r^2 = 0.96$)	0.041 ± 0.004 ($r^2 = 0.94$)
	0.041 ± 0.01 ($r^2 = 0,82$)	0.078 ± 0.01 ($r^2 = 0.93$)
Pseudomonas fluorescens strain P17	0.040 ± 0.007 ($r^2 = 0.82$)	0.071 ± 0.012 ($r^2 = 0.85$)
	0.163 ± 0.006 ($r^2 = 0.99$)	0.226 ± 0.03 ($r^2 = 0.94$)
	0.122 ± 0.01 ($r^2 = 0.97$)	0.147 ± 0.02 ($r^2 = 0.95$)
Mixed microbial community from GAC	0.0066 ± 0.004 ($r^2 = 0.59$)	0.035 ± 0.002 ($r^2 = 0.99$)
	0.054 ± 0.01 ($r^2 = 0.94$)	0.101 ± 0.02 ($r^2 = 0.82$)

$91 \pm 12\%$ ($n = 4$) for GAC from a filter bed. The average recovery of ATP observed after standard addition ($97 \pm 3\%$; $n = 48$) further demonstrated that carbon fines produced during sonication of GAC had no significant effect on the ATP measurement.

3.3. Removal of microorganisms from GAC

Three samples from GAC filters and two sand samples from RSF filters were added to autoclaved tap water (0.1–0.3 g filter material cm^{-3}) and subjected to LES or HES at 40 W for 2 min in a series of 6–20 treatments of 2 min each. The cumulative ATP yield of the biomass obtained with LES was $50 \pm 8\%$ ($n = 5$) of the biomass obtained with HES. GAC samples from two different filter beds and samples of PVC-P with attached microorganisms were treated with HES at power inputs of 10–40 W. Maximum removals were achieved at power inputs above 20 W (Fig. 2a). Subsequently, the effects of sequential HES treatments at 40 W on biomass removal was determined for GAC sampled from seven different filter beds. Four samples had ATP yields below 1000 ng ATP cm^{-3} of GAC and in 3 samples the ATP yield varied between 1000 and 3200 ng ATP cm^3 GAC. Typical examples of cumulative ATP yields are shown in Fig. 2b. The data fitted well with the saturation kinetics as described by the Michaelis–Menten equation ($0.91 \leqslant r^2 \leqslant 0.99$; $n = 7$). In 5 of 7 samples the cumulative ATP yield as measured after six treatments was $90 \pm 3\%$ of the maximum values calculated with this equation. GAC particles that had been sonicated ten times at 40 W cm^{-3} were stained with acridine orange and observed using fluorescence microscopy. The untreated particles emitted a high level of fluorescence but no fluorescent objects were observed on the treated particles

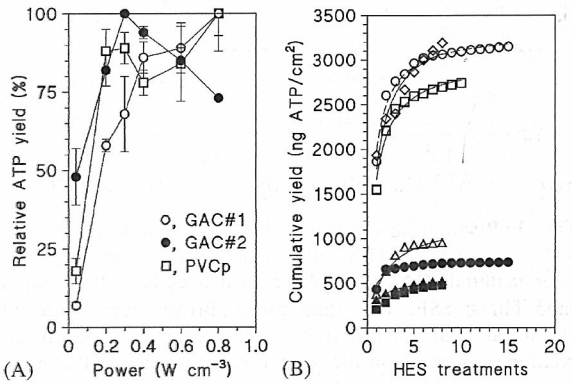

Fig. 2. A. Removal of attached bacteria from GAC and plasticised (PVC) PVCp at different power inputs of HES; B. Cumulative ATP-yield as a function of the number of sonications in seven different GAC samples. The relative ATP yield (A) maximum observed value at applied power range.

(data not shown). Obviously, ten treatments had removed almost all attached biomass.

3.4. Active biomass in GAC filters, rapid sand filters and slow sand filters

From a total of nine water treatment plants in The Netherlands samples were collected from 30 different GAC filters, nine rapid sand filters (RSF) and three slow sand filters (SSF) and analysed for ATP, HPC and TDC, respectively. These samples were selected to include different conditions of filtration, viz. raw water source, DOC concentration, pretreatment, filter run time, filtration rate and carbon type (Table 2). Measurements on GAC samples were all conducted in duplicate. The median value of the variation coefficient for the ATP analysis (6%) was slightly lower than the variation

Table 2
Operational parameters of filtration units and ranges of biomass concentrations on filter material

Filter type (number of samples)	Run time (days)	Contact time (min)	Filtration rate ($m\,h^{-1}$)	DOC ($mg\,l^{-1}$)	ATP ($ng\,cm^{-3}$)	HPC ($CFU\,cm^{-3}$)	TDC ($Cells\,cm^{-3}$)
SSF ($n=3$)	675–2000	30–240	0.25–0.5	1.4–3.2	18–93	$4\,10^5$–5×10^5	3×10^7–2×10^8
RSF ($n=9$)	820–7300	5–20	3–11	2.0–3.2	16–2592	$4\,10^5$–1×10^8	3×10^7–2×10^{10}
GAC($n=30$)[a]	7–1580	10–45	3–10	1.8–5.4	24–5067	1×10^7–1×10^9	1×10^9–4×10^{10}

[a]number of samples tested for ATP concentration

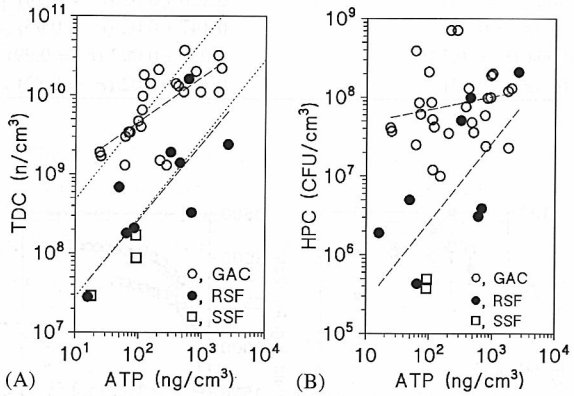

Fig. 3. Relationships between the ATP concentrations and TDC values (A) and HPC values (B), respectively in samples of filter material. GAC, granular activated carbon; RFS, rapid sand filters; SSF, slow sand filters. Broken lines represent calculated relationships; dotted lines represent proportional relationships based on the median values of the ATP content per cell (A).

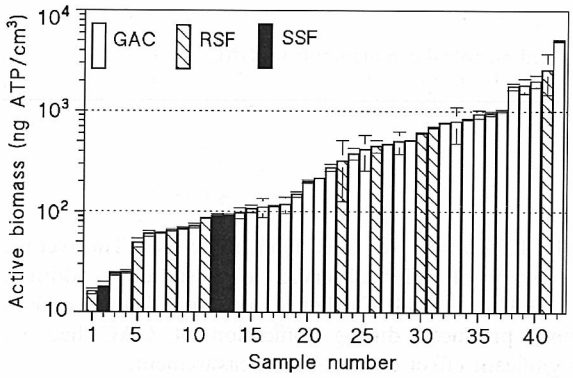

Fig. 4. Active-biomass concentrations in GAC, RSF and SSF samples from 9 water treatment plants in the Netherlands.

coefficients for TDC (14%) and HPC (15%), respectively. Significant correlations were observed between values of ATP and TDC ($p<0.05$) in GAC filters and in RS filters ($p<0.05$), respectively, but not between values of ATP and HPC (Fig. 3).

The ATP concentrations in the GAC samples ranged from 25 to 5000 $ng\,cm^{-3}$, thus covering more than two orders of magnitude. The ATP concentrations of the RSF samples also covered a wide range but values were below 100 ng ATP cm^{-3} in the SSF samples (Fig. 4). ATP concentrations below 100 $ng\,cm^{-3}$ were observed in GAC filters that had been in operation for a few weeks (samples 3 and 4). After 330 days the ATP concentration in these filters had increased tenfold (samples 21 and 22). The ATP concentration in GAC filters supplied with ozonated water (samples 15 and 40) was about 2 to 3 times higher than in filters operating under similar conditions but supplied with non-ozonated water (samples 7 and 32). At two of the nine investigated locations, two-stage GAC filtration was applied. The ATP concentration in the first stage filters at both locations (samples 20 and 37) was higher than in the second stage filters (samples 19 and 18).

4. Discussion

4.1. Method development

Removal of attached biomass from the GAC surface and its subsequent quantification by ATP are essential steps in the determination of the concentration of active biomass on GAC with the method developed in this study. LES treatment did not affect the ATP concentration and cultivability of the sonicated bacteria but was clearly less effective in biomass detachment than HES treatment. The first HES treatment incidentally caused some increase of the ATP concentration but subsequent sonications slightly decreased the ATP concentration, with the total decrease remaining less than 10% after ten HES treatments. The slight initial increase of the ATP concentration and the HPC values after HES treatment may be attributed to a disintegration of clusters of bacteria during sonication and a subsequent better performance of nucleotide releasing reagent on individual bacteria. However, further HES treatment clearly damaged bacteria, as is demonstrated by the decreasing HPC values with increasing number of sonications

(Fig. 1). The effect depends on the nature of the bacteria and the intensity of sonication, with HES treatments at 40 W damaging significantly more bacteria than treatment at 20 W (Table 1). The observed inactivation of strain NOX and the mixed microbial community of 3–9% for each minute of HES treatment at 40 W agrees well with the inactivation (7–10%) reported for *Escherichia coli* for each minute of HES treatment (Hua and Thompson, 2000).

Both virgin and colonised GAC did not effect the concentration of added ATP when the measurement was performed 5 min after addition. This observation demonstrates that free ATP (i) is relatively stable in the presence of GAC, (ii) does not adsorb rapidly onto GAC and (iii) is not rapidly utilised by the attached microorganisms. Also carbon fines eroding from GAC during sonication did not have a significant effect on the light yield at the applied sample volumes. The removal of attached bacteria depends on the power input of ultrasound. Maximum removal of attached bacteria from GAC is obtained with HES treatment at power inputs above 20 W (Fig. 2a). Six to eight HES treatments removed about 90% of the maximum biomass yield as calculated with the Michelis–Menten equation (Fig. 2b), but these treatments may significantly affect the cultivability of the bacteria (Fig. 1). Based on these observations the following procedure for removal of bacteria from GAC was established. A total of 2–5 g of wet filter material is added to 50 cm^3 of autoclaved tap water and treated with a series of six HES treatments at 40 W of 2 min each. After each HES treatment, the detached bacteria are separated from the material to avoid the damaging effect of subsequent treatments and stored on ice (<30 min) prior to biomass analysis (ATP, HPC).

4.2. Biomass concentration in GAC filters

The significant correlation with TDC values and the low value of the variation coefficient of the ATP measurement confirmed that ATP is a suited parameter for the quantification of active biomass in GAC filters. Attractive properties of the ATP analysis include the short time required for analysis, the low detection level and the knowledge about the ecological and physiological significance of ATP Limitations of the parameter are the variations of the ATP content of cells depending on the growth phase and temperature and the sensitivity of the enzymatic reaction for certain inorganic compounds, e.g. copper and calcium (Karl, 1980). Hence, the analytical procedures must be conducted under strictly defined conditions.

The concentrations of ATP in most GAC samples in this study were clearly higher than the values (60–200 ng ATP cm^{-3} GAC) reported by Van Leeuwen et al. (1985). The ATP concentration of 80 ng ATP g^{-1} dry weight sand as reported for an SSF sample (Seger and Rothman, 1996) was similar to the values found in this study. The low biomass concentrations in SSF can be attributed to the low load with biodegradable compounds, viz. a low filtration rate (0.25–0.5 m h^{-1}) in combination with a low concentration of biodegradable compounds in the influents of these filters which are at the end of the treatment chain. The similarities between concentrations of active biomass in GAC and RS filters may be explained by similarities in operational process parameters viz. filtration rate (3–10 m h^{-1}) and water quality. This study showed that GAC filters supplied with ozonated water contained about 2 to 3 times more active biomass than filters fed with non-ozonated water operating under the same hydraulic conditions. This observation is consistent with the observation that biomass levels as determined with INT in biofilter systems operated with ozonated water were about 50% higher than in identical systems operating with non-ozonated water. (Fonseca et al., 2001). Furthermore, a 50% reduction of biomass (measured as phospholipids) on GAC was observed after switching from ozonated to non-ozonated the influent water (Wang et al., 1995). Hence, preoxidation, running time and also the position of the filter beds in water treatment are dominant parameters affecting the biomass concentration in GAC filters. Quantification of these effects, and also the impact of carbon type, requires further investigation.

The surface area which is important for the transport of substrate from the water phase to the GAC surface can be estimated at approximately 50–100 $cm^2 g^{-1}$ when spherical shapes are assumed for the GAC particles (Sontheimer et al., 1985). The observed values of 25–5000 ng ATP cm^{-3} of GAC, with an average dry weight of about 0.5 g cm^{-3}, thus correspond with $500–2 \times 10^5$ (median: 10^4) pg ATP cm^{-2} of this surface. These values are in the same range as biomass concentrations in fouling spiral wound membranes used in water treatment and on plastic materials in contact with biologically stable water but much higher than most biofilm concentrations observed in distribution systems in the Netherlands (Van der Kooij et al., 1999; Vrouwenvelder et al., 1998). The concentrations of biomass carbon on GAC, as calculated with the conversion factor C = 250 x ATP (Karl, 1980), ranged from 6.3 to 1250 (median: 80) μg C cm^{-3} of GAC. These values clearly exceed the biomass concentrations previously reported for GAC (<10 μg C cm^{-3}) used in water treatment (Servais et al., 1991) and are also higher than the value for the maximum capacity of GAC for bacterial fixation (20 μg C cm^{-3}) and the maximum adsorption capacity for bacteria (40 μg C cm^{-3}) as defined in the Chabrol model (Billen et al., 1992). Most biomass carbon concentrations estimated for the surface of spherical GAC particles (0.13–50 μg C cm^{-2}) are also higher than those (0.1–1.2 μg C cm^{-2}) reported for

drinking water distribution systems (Niquette et al., 2000). The surface of GAC potentially available to microbial attachment, which includes the surfaces of all pores larger than 1 μm, is about 20 times larger than the surface of the spheres (Wheeler et al., 1983). The average biomass concentrations calculated for this surface (25–10,000 pg ATP cm^{-2}; 0.01–2.5 μg C cm^{-2}) are similar to concentrations observed on surfaces exposed to treated water.

4.3. Relationships between HPC, TDC and ATP analysis

The HPC values in GAC samples measured in this study correspond well with values reported earlier (Klotz et al., 1975, Van der Kooij, 1983; Cairo et al., 1979) and also with the numbers of active bacteria calculated from the respiration of radiolabeled substrate (Servais et al., 1994). Only small fractions of all bacteria (0.02–9%) produced colonies on R_2A medium. The impact of sonication on cultivability is limited in comparison to these low percentages. The observed TDC values (up to 4×10^{10} cells cm^{-3} GAC) are clearly higher than the values obtained with scanning electron microscopy (10^8 cells g^{-1} wet GAC; Klotz, 1979) and fluorescence microscopy (10^9 cells g^{-1} wet GAC; DiGiano et al., 1992). The relatively high TDC values may be explained by the release of microbial cells from the pore surfaces, which are not visible with microscopy. From the TDC values it can be derived that the present cells do not form a real biofilm on the GAC surface. The average coverage of the total accessible surface (up to 4000 cm^2 cm^{-3} GAC) is less than 5% at TDC values below 10^{10} cells cm^{-3}. This low percentage indicates that the growth of bacteria on the GAC surface of the pores larger than 1 μm is limited by the availability of biodegradable compounds.

The average ATP content per cell, which is a measure for metabolic activity, ranged from 7×10^{-9} to 2×10^{-7} ng AT $cell^{-1}$ for the filter bed materials (Table 2). The median ATP content of cells on GAC (2.1×10^{-8} ng ATP $cell^{-1}$) is at the low range of the ATP content reported for groundwater bacteria ($2\ 10^{-8}$–4×10^{-7} ng ATP $cell^{-1}$; Jensen, 1989; Metge et al., 1993) and bacteria on membranes used in water treatment (2×10^{-8}–7×10^{-7} ng $cell^{-1}$; Vrouwenvelder et al., 1998). This median value is also lower than 10^{-7} ng ATP $cell^{-1}$ reported for starving cells (Webster et al., 1985), indicating rather low metabolic activities of the cells on GAC. The median value of the ATP content of the cells in RS filters ($3.6\ 10^{-7}$ ng $cell^{-1}$) was about 10 times higher than the value obtained for the GAC filters. These observations indicate that (i) the cells in GAC filters were smaller and/or less active than those in RS filters and (ii) TDC values are not suited for quantitative assessment of the biomass concentration but give valuable information when used in combination with measurements of ATP or cell size. The high ATP content of the cells in RS filters suggests a relatively high growth rate, which may be explained by the frequent and intensive backwashing of these filters. The effects of the difference in cell activities (and growth rates) between GAC and RS filters on biodegradation processes are not yet clear. The biomass activity in GAC filters will be further investigated by determining the identity and physiological properties of the predominating bacteria.

5. Conclusions

1. HES treatment is more effective than LES treatment for the removal of biomass from GAC; it does not affect the concentration of free ATP, but reduces cultivability. Modeling indicates that more than 90% removal of attached biomass (ATP) is achieved with a series of six to eight HES treatments at 40 W power input.
2. The uptake of free ATP by cells or GAC is negligible in the applied procedure when the analysis is conducted 5 min after sonication and ATP analysis is not affected by carbon fines released during sonication. ATP concentrations in GAC filters and in RS filters correlate significantly with the TDC values in each of these filter types, but the median value of the ATP content of the cells in GAC filters is about 10 times lower than in RS filters;
3. ATP concentrations in GAC filters in water treatment cover a wide range of concentrations. Biomass concentration on GAC was affected by running time and pretreatment with ozone. The concentrations of active biomass on GAC calculated for the total accessible surface are at the same range as concentrations of biofilms in distribution systems, indicating a limited availability of growth substrates.

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Removal of AOC and NOM from water with Nanofiltration

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Abstract Assessment of the utility of nanofiltration (NF) for reducing the microbial growth potential of water requires information about the removal efficiency of low molecular-weight biodegradable organic compounds. For this purpose, the effect of NF on easily assimilable organic carbon (AOC) and NOM was tested using three types of membranes, one with a molecular-weight cut-off (MWCO) of 1000 D and two with MWCO of 200 D, in a single-element test unit (SETU) at different pH values without and with dosage of low concentrations of acetate and ethanol to the feed water. NOM was effectively removed by these membranes at pH 8, but the removal of acetate ranged from very limited (at MWCO of 1000 D) to >90% (at MWCO of 200 D). The membranes with an MWCO of 200 D showed a poor removal of acetate and ethanol at pH 5. One of the membrane types with MWCO of 200 D was also used in a SETU fed with ozonated water with an elevated AOC concentration at pH 8. AOC concentrations were reduced from 67 to 1.8 µg AOC-C/l (site 1) and from 40 to 1.0 µg C/l (site 2), respectively. Also, NOM was largely removed (DOC > 85%, UV absorbance > 92%). Biofouling may however limit the direct application of spiral-wound NF membrane systems on water with elevated AOC concentrations. Consequently, NF may be used after biological filtration to achieve water with a high degree of biological stability.

Keywords AOC, biological stability, nanofiltration, water treatment

Introduction

Continuing discussions about further improvement of drinking water quality and the decreasing costs of membrane technology are stimulating research into the application of membranes in water treatment. In the Netherlands, the water supply companies aim at distributing biologically-stable drinking water without a disinfectant residual (Van der Kooij et al. 1999). Increased concerns about the multiplication of *Legionella* in plumbing systems in buildings and households have raised questions about the effects of water quality on biofilm formation and growth of *Legionella* in such systems. Nanofiltration (NF) removes natural organic matter (NOM) and inorganic compounds, organic and inorganic contaminants and also is a barrier against microorganisms. Hence, a considerable improvement of water quality may be achieved with NF. However, limited information is available on the removal efficiency of NF for biodegradable compounds. Escobar and Randall (1999) reported that NF did not remove AOC from acidified groundwater, although BDOC and DOC concentrations were greatly reduced. Sibille et al. (1997) also observed a considerable BDOC reduction by NF, but still some regrowth occurred in the system supplied with this water.

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To elucidate the effects of NF on concentrations of AOC and NOM three types of commercially available NF membranes with different pore sizes were used in a single element test unit (SETU) with and without dosage of selected compounds to the NF feed water at different pH values. Additionally, the SETU with a selected membrane was also supplied with ozonated water with an elevated AOC concentration in two water treatment plants.

Methods

The AOC test as applied on the feed and product water, is based on determining the maximum colony counts of two selected bacterial cultures growing in a sample of pasteurised water, contained in thoroughly cleaned glass-stoppered Erlenmeyer incubated flasks (in duplicate) at 15°C. The selected bacterial cultures, which had been isolated from drinking water, are *Pseudomonas fluorescens* strain (P17) which can utilise a wide range of organic compounds and *Spirillum* sp. strain NOX, which only utilises organic acids (Van der Kooij and Hijnen, 1984). The AOC concentration is calculated as µg of acetate-C equivalents/l from the maximum colony counts of the individual strains and their yield factor for acetate (Van der Kooij, 1992). Additional water quality parameters included: DOC, UV absorbance (at 254 nm) conductivity and pH. These analyses were conducted with standardised procedures.

The investigations were carried out with three different types (M1, M2 and M3) of spiral-wound NF membranes (Ø 4 inch) in a SETU at a recovery of ± 10% and a flux of ± 20 l/(m^2.h). Water temperature ranged from 12 to 22°C. M1 is a Hydranautics CoRe membrane with a molecular-weight cut-off (MWCO) of 1000 D, M2 is a Trisep TS-80 membrane and M3 is a Koch TFC-S membrane, both with an MWCO of 200 D. The feed water (treated ground water; DOC = 1.9 mg/l) was supplemented with 100 µg C/l of either acetate or ethanol from stock solutions. Before and after 2 hours of dosage, samples were taken of the feed and product water for analysis. The SETU with a selected membrane (M2) was also supplied with ozonated water with an elevated AOC concentration at two water treatment plants. The pre-treatment at one treatment plant (site 1) using river Rhine water was: coagulation, sedimentation, rapid sand filtration, transport, dune passage, abstraction, aeration, rapid sand filtration and ozonation. The ozone dosage was 0.5 to 2.0 g O_3/m^3 at a minimum contact time of 15 minutes. Pre-treatment at site 2 with river Meuse water was: reservoir storage, coagulation, sedimentation, ozonation and dual media filtration (DMF, anthracite and sand). The DMF effluent was fed to the SETU. The ozone dosage was 1 to 2 g O_3/m^3 at a minimum contact time of 7 minutes.

Results

Removal of added biodegradable compounds and NOM

Figure 1 shows the results of the study with dosages of about 100 µg C/l to the feed water of a SETU. The membrane types were all tested with dosages of acetate, but the M2 membrane was also supplied with 100 µg ethanol-C/l. Filtration with the relatively 'open' M1 membrane showed a limited reduction of conductivity (<10%), a reduction of UV absorbance of 70 to 80% and a DOC removal of 77%, respectively The removal of AOC with the M1 membrane also was <50% (Fig. 1). The M2 and M3 membrane types both demonstrated a good conductivity reduction (>92%) and effective removals of UV (>95%), DOC (88% and 91%) and also AOC (90%). Lowering the pH of the feed water from 8 to 5

with the M3 membrane did not affect NOM removal (UV, DOC), but decreased the conductivity reduction to 82% and clearly limited AOC removal (Fig. 1 D). The M2 membrane supplied with ethanol at pH 5 also showed a limited AOC removal but effective removals of DOC, UV, and conductivity, respectively,. Experiments with ethanol at pH 8 were not performed.

Comparison of the feed and product water characteristics prior and during addition of 100 µg C/l showed similar values for DOC, conductivity and UV-absorbance. AOC concentrations of the feed water prior to dosage ranged from 3 to 14 µg C/l and concentrations in the product water ranged from 0.7 to 2 µg C/l, respectively. Dosage of acetate and ethanol gave elevated concentrations of AOC (≥6.7 µg C/l) in the product, demonstrating that part of the dosed compounds passed the membrane.

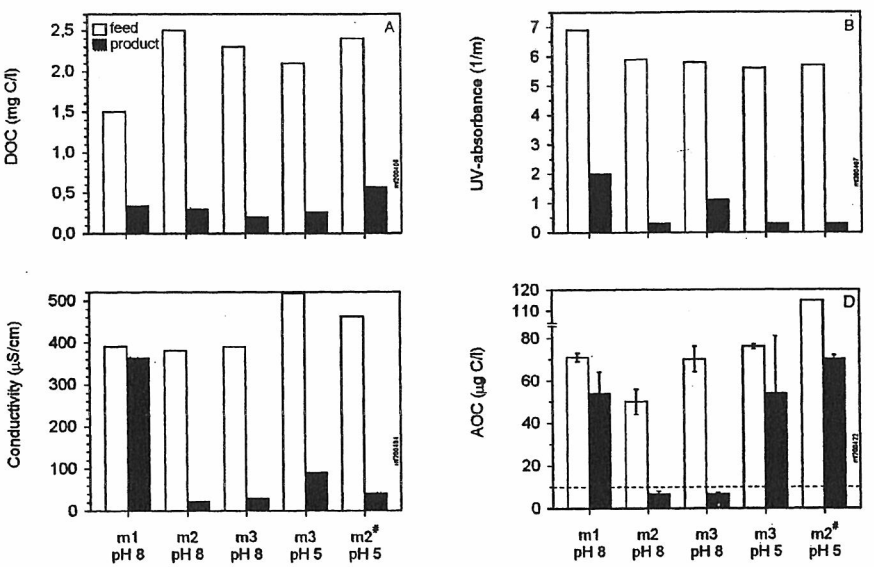

Figure 1: Effects of three NF membrane types (M1, M2 and M3) on selected water quality parameters at pH 8 and 5. (A) Dissolved organic carbon (DOC); (B) UV absorbance at 254 nm; (C) conductivity, and (D) assimilable organic carbon (AOC). # dosage of 100 µg ethanol C/l.

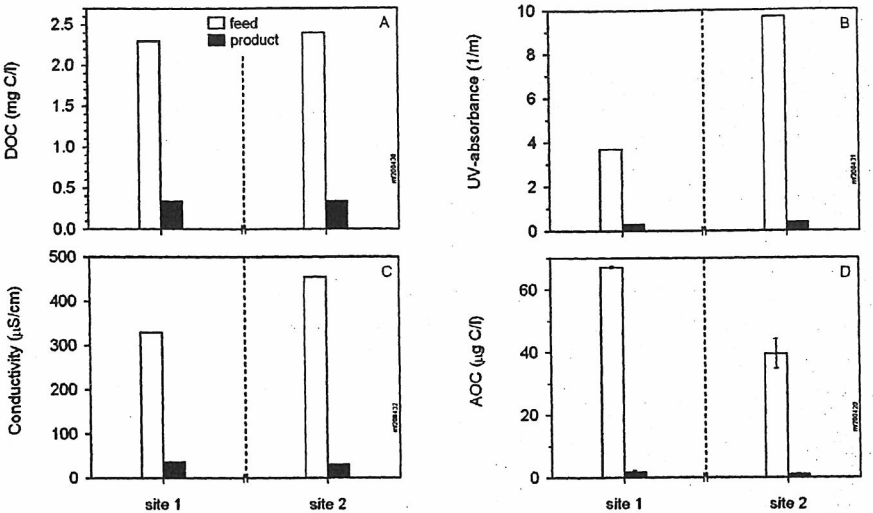

Figure 2: Effect of membrane type M2 on selected quality characteristics of ozonated water (pH 8) at two water treatment plants. (A) Dissolved organic carbon (DOC); (B) UV absorbance at 254 nm; (C) conductivity, and (D) assimilable organic carbon (AOC).

Removal of AOC and NOM from ozonated water

Figure 2 depicts the results of the study with the M2 membrane type in a SETU supplied with water after ozonation at pH 8 (no addition of biodegradable compounds). AOC concentrations were reduced from 67 to 1.8 µg AOC-C/l (site 1) and from 40 to 1.0 µg C/l (site 2), respectively. Also clear reductions of the conductivity (> 90%), the concentration of DOC (> 85%) and UV absorbance (> 92%) were observed.

Discussion

SETU experiments with and without added low molecular weight biodegradable compounds, (acetate, MW 82; ethanol, MW 46) proved to be well suited for studying the removal of AOC and NOM by NF. The obtained results clearly show that the removal capacity of NF membranes for the selected water quality parameters is affected by (i) the MWCO of the membrane used and (ii) the pH of the feed water. The conductivity reduction is related to the MWCO characteristic of the membrane, with only a limited effect at MWCO = 1000 D. This membrane type (M1) also gave a limited removal of AOC, although most NOM was removed. Lowering the pH of the feed water clearly reduced the acetate removal by the M3 membrane with a MWCO value of 200 D. Obviously, undissociated acetic acid molecules (MW 60) pass this NF membrane. A change in membrane charge at lower pH may also affect AOC removal. AOC removal from ozonated water at pH 8 was very effective (>95%) with MWCO of 200 D.

The presented results are in agreement with the observations of Escobar and Randall (1999) who showed that NF did not remove AOC from acidified groundwater (pH not reported), but DOC and BDOC concentrations were clearly reduced (> 90%). In their study at two plants without ozonation the AOC concentrations of the feed water ranged from 60 to 250 µg C/l and the MWCO value (200 D) of the Fluid Systems TFCS membrane was similar

to the MWCO of the M2 and M3 membrane types. In another study on NF (MWCO and pH not reported) the AOC-concentration of pre-treated water was reduced from 25 to 4 µg C/l (Yeh et al. 2000). Sibille et al (1997) also observed that NF (pH 8, MWCO not specified) considerably reduced the concentrations of DOC (85%) and BDOC (>70%) of water after ozonation and GAC filtration. However, this treatment only gave a limited reduction of the growth of microorganisms in an experimental distribution system.

A study on integrated membrane systems for evaluation of conventional pre-treatment processes followed by NF or reverse osmosis (RO) showed that at locations without biofouling AOC values of feed water ranged from 3 to 10 µg C/l and AOC values of the product were ≤ 1 µg C/l (Schippers et al., 2004). Biofouling of membranes was observed at an AOC concentration of about 27 µg C/l (Vrouwenvelder, 1998). Dosage of certain antiscalants can increase the growth potential of the feed water and cause biofouling (Vrouwenvelder 2000). Reverse osmosis gave a 94% reduction of an AOC concentration of about 1000 µg C/l in a test period of 3 hours (Ng et al., 2001), but biofouling is inevitable with such a high AOC concentration in the feed water. Consequently, the application of spiral-wound (NF) membranes for the removal of elevated AOC concentrations may be limited because AOC present in the feed water may cause biofouling, resulting in operational problems. Hence, pre-treatment is needed to achieve relatively low AOC concentrations in the feed water and NF will further reduce these AOC concentrations to a very low level. Another option is to improve cleaning procedures for periodic removal of biomass from the membrane.

Further studies will be conducted to define the conditions required for optimal use of NF filtration to improve biostability. These studies will include (i) SETU experiments with dosages of selected nutrients at different pH values and (ii) investigations with model distribution systems supplied with NF treated water to assess the impact on regrowth.

Conclusions

- NF membranes cause significant reductions of NOM concentrations, but the reduction of conductivity and the removal of AOC is limited at low pH-values and/or MWCO of 1000 D;
- Biofouling may hamper the use of NF for direct AOC removal thus requiring biological pre-treatment and/or effective cleaning techniques;
- Further studies are needed to demonstrate the effects of NF on limiting regrowth in distribution systems and water installations at different water types and pretreatment scenarios.

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Elucidation and control of biofilm formation processes in water treatment and distribution using the unified biofilm approach

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Abstract Controlling biological processes in water treatment and distribution is a major challenge to water supply companies. In the Netherlands, the use of chlorine-based disinfectants in water treatment is limited as much as possible and treated water is distributed without disinfectant residual in most cases. Biofilm formation processes in water treatment and distribution are studied using adenosinetriphosphate (ATP) as the parameter for active biomass. ATP measurements are applied to assess biofilm concentrations in distribution systems, in the biofilm monitor to determine the biofilm formation rate of treated water, in the biomass production potential test to determine the effect of pipe materials on microbial growth and in membrane systems to quantify biofouling. The use of a single parameter enables to compare biofilm concentrations in all situations and contributes to the understanding and control of biofilm formation processes in water treatment and distribution. This approach has been designated as the Unified Biofilm Approach.

Keywords Adenosinetriphosphate; biofilm formation rate; biofilms; biological stability; biomass production potential; unified biofilm approach

Introduction

Biofilms play an important role in water treatment and distribution. During soil passage and in filtration processes, biofilms contribute to the removal of organic and inorganic biodegradable compounds and also play a role in the removal of pathogens. Under certain conditions however, biofilm formation seriously hampers treatment process, e.g. biofouling of membranes and clogging of filter beds (Ridgway and Flemming, 1996; Hijnen and van der Kooij, 1992). Also abstraction wells or recharge wells may become clogged due to biological processes (Kawanishi *et al.*, 1990). In water distribution, excessive biofilm formation leads to a deterioration of the microbiological quality of treated water. Major disadvantages include: (i), regrowth of coliforms (LeChevallier *et al.*, 1987); (ii), multiplication of opportunistic pathogens such as *Mycobacterium* spp., *Legionella* spp., *Aeromonas* spp. and *Pseudomonas* spp. (Engel *et al.*, 1980; Havelaar *et al.*, 1990; Tobin *et al.*, 1980; Von Reyn *et al.*, 1994). *Legionella* especially multiply at elevated temperatures in plumbing systems (Wadowsky *et al.*, 1982). Other problems include increased heterotrophic plate counts, complaints about invertebrates (van Lieverloo *et al.*, 1994), color, taste and odor and microbially induced corrosion (MIC) (Lee *et al.*, 1995). Controlling biofilm formation is therefore a precondition in all stages of water supply, aiming at optimal use on one side and effective limitation on the other. This is particularly the case in the Netherlands where biological processes are applied in water treatment, and treated water is distributed without a disinfectant residual. The aspect of biostability, which is related to both biofilms in water treatment and in distribution, has been studied for long period (van der Kooij *et al.*, 1999).

Biofilm formation processes have attracted much attention in the past few decades, because water-exposed surfaces are the sites where microbiological activity in water systems is located. The formation of biofilms is caused by attachment of microorganisms to

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surfaces followed by growth. Attachment processes have been studied in detail by many investigators and also the various stages of growth, viz. exponential growth, linear growth and stationary phase (Characklis, 1973; LaMotta, 1976). These phases are schematically presented in Figure 1. Elucidation of the factors determining these processes in the various phases requires techniques to determine the concentration and nature of the micro-organisms contributing to biofilm formation. Parameters most commonly used to determine the concentration of attached biomass include: heterotrophic plate count (HPC) on various culture media and total direct cell count using epifluorescence microscopy. Typical biomass components such as proteins, carbohydrates and total biomass can be analyzed at higher biofilm concentrations. The parameters mentioned all have their typical advantages and limitations, e.g. HPC techniques only detect (an unknown fraction of the) culturable microorganisms whereas total cell counts and biomass parameters (proteins, carbohydrates) do not differentiate between dead and live organisms. The use of adenosinetriphosphate (ATP) as a biomass parameter has a number of advantages, including: (i), ATP, is an energy carrier in all active biomass and is not present in dead microorganisms (Holm-Hansen and Booth, 1966); (ii), ATP analysis is rapid and accurate, even at very low concentrations. For the ratio between cell carbon and ATP, generally a value of 250 is used (Karl, 1980; Holm-Hansen and Booth, 1966). The ATP concentration thus gives quantitative information about the concentration of active biomass, either attached or suspended. Attached active biomass reflects the response of the biofilm to the supply of energy sources, both organic and inorganic. Determining biofilm concentrations with ATP enables a simple comparison of biofilms in a variety of situations prevailing in water treatment and distribution, but also in other man-made and natural situations. This paper describes the use of ATP analysis for the quantitative assessment of biofilms in systems developed for monitoring the biofilm formation characteristics of treated water and materials and on surfaces exposed to water during treatment and distribution.

Biological stability of treated water

AOC test. Biofilm development is promoted by microbial utilization of biodegradable compounds, either originating from treated water or from the exposed material. AOC and BDOC tests are commonly used to assess the concentration of growth-promoting organic compounds present in water (van der Kooij *et al.*, 1982; Joret and Levy, 1985). The AOC test is based on determining the maximum level of growth of two pure bacterial cultures inoculated in pasteurized samples of water to be tested. These water samples (600 ml) are incubated in thoroughly cleaned glass-stoppered Erlenmeyer flasks with a volume of 1 L. The test strains are *Pseudomonas fluorescens* strain P17, which is capable of utilizing a

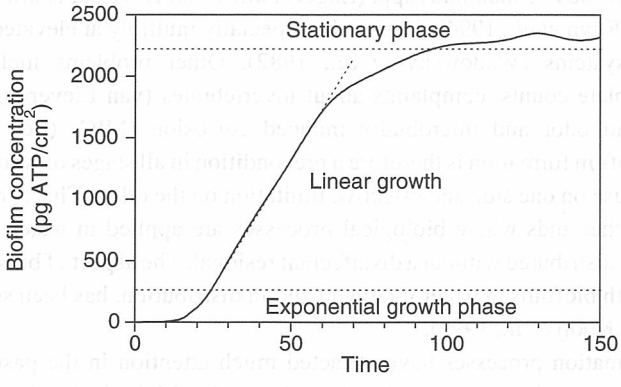

Figure 1 Biomass concentration on a surface ("biofilm") exposed to flowing water (theoretical). ATP gives the concentration of active biomass

wide range of compounds at very low concentrations (van der Kooij *et al.*, 1982a) and *Spirillum sp.* strain NOX, which only utilizes carboxylic acids (van der Kooij and Hijnen, 1984). AOC concentrations are calculated from the maximum colony counts of these two strains grown as a mixed culture in the water samples using the yield values of these organisms for acetate. AOC concentrations in treated water in the Netherlands range from less than 10 $\mu g\ C\ L^{-1}$ in slow sand filtrates and in groundwater supplies to values of about 50 $\mu g\ C\ L^{-1}$ in surface water supplies with ozonation in water treatment (Van der Kooij, 1992). The AOC fraction utilized by strain P17 in the presence of strain NOX is usually less than a few μg of $C\ L^{-1}$, indicating that the major fraction of AOC in treated water consists of carboxylic acids. AOC values below 10 $\mu g\ C\ L^{-1}$ hardly decline in drinking water during distribution, indicating that AOC uptake is very limited at these low concentrations. Also HPC values do not increase at these concentrations and therefore an AOC level of 10 $\mu g\ C\ L^{-1}$ has been derived as a reference value for biostable drinking water in which multiplication of bacteria contributing to HPC values is very limited.

Biofilm formation rate (BFR). Observations on increasing numbers of aeromonads in a number of groundwater supplies at AOC concentrations below 10 $\mu g\ C\ L^{-1}$ revealed that the definition of biostable water had to be extended. Furthermore, AOC tests and also BDOC tests do not provide information about the rate of uptake nor about the effects of non-organic growth-promoting compounds, e.g. ammonia, sulfides. For these reasons, a simple tool, the biofilm monitor, had been developed to determine the biofilm formation characteristics (biofilm formation rate, BFR; biofilm formation potential, BFP) of drinking water (Van der Kooij *et al.*, 1995). This biofilm monitor consists of a vertically-placed glass column containing glass cylinders (surface area: 17 cm^2) on top of each other. The water to be investigated is flowing through the column at a rate of 0.2 m/s. Biofilm formation is determined as a function of time by collecting the glass cylinders from the column at regular intervals and determining the biomass concentration on the surface of these cylinders. Attached biomass is released from the glass surface by a series of three low energy sonications, of 2 min each. Subsequently, the ATP concentration is determined in the obtained biomass suspension and the biofilm concentration is calculated. The BFR (pg ATP $cm^{-2}\ d^{-1}$) of the water can be calculated as the slope of the linear increase of the biofilm concentration with time (see Figure 1). The BFP (pg ATP cm^{-2}) of water is defined as the maximum biofilm concentration, but in many cases this value is not reached within the applied exposure period of 150 days. Figure 2 gives a typical example of biofilm formation in the biofilm monitor supplied with treated ground water (temperature: 12.5°C; DOC = 2.4 $mg\ L^{-1}$; AOC = 5.0 $\mu g\ C\ L^{-1}$). Biofilm formation was determined with ATP analysis, total direct cell counts (TDC) and HPC values on R2A-medium (10 days incubation at 25°C). The BFR value as calculated from the linear increase of the ATP concentrations was 12.5 ± 0.7 pg ATP $cm^{-2}\ d^{-1}$. The biofilm concentration was about 1,850 pg ATP cm^{-2} after 150 days, but the maximum level had not been reached. TDC values reached a level of about 3×10^7 cells cm^{-2}. TDC values were proportional with the ATP values (Figure 2C) and an average ATP concentration of 7.4×10^{-11} μg ATP per cell was calculated. This value is lower than the ATP content of bacteria grown as pure culture (Hamilton and Holm-Hansen, 1967) and may reflect the effect of the nutritional status of the bacteria growing in the biofilm. HPC values attained a maximum level of about 10^5 CFU cm^{-2} and initially were about 1% of the TDC values, but this percentage had decreased to less than 0.5% after 150 days. These results demonstrate that different biomass parameters give different information about biofilm formation. For reasons given above, ATP had been selected as the parameter of choice for determining biofilm formation.

BFR values in treated water in the Netherlands usually are below 30 pg ATP $cm^{-2}\ d^{-1}$,

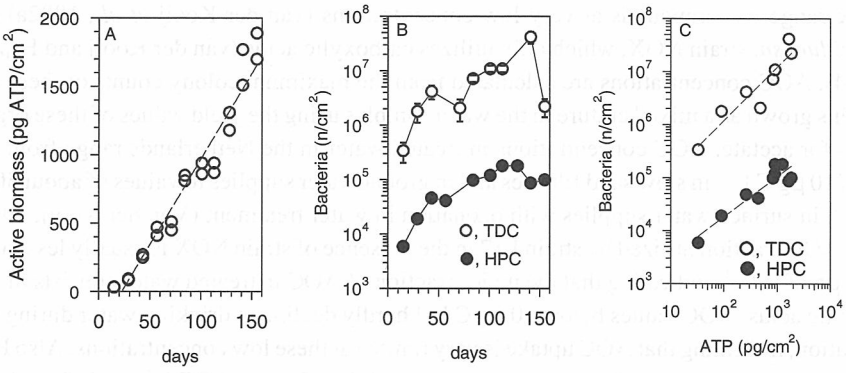

Figure 2 Biofilm formation in the biofilm monitor supplied with treated groundwater at a flow rate of 0.2 m/s. A, ATP as parameter for attached biomass (biofilm); B, TDC and HPC as biomass parameters; C, relationship between ATP concentrations and values for TDC and HPC in the biofilm

ranging from less that 1 pg ATP cm^{-2} d^{-1} for slow sand filtrates to about 100 pg ATP cm^{-2} d^{-1} for drinking water prepared from ground water containing ammonia and methane. Furthermore, a linear relationship has been observed between low concentrations of acetate-C added to drinking water and the BFR value. From this relationship a BFR value of 35 pg ATP cm^{-2} d^{-1} for 1 µg of added acetate-C L^{-1} was obtained (van der Kooij *et al.*, 1995a). Consequently, the BFR values observed for treated water correspond with concentrations of acetate-C (equivalents) which were clearly below 1 µg of C L^{-1} in most cases. These calculated acetate-C concentrations were usually a small fraction of the AOC concentration, which was less that 10 µg C L^{-1} in most types of treated water. Combining the results of the two methods gives a two dimensional scheme for defining the biological stability of drinking water, with the AOC concentration on the x-axis and the BFR-value on the y-axis.

Biofilm concentrations on pipe walls. Typical HPC values on surfaces range from less than 100 CFU cm^{-2} to more than 10^6 CFU cm^{-2} in chlorinated supplies (LeChevallier *et al.*, 1987) and values from 10^3 to 10^6 CFU cm^{-2} were observed in an experimental reactor supplied with treated water (Volk and LeChevallier, 1999). Data about biofilm concentrations have also been determined in unchlorinated supplies in the Netherlands. For this purpose, segments of pipes of unplasticized PVC, which is the main (more than 40%) pipe material, have been collected from a series of water supplies and biofilm concentrations were determined using ATP analysis. Biofilm concentration on these surfaces ranged from less than 100 pg ATP cm^{-2} to about 6000 pg ATP cm^{-2} with a median value of 700 pg ATP cm^{-2} (van der Kooij *et al.*, 1999). The lowest biofilm concentration values were observed in supplies distributing aerobic groundwater with a low concentration of organic compounds and a low BFR value (<1 pg ATP cm^{-2} d^{-1}). The highest biofilm concentrations were observed in supplies distributing drinking water derived from anaerobic groundwater containing ammonia and methane and BFR values greater than 50 pg ATP cm^{-2} d^{-1}. These results demonstrate the effect of biostability (BFR values) on biofilm formation.

Biomass production potential (BPP) of materials in contact with treated water. Synthetic pipe materials in contact with treated water may enhance biofilm formation by releasing biodegradable compounds. Methods have been developed in the United Kingdom and in Germany to test the growth-promoting properties of such materials (Colbourne and Brown, 1979; Schoenen and Schöler, 1983). In the absence of a disinfectant residual high demands must be made on the biostability of materials. Therefore, a sensitive test based on the use of

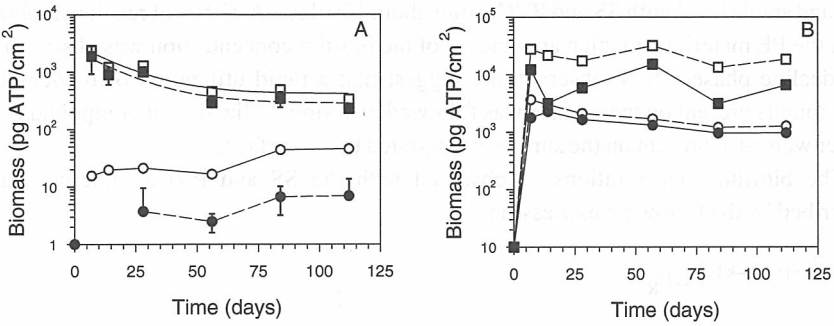

Figure 3 Biomass production in the BPP test. A. Silicone rubber (■, attached biomass; □, attached and suspended biomass) and glass (closed circles: attached biomass; open circles, total biomass); B, plasticized PVC (■, attached biomass; □, attached and suspended biomass) and glass-fiber reinforced epoxy (●, attached biomass; ○, total biomass). BPP values are calculated as averages of the biomass concentrations in the period from 8 to 16 weeks

ATP as biomass parameter has been developed to assess the BFP of materials (van der Kooij and Veenendaal, 1994). In this test material samples are incubated in biostable water (slow sand filtrate) at 25°C and the biomass concentration on the surface of these materials is determined as a function of time. More recently, this method has been adapted to the BPP method, where the BPP is defined as the sum of the BFP and the suspended biomass production (SBP). These parameters are expressed as pg ATP cm^{-2} (van der Kooij and Veenendaal, 2001). The concentrations of active biomass on the material surface and in the water are determined with ATP analysis during a period up to 16 weeks. Initially, the concentrations of biofilm and suspended biomass are relatively high (depending on the type of material), but decrease to a stable level with most materials after about 50 days. Typical examples of such curves are presented in Figures 3 and 4. BPP values range from values less that 100 pg ATP cm^{-2} (glass, stainless steel) to values greater than 10,000 pg ATP cm^{-2} for plasticized PVC and natural rubber. Unplasticized PVC (PVCu) materials also have BPP values below 100 pg ATP cm^{-2}. Figure 4 demonstrates that maximum concentrations of attached biomass were reached within a few weeks on stainless steel (SS), PVCu and polyethylene (PE). Subsequently, the biofilm concentration declined rapidly and reached a

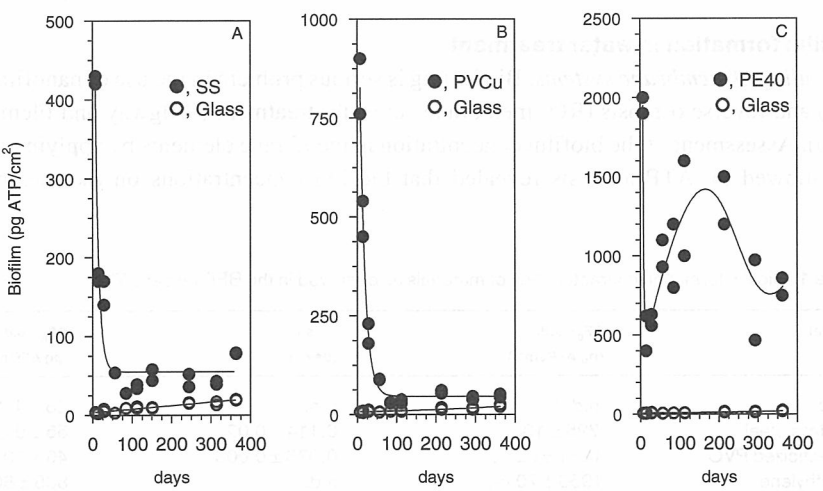

Figure 4 Biofilm concentration on materials incubated in slow sand filtrate at 25°C (surface to volume ratio = 0.166 cm^2 cm^{-3}) without replacement of the water

low and stable level with SS and PVCu after about 100 days. A different result was obtained with the PE material on which an increase of the biofilm concentration was observed after the decline phase. These observations suggest that a rapid utilization of biodegradable compounds present on the surface was followed by a slow utilization of compounds which either were also present on the surface or migrated to the surface.

The biofilm concentrations as observed with the SS and PVCu materials can be described by the following expression:

$$BF_T = BF_0 . e^{-kT} + BF_R \quad (1)$$

where BF_T is the biofilm concentration at time T (days) and BF_0 is the (hypothetical) biofilm concentration at time 0. In reality, the concentration of biomass on the surface of the material is zero at day zero, but the biomass concentration increases rapidly within a few days with many materials. BF_R is the residual biofilm concentration, which may represent the biofilm formation potential in practice. The values of the characteristics as obtained from the experiment shown in Figure 4 are presented in Table 1. Biofilm formation on glass and PE did not follow Eq. (1), and for these materials the observed values are presented.

BFR levels < 100 pg ATP cm^{-2} are low in relation to the biofilm concentrations as observed on the surfaces of pipe materials. Hence: (i), the predominant pipe material used in the Netherlands has a high degree of biostability; and (ii), biofilm formation on this material in distribution systems is mainly caused by the utilization of biodegradable compounds from the water, confirming the observations with the biofilm monitor.

Inclusion of *Legionella pneumophila* in BPP tests revealed a highly significant relationship between the BPP values and the extent of growth of *Legionella* on a number of materials tested at 25°C (van der Kooij *et al.*, 2002). Further research is needed to elucidate this relationship in more detail at incubation temperatures, which are more favorable for the growth of *L. pneumophila*.

The use of different methods in different countries in Europe is hampering a European policy regarding testing and selecting materials in contact with treated water. In the framework of the development of the European Acceptance Scheme (EAS) for construction products in contact with drinking water (CDPW), a research project is being conducted aiming at harmonizing the test method for determining the microbial growth potential.

Biofilm formation in water treatment

Biofouling of membrane systems. Biofouling is serious problem in the use of nanofiltration (NF) and reverse osmosis (RO) membranes in water treatment (Ridgway and Flemming, 1996). Assessment of the biofilm concentration in membrane elements by applying autopsy followed by ATP analysis revealed that biofilm concentrations on the membranes

Table 1 Biofilm formation characteristics of materials as observed in the BFP test at 25°C

Material	$BF_0 \pm$ s.d. (pg ATP cm^{-2})	$k \pm$ s.d. (day^{-1})	$BF_R \pm$ s.d. (pg ATP cm^{-2})
Glass	n.d. *	n.d.	23 ± 4 (**)
Stainless steel	795 ± 138	0.114 ± 0.02	55 ± 9.5
Unplasticized PVC	1331 ± 82	0.076 ± 0.006	46 ± 10
Polyethylene	1950 ± 70 (#)	n.d.	805 ± 80 (**)

*, n.d. not determined; **, observed value on day 362; #, value observed on day 7; ##, value observed on day 362

(including spacer) from a large number of installations ranged from less that 100 pg ATP cm^{-2} to more than 10,000 pg ATP cm^{-2}. An increase of the pressure drop was generally observed at biofilm concentrations above 1000 pg ATP cm^{-2}. (Vrouwenvelder *et al.*, 2000; Vrouwenvelder and van der Kooij, 2001). The rate of biofouling depends on the growth-promoting properties (AOC, BFR) of the feed water, which in turn depend on the composition of the raw water and the applied water treatment. Addition of an antiscalant can also affect biofouling when the added compound stimulates microbial growth. Testing of a series of antiscalants with AOC analysis and BFR measurements showed a wide range of growth promotion (Vrouwenvelder *et al.*, 2000).

Biological processes in filter beds. AOC and BFR measurements are also used to investigate treatment processes. Concentrations of biomass in filter bed materials (sand, granular activated carbon) can be determined using ATP analysis (Magic and van der Kooij, 2002).

Conclusions

ATP is an attractive parameter for determining the concentration of active biomass (biofilm) on water exposed surfaces. It can also be used for determining the concentration of suspended biomass in treated water and in experimental systems. Collecting data with this parameter about biofilms as present in water treatment, in distribution systems, in biofilm monitoring devices and in materials testing gives a framework for evaluation of the observed concentrations. This framework has been designated as the Unified Biofilm Approach (van der Kooij *et al.*, 1999). This approach is used in the Netherlands in combination with AOC and BFR measurements to elucidate microbiological processes in water treatment and distribution. The objectives are: (i), to control biofilm formation in water treatment; and (ii), to maintain biological stability in the distribution system.

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11

Managing regrowth in drinking-water distribution systems

D. van der Kooij

11.1 INTRODUCTION

Colony counts of bacteria on or in solid media containing organic compounds as sources of energy and carbon give information about the concentration of culturable heterotrophic bacteria in water or other environments under investigation. This so-called heterotrophic plate count (HPC), originally developed in 1881 by Robert Koch, was the first tool for monitoring the microbial quality of (treated) water. Within a few years after its introduction, the method was used in many European countries, and soon data came available on HPCs in both raw and treated water (Frankland and Frankland 1894).

A value of 100 cfu/ml had been defined as the first microbial water quality criterion (Koch 1893). However, storage of samples of treated water gave increased plate counts, and it was found that bacteria contributing to HPC values were able to

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grow in treated water at very low concentrations of organic compounds (Frankland and Frankland 1894). Many studies were conducted at the end of the 19th century to identify bacteria present in drinking-water and to elucidate their public health significance. For this purpose, pure cultures were inoculated into animals. The results of these studies and the inability of most bacteria to multiply at body temperature demonstrated that HPC values of water had no direct hygienic significance (Zimmermann 1890; Frankland and Frankland 1894; Kayser 1900; Haenle 1903).

At the beginning of the 20th century, the concept of testing water for bacteria of faecal origin was introduced to assess the hygienic safety of treated water (Eijkmann 1904). Methods and media for the detection of faecal indicator bacteria were developed and improved continuously in the course of the century. The Milwaukee outbreak of cryptosporidiosis in 1993 convincingly demonstrated that absence of coliforms does not always ensure microbial safety (Craun *et al*. 1997). Methods for the detection of pathogens are becoming more important for assessing the safety of water treatment. The focus on detection methods for pathogens is the result of both the limitations of the indicator bacteria concept and developments in the field of molecular microbiology, enabling the design of methods for specific and rapid detection of a large variety of bacteria, viruses and protozoa.

Despite the developments in assessing the hygienic safety of treated water, the HPC method has remained a generally applied water quality parameter, and criteria are included in legislation related to the quality of treated water in many countries (e.g., European Union 1998). HPC values provide information about the level of microbial activity in water and therefore can be used to control and optimize treatment processes, procedures and good engineering practices related to water treatment and distribution.

The objective of this chapter is to describe measures for controlling regrowth, with specific emphasis on systems in which treated water is distributed routinely with no or a low disinfectant residual. Brief descriptions are given of the problems caused by biological processes in distribution systems ("regrowth"), methods for determining microbial activity and methods for the assessment of the growth potential (biological stability) of water and materials.

11.2 PROBLEMS RELATED TO MICROBIAL ACTIVITY

11.2.1 Regrowth, biofilms and microbial activity

An increase of HPC values in treated water during distribution is generally described as regrowth or aftergrowth. These descriptions suggest that microorganisms start multiplying in water some time after leaving the treatment facility (Brazos and O'Connor 1996). However, multiplication in distribution

systems mainly takes place on the water-exposed surfaces of the pipes and in sediments, even in the presence of a disinfectant residual. The increase of HPC values in water during distribution thus is mainly due to bacteria originating from biofilms and sediments (LeChevallier *et al*. 1987; van der Wende *et al*. 1989). However, these HPC values are not suited for the identification of water quality problems as may be caused by the multiplication of microorganisms in distribution systems. A number of water quality problems related to microbial activity are described below. More detailed descriptions have been given in earlier reviews (e.g., Olson and Nagy 1984; LeChevallier 1990) and in other chapters in this book.

11.2.2 Coliforms

Multiplication of coliforms in distribution systems has been reported since the beginning of the 20th century. Baylis (1930) found that these organisms grew in sediments accumulating in the distribution system. Howard (1940) also reported multiplication of coliforms in a distribution system during summer. Redwood reservoirs were found to stimulate growth of *Klebsiella* (Seidler *et al*. 1977). Also, coatings were found to stimulate coliform growth (Ellgas and Lee 1980). Wierenga (1985) reported coliform occurrences in distribution systems in the presence of a free chlorine residual. A national survey in the USA revealed that about 18% of the responding companies experienced non-compliance with coliforms, most likely due to multiplication of these organisms in the distribution system (Smith *et al*. 1990). Coliforms can multiply at low substrate concentrations (van der Kooij and Hijnen 1988b; Camper *et al*. 1991). Growth-promoting conditions include concentration of available substrates, water temperature, corrosion, presence of sediments and disinfectant residual (LeChevallier 1990; LeChevallier *et al*. 1996) and are described below and also in chapter 10 of this book.

11.2.3 Opportunistic pathogens

In recent decades, concern has increased about the multiplication of opportunistic pathogens in distribution systems and in plumbing systems. Such organisms include *Aeromonas* spp., *Flavobacterium* spp., *Legionella* species, especially *L. pneumophila*, *Mycobacterium* spp. and *Pseudomonas* spp., especially *P. aeruginosa*. A few characteristics of these organisms are described below. Detailed descriptions of the significance of *Aeromonas* (WHO 2002), *Legionella* (WHO, in revision) and *Mycobacterium* (some non-tuberculous mycobacteria, including *Mycobacterium avium* complex, are the subject of a

separate book in the same series as this volume) in relation to drinking-water safety either have been given elsewhere or are being prepared.

Aeromonas is a common component of the bacterial population of drinking-water in distribution systems but comprises only a small fraction of the heterotrophic population (Leclerc and Buttiaux 1962; Schubert 1976; van der Kooij 1977; LeChevallier *et al*. 1982; Havelaar *et al*. 1990). Reports of Burke *et al.* (1984) caused concern about the possible health effects of *Aeromonas* in drinking-water. In a national survey in the Netherlands, no evidence was obtained that the aeromonads present in drinking-water were enteric pathogens (Havelaar *et al*. 1992). Still, in drinking-water legislation in the Netherlands, a maximum value for *Aeromonas* of 1000 cfu/100 ml is included, aiming at limiting the exposure of the consumer to this organism (VROM 2001).

Pigmented bacteria, including *Flavobacterium* spp., constitute a significant proportion of the HPC values in treated water (Reasoner *et al*. 1989). Certain *Flavobacterium* spp. have been identified as opportunistic pathogens (Herman 1978).

Of the potential pathogens, *Legionella* has attracted most attention, particularly after its discovery in plumbing systems in connection with disease (Tobin *et al*. 1980; Cordes *et al.* 1981; Wadowsky *et al*. 1982). Numerous reports are available about cases of legionellosis caused by exposure to aerosols of warm tap water containing *Legionella*. Certain protozoans grazing on bacteria in biofilms and sediments can serve as hosts for *Legionella* (Rowbotham 1980; Abu Kwaik *et al.* 1998).

Mycobacterium spp., including *M. kansasii*, *M. avium*, *M. chelonae* and *M. fortuitum*, originating from water supplies have been associated with lung infections (McSwiggan and Collins 1974; Engel *et al*. 1980; Kaustova *et al*. 1981; Von Reyn *et al*. 1994). These bacteria, which are highly resistant to chlorine (Carson *et al*. 1978; Haas *et al*. 1983; Taylor *et al*. 2000), can multiply in dead ends of distribution systems and in biofilms (Schulze-Röbbecke and Fischeder 1989; Fischeder *et al*. 1991; Falkinham *et al.* 2001).

P. aeruginosa is not a normal constituent of the bacterial population of treated water (Lantos *et al*. 1969; Hoadley 1977; Hardalo and Edberg 1997), probably because it cannot compete effectively with the related species *P. fluorescens*, which grows at lower temperatures (van der Kooij *et al*. 1982b). Even the fluorescent pseudomonads, which in most cases are unable to multiply at 37 ºC, constitute only a small part of the bacterial population of tap water (van der Kooij 1977).

The opportunistic pathogens mentioned above usually remain undetected with the media used for HPC determination, because the organisms either cannot produce colonies on these media or are typically only a very small

fraction of the HPC values. Their detection therefore requires selective media or molecular methods (Manz *et al.* 1993; Schwartz *et al.* 1998).

11.2.4 Increased HPC values

In the second half of the 20th century, granular activated carbon filtration and ozonation were introduced to limit concentrations of undesirable organic compounds in water. These developments and more detailed definitions of microbial water quality criteria increased the focus on HPC values in treated water during distribution. Geldreich *et al*. (1972) concluded that the risk of pathogen contamination increases as the general bacterial population increases and that HPC values (two days, 35 ºC) above 500 cfu/ml hampered coliform detection. A problem with HPC values is the diversity of methods used in practice. Typical methods are pour plate count incubated at 35 ºC (or 37 ºC) for one or two days or at 20–22 ºC for two or three days and 20–25 ºC spread plate count on diluted agar medium incubated for 7–14 days. Distribution of water treated with ozone as a final treatment step (followed by post-chlorination) gave increased HPC values (three days, 20 ºC) ranging from 10^3–10^4 cfu/ml in distribution pipes (Berger 1970; Dietlicher 1970; Stalder and Klosterkötter 1976; van der Kooij *et al*. 1977). Incubation of ozonated water in batch tests gave HPC values (three days, 22 ºC) above 10^5 cfu/ml, which clearly demonstrated that ozonation increased the growth potential of water (Snoek 1970). In chlorinated supplies, increases of HPC values to more than 10^4 cfu/ml have been reported, usually in situations where chlorine residual became less than 0.1 mg/litre (Rizet *et al*. 1982; Maul *et al*. 1985; Prévost *et al*. 1997, 1998). With the use of R2A medium (seven days, 22 ºC), values up to 10^5 cfu/ml were observed (Reasoner and Geldreich 1985; Maki *et al.* 1986). LeChevallier *et al.* (1987) reported HPC values on R2A medium ranging from 320 to 1.3×10^7 cfu/ml in one supply. In a survey in the Netherlands in a summer–autumn period, median HPC values on diluted broth agar medium (14 days, 25 ºC) in 19 different water supplies, nearly all without disinfectant residual, ranged from about 150 cfu/ml to 1.6×10^4/ml. On plate count agar medium (three days, 25 ºC), HPC values ranged from 3 to 550 cfu/ml (van der Kooij 1992) (Figure 11.1).

Obviously, some increase of HPC values in treated water during distribution is quite common. The extent of the increase depends on the medium used, the disinfectant residual and the growth-promoting conditions in the systems, as will be discussed below.

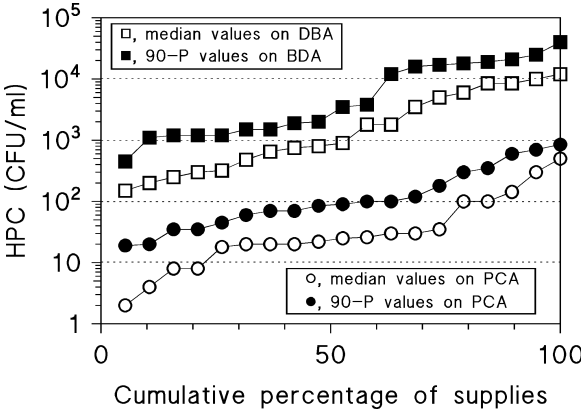

Figure 11.1. HPC values in 19 distribution systems in the Netherlands in a summer–autumn period. PCA, plate count agar, incubated three days at 25 ºC; DBA, diluted broth agar, incubated for 10 days at 25 ºC. 90-P, 90th-percentile value of the sample series (adapted from van der Kooij 1992).

11.2.5 Nuisance organisms

Discoloured water containing iron bacteria is one of the earliest described problems related to the activity of microorganisms in treated water. De Vries (1890) studied the presence of the iron-precipitating organism *Crenothrix* in the distribution system of the water supply of Rotterdam. This organism had also been observed in other supplies in Europe, even before techniques for culturing bacteria had been developed. Many investigators since then have reported on iron-accumulating bacteria, including *Gallionella* and *Lepthothrix*, in relation to corroding pipes (Clark *et al*. 1967; McMillan and Stout 1977; Tuovinen *et al.* 1980; Ridgway and Olson 1981; Ridgway *et al.* 1981).

Fungi and actinomycetes are usually present in low numbers in distribution systems (Silvey and Roach 1953; Burman 1965; Dott and Waschko-Dransmann 1981; Nagy and Olson 1982, 1985, 1986). These organisms have been associated with taste and odour complaints. Certain actinomycetes are able to degrade natural rubber sealing rings, which may lead to leakage (Leeflang 1968). Water supplies using anaerobic groundwater as a raw water source may contain methane-utilizing bacteria (Schweisfurth and Ruf 1976; Tuschewitski *et al.* 1982). These bacteria do not contribute to HPC values, but their biomass may lead to fouling of the system and serve as food source for protozoa and invertebrates. Also, nitrifying bacteria may be found in such water types when

ammonia removal is incomplete or when monochloramine is used as a disinfectant in distribution systems (Wolfe *et al.* 1990; Skadsen 1993). Growth of these bacteria results in nitrite formation and in an increase in the HPC counts, because components of the biomass of the nitrifying bacteria serve as a food source for heterotrophs. In corroding pipes, sulfate-reducing bacteria are present. These bacteria play a role in microbially induced corrosion, which results in complaints about discoloured water (O'Connor *et al.* 1975; Lee *et al.* 1980; Tuovinen *et al.* 1980; Victoreen 1984; Lee *et al.* 1995). Where bacteria multiply, protozoans may also be present (Michel *et al.* 1995). At elevated temperatures, protozoans with pathogenic properties (*Acanthamoeba*, *Naegleria*) may multiply (de Jonckheere 1979).

The presence of invertebrates in water used for consumption also had attracted attention before bacteriological techniques were used to assess water quality (de Vries 1890). In 1928, Heymann in the Netherlands described the sequence of natural biological processes in distribution systems — namely, multiplication of bacteria, followed by protozoans and subsequently the development of a population of small and larger animals, including *Asellus* (Heymann 1928). Heymann concluded that iron bacteria were a main food source for *Asellus*. In the second half of the century, these animals were studied in a number of other countries (Smalls and Greaves 1968; Levy *et al*. 1986). In the Netherlands, extensive studies have been conducted to obtain information about numbers of invertebrates in unchlorinated water supplies. Asellids comprised the main proportion (>75%) of invertebrate biomass in water flushed from mains, with maximum numbers of *Asellus* ranging from less than $1/m^3$ to about $1000/m^3$. Higher maximum numbers (between 10^3 and $10^4/m^3$) were observed for cladocerans and copepods. For nematodes and oligochaete worms, these levels were usually below 10 and 100 organisms/m^3, respectively (van Lieverloo *et al*. 1997).

11.3 ASSESSMENT OF MICROBIAL ACTIVITY

11.3.1 Monitoring tools needed

Biological processes in distribution systems may cause a variety of water quality problems and therefore should be limited. Reliable analytical tools are needed to monitor the extent of the problem, the effects of control measures and the factors promoting microbial activity. Monitoring of HPC values using standard plate count methods is needed because criteria for such HPC values are defined in legislation. However, improved HPC methods and other techniques are

available to elucidate the nature and the extent of the microbial problems and processes.

11.3.2 Heterotrophic plate counts

The HPC value usually represents only a small fraction of the microbial population in water. Major factors affecting the yield of the method include the composition of the medium, the mode of use (spread or pour plate), incubation temperature and incubation time. The medium prescribed for routine monitoring of HPC values contains high concentrations of substrates (beef extract, peptone), and, after a short incubation period (24–48 h), only bacteria growing rapidly on these compounds are enumerated. A large variety of HPC media have been developed since the end of the 19th century, and, in combination with various incubation temperatures and/or incubation periods, different fractions of the community of heterotrophic bacteria can be enumerated. The highest HPC values are obtained with the streak plate method on non-selective media with low substrate concentrations in combination with a long incubation period (Foot and Taylor 1949; Jones 1970; Fiksdal *et al.* 1982; Maki *et al.* 1986). Also, Reasoner and Geldreich (1985) demonstrated the effect of medium composition and incubation time on the HPC yield. The R2A medium, with a relatively low substrate concentration, gave the highest yield after 14 days of incubation at 20 ºC. Figure 11.1 shows that diluted broth agar medium gave much higher HPC values than plate count agar medium (van der Kooij 1992). However, even despite these improvements, HPC values on solid media are usually a small fraction (in many cases <1%) of the total bacterial population as enumerated with microscopic techniques (Maki *et al.* 1986; McCoy and Olson 1986; Servais *et al.* 1992). The difference between total direct counts and HPC values is caused by the inability of a majority of bacteria to produce colonies on the applied solid medium, the presence of chemolithotrophic bacteria and the presence of dead cells.

One specific culture medium will never detect all viable heterotrophic bacteria. The best approach for monitoring the multiplication of heterotrophic bacteria in the distribution system is the use of a standardized HPC method with a high yield. Additionally, selective culture methods for the detection of opportunistic pathogens and nuisance organisms can be applied when needed.

11.3.3 Total direct counts

Several techniques are available for enumerating the total number of bacteria in water. The most commonly applied method includes membrane filtration to concentrate bacteria, staining with a fluorescent dye (acridine orange) and

microscopic observation (Hobbie *et al.* 1977). The total direct count (TDC) value obtained in this way is an indicator for bacterial biomass, and observations of specific morphological types of organisms give additional information. Furthermore, the information is available within a short period. TDC values between 10^4 and 10^5 cells/ml have been observed in the distribution systems of Paris (Servais *et al.* 1992) and Metz (Matthieu *et al.* 1995). Prévost *et al.* (1997, 1998) reported values above 10^5 cells/ml for treated water in two Canadian distribution systems and in water from services lines. Concentrations of about 10^6 cells/ml were observed in treated surface water entering a distribution system (Brazos and O'Conner 1996). TDC methods give information about the concentration of cells, but not about the concentration of active biomass, because not all detected organisms are active and because cells have large differences in size. Special methods are available for directly determining the number of viable cells (Coallier *et al.* 1994; McFeters *et al.* 1999).

11.3.4 Adenosine triphosphate

For determining the concentration of active microorganisms, the adenosine triphosphate (ATP) assay has been developed. ATP is an energy-rich compound present in active biomass. The first applications of the ATP analysis for determining microbial activity in water were described by Holm-Hansen and Booth (1966). Values of 250–300 have been reported for the ratio between concentrations of biomass estimated as particulate organic carbon and ATP (Karl 1980). Attractive properties of this analytical method include the following:

- rapidity: the analysis can be conducted within a few minutes;
- low detection level: a concentration of 1 ng ATP/litre can be detected without concentration techniques;
- inclusion of all types of active (micro)organisms;
- ease of interpretation, because ATP concentration is directly related to activity;
- automation: enables the analysis of large series of samples; and
- on-site analysis, using portable equipment.

Improvements of the chemicals and equipment will lead to further decreases in detection limits and improve ease of operation.

ATP analysis is used as a research tool for assessing the presence of microorganisms in drinking-water. In a study conducted in 19 water supplies in the Netherlands, it was found that ATP concentrations in treated water collected

from the distribution systems (mostly without chlorine residual) were usually below 10 ng/litre (Figure 11.2). The HPC/ATP ratio in groundwater supplies ($10^5 – 3 \times 10^5$ cfu/ng) was lower than in surface water supplies ($10^6 – 3 \times 10^6$ cfu/ng), probably because of the presence of nitrifying bacteria coming from filter beds used in groundwater treatment (van der Kooij 1992). Deininger and Lee (2001) observed a high correlation between ATP concentrations and HPC values in 120 samples collected from various systems in the USA. Relatively high ATP concentrations (up to 50 ng/litre) have been reported for a distribution system receiving ozonated water (Bourbigot *et al.* 1982). A survey of all supplies in the Netherlands showed that ATP concentrations in water leaving the treatment plant were below 1 ng/litre in 15% of the samples, with 2.5 ng/litre and 8 ng/litre as median value and 90th-percentile value, respectively (Figure 11.3). Hence, a database for this parameter in treated water is available for reference.

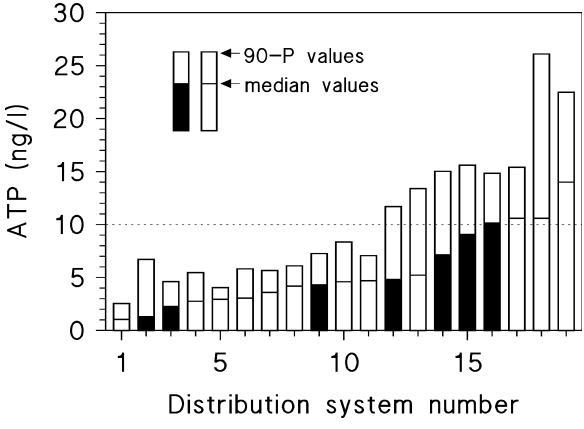

Figure 11.2. ATP concentrations in drinking-water during distribution in 19 water supplies in the Netherlands sampled during summer–autumn. Open bars (bottom) indicate groundwater supplies, black bars indicate surface water supplies. Nos. 2, 3 and 12 have slow sand filtrate as final treatment (adapted from van der Kooij 1992).

11.3.5 Other methods

For determining microbial activity, a large number of enzymatic methods and methods based on the incorporation of radioactive compounds in biomass are available, but these techniques will not be reviewed here. A new and very promising development is the use of molecular methods based on polymerase chain reaction (PCR) or fluorescence *in situ* hybridization (FISH) methods

(Manz *et al.* 1993; Schwartz *et al.* 1998). Such techniques are especially useful in determining the concentrations of specific bacteria that are difficult to culture — e.g., nitrifying bacteria and sulfate-reducing bacteria — but also other types of microorganisms. Developments in this area are fast, and it is expected that rapid molecular techniques will be available in the near future for the quantitative detection of many types of organisms (see chapter 9 of this book).

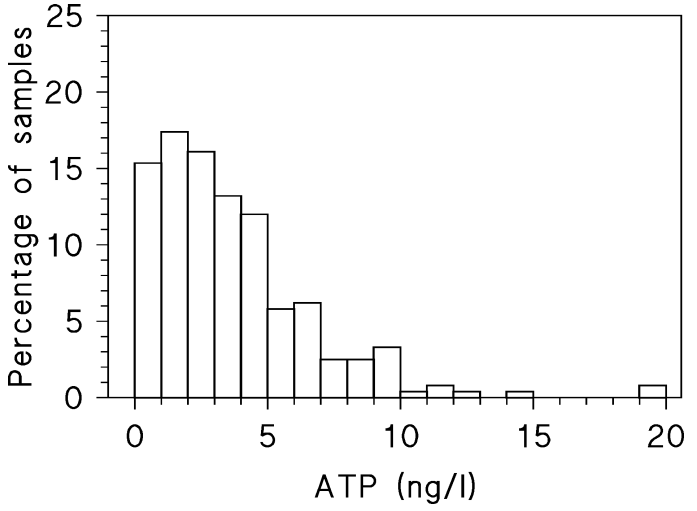

Figure 11.3. Frequency distribution of ATP concentrations in treated water of 243 treatment facilities in the Netherlands. ATP concentrations were above 20 ng/litre in five locations, with a maximum value of 46 ng/litre (unpublished data).

11.3.6 Suite of methods

Monitoring or elucidating microbial activity in distribution systems is best done by using a suite of parameters, namely:

- HPC on a nutrient-poor solid medium, e.g., R2A agar, incubated at 20–25 ºC for 7–14 days;
- selective media for detection and isolation of undesirable bacteria, such as *Aeromonas*, *Legionella*, *Mycobacterium*, *Pseudomonas*, etc., when needed;
- ATP, for rapid determination of the total microbial activity; and

- molecular methods (PCR, FISH) for the selective detection of specific microorganisms.

ATP analysis and molecular methods enable a rapid assessment of the microbial water quality, but general application requires further investigations to obtain standardized methods and well defined criteria.

11.4 FACTORS PROMOTING MICROBIAL ACTIVITY

11.4.1 Energy sources in water

11.4.1.1 Power of multiplication

Microbial activity depends on the availability of sources of energy and carbon for formation and maintenance of biomass. The major energy source in treated water is organic carbon, but ammonia may also be present in certain water types. Already in 1885, it was observed that bacteria multiplied in treated water when samples were not processed immediately (Frankland and Frankland 1894). In the early days of microbiology, these observations on the "power of multiplication" of microorganisms caused much excitement, and investigations were conducted for explanation. It was found that even water with a high purity promoted growth of bacteria when stored in a bottle. This power of multiplication was also demonstrated with pure cultures. Attempts were made to quantify the growth potential of water for microorganisms, but in many cases these tests were hampered by the use of flasks plugged with cotton wool, allowing the diffusion of growth-promoting volatile compounds into the test water. Beijerinck (1891) suggested that growth tests with pure cultures of microorganisms should be conducted in boiled water, but results of such tests do not seem available. Heymann (1928) published a method for determining the concentration of assimilable organic compounds in water based on the reduction of the potassium permanganate value after one or more passages of water through a sand filter at a rate of 1 cm/h. With this method, he observed potassium permanganate reductions in raw water above 50% and 20–40% in treated (rapid sand filtration followed by slow sand filtration) water and in groundwater, respectively. These values demonstrate that even after extended treatment or after soil passage, a substantial part of the organic compounds in water remains available to microbial activity, provided that enough contact time is given.

In the 1970s, the increased interest in microbial water quality and the introduction of new treatment methods strengthened the focus on the assessment of the microbial growth potential of treated water. A number of methods have been developed in European countries and in the USA (Table 11.1).

Table 11.1. Methods for determining the microbial growth potential or the concentration of biodegradable organic compounds in treated water

Method (units)[1]	Key parameter	Mode	References
AOC (µg carbon/litre)	Biomass (cfu)	Batch	van der Kooij *et al.* 1982c; van der Kooij 1992; Kaplan *et al.* 1993; LeChevallier *et al.* 1993b
BDOC (mg carbon/litre)	DOC	Batch	Joret and Levi 1986; Servais *et al.* 1987
BDOC (mg carbon/litre)	DOC	Flow-through	Lucena *et al.* 1990; Ribas *et al.* 1991; Kaplan and Newbold 1995
BFR (pg ATP/cm^2 per day)	Biomass (ATP)	Flow-through	van der Kooij *et al.* 1995b

[1] BDOC, biodegradable dissolved organic carbon; AOC, assimilable organic carbon; BFR, biofilm formation rate (expressed as amount of ATP per cm^2 of exposed surface and per day); DOC, dissolved organic carbon.

11.4.1.2 Assimilable organic carbon

Assessment of the assimilable organic carbon (AOC) concentration is based on growth measurements with a mixture of two selected pure cultures in a sample of pasteurized water contained in a thoroughly cleaned glass-stoppered Erlenmeyer flask (van der Kooij *et al.* 1982c). The strains used in the AOC test are *Pseudomonas fluorescens* strain P17, which is capable of utilizing a wide range of low-molecular-weight compounds at very low concentrations (van der Kooij *et al.* 1982a), and a *Spirillum* sp. strain NOX, which utilizes only carboxylic acids (van der Kooij and Hijnen 1984). The AOC concentration is calculated from the maximum colony counts of these strains, using their yield values for acetate. Consequently, AOC concentrations are expressed as acetate-carbon equivalents/litre. AOC concentrations in treated water in the Netherlands usually are below 10 µg carbon/litre, but values up to about 60 µg carbon/litre have been observed in surface water supplies with ozonation included in water treatment (van der Kooij *et al.* 1989; van der Kooij 1992). In all types of treated water, the fraction available to strain NOX was the largest proportion of the AOC concentration, indicating that carboxylic acids were the predominating growth substrates. The AOC concentration utilized by strain P17 was less than 1 µg carbon/litre in most types of treated water. With this technique, effects of water treatment and distribution have been determined (van der Kooij 1984, 1987, 1992). The method is used in other countries, usually after modification (Kaplan *et al.* 1993; LeChevallier *et al.* 1993b; Miettinen *et al.* 1999). Table

11.2 shows that AOC values reported for treated water in the USA and in Finland ranged from about 20 to more than 400 µg carbon/litre, with median values of about 100 µg carbon/litre (Kaplan *et al.* 1994; Miettinen *et al.* 1999; Volk and LeChevallier 2000). These values are much higher than those observed in the Netherlands.

Table 11.2. Ranges of concentrations of DOC, BDOC and AOC in treated water as observed in a few surveys (mean values are given in parentheses)

Country	Systems	DOC (mg carbon/litre)	BDOC (mg carbon/litre)	AOC (µg carbon/litre)[1]	Reference
Netherlands	20	0.3–8.6 (3.3)	ND[2]	1.1–57 (8.1)	van der Kooij 1992
USA	79	0.2–4.3 (2.0)	0.01–0.97 (0.24)	18–322 (110)	Kaplan et al. 1994
Finland	24	0.6–5.0 (2.7)	ND	45–315 (130)	Miettinen et al. 1999
USA	31 (95)[3]	0.6–4.5 (2.0)	0.03–1.03 (0.32)	14–491 (94)	Volk and LeChevallier 2000

[1] Acetate-carbon equivalents/litre.
[2] ND, not determined.
[3] Number of plants for which AOC tests were conducted in parentheses.

11.4.1.3 Biodegradable dissolved organic carbon

The biodegradable dissolved organic carbon (BDOC) method, as developed by Joret and Levi (1986), determines the decrease of the dissolved organic carbon (DOC) concentration in water samples incubated for several days with sand from a biological filter. The BDOC method developed by Servais *et al.* (1987) determines the DOC decrease in water as caused by the indigenous microbial community after an incubation period of 30 days. Table 11.2 shows that typical BDOC values in treated water in the USA range from less than 0.1 mg/litre to about 1 mg/litre, with median values of 0.24–0.32 mg/litre (Kaplan *et al.* 1994; Volk and LeChevallier 2000). These values were clearly higher than the AOC values reported for the same water types. The difference between BDOC and AOC values is caused by using a relatively high concentration of biomass of an adapted microbial community in the BDOC test, whereas low numbers of two pure cultures are used in the AOC test. A rapid assessment of the BDOC value can be obtained from changes in DOC concentrations following the passage of water through a column containing a support with an adapted microbial community (Lucena *et al.* 1990; Ribas *et al.* 1991).

11.4.1.4 Biofilm formation rate

The biofilm formation rate (BFR) value is determined with the use of a biofilm monitor, consisting of a vertical glass column containing glass cylinders (with an external surface of about 17 cm^2) on top of each other. This column is supplied with the water to be investigated at a flow of 0.2 m/s (empty column). Cylinders are sampled periodically from the column, and the biomass concentrations of the glass surface are determined with ATP analysis. Subsequently, BFR values are calculated from the biomass increase in time and expressed as pg ATP/cm^2 per day (van der Kooij *et al.* 1995b). BFR values of treated water in the Netherlands typically range from less than 1 pg ATP/cm^2 per day in slow sand filtrate to values between 30 and 50 pg ATP/cm^2 per day in drinking-water prepared from anaerobic groundwater. The system has been calibrated with acetate added to treated water. A concentration of 10 µg acetate-carbon/litre gave a BFR value of 360 pg ATP/cm^2 per day, and a BFR value of 35 pg ATP/cm^2 per day corresponds with 1 µg carbon/litre of easily available carbon compounds (van der Kooij *et al.* 1995a).

These observations demonstrate that very low concentrations of readily biodegradable compounds may affect biofilm formation. From the observed BFR values, it can be derived that the concentration of such compounds is less than 1 µg carbon/litre in most supplies with AOC concentrations below 10 µg carbon/litre. Combining the results of the AOC test and the BFR values gives a two-dimensional approach for evaluating the biological (in)stability of treated water.

11.4.2 Materials and sediments

11.4.2.1 Materials

Many reports are available about microbial growth promotion induced by materials in contact with treated water. Such materials included coatings, rubbers and pipe materials (Speh *et al.* 1976; Colbourne and Brown 1979; Ellgas and Lee 1980; Schoenen and Schöler 1983; Frensch *et al.* 1987; Bernhardt and Liesen 1988) and also wood used in service reservoirs (Seidler *et al.* 1977). Certain chemicals used in water treatment — e.g., coagulant or filtration aids — and lubricants can also enhance microbial growth (van der Kooij and Hijnen 1985; White and LeChevallier 1993). A number of materials in contact with treated water can promote the growth of opportunistic pathogens — e.g., *Legionella* and *Mycobacterium* (Colbourne *et al*. 1984; Niedeveld *et al*. 1986; Rogers *et al*. 1994; Schulze-Röbbecke and Fischeder 1989). In the United Kingdom, materials in contact with treated water are tested in the mean dissolved oxygen difference (MDOD) test (Colbourne and Brown 1979).

Materials with an MDOD level above 2.3 mg/litre are considered unsuitable for use in contact with treated water (Colbourne 1985). In Germany, a test method based on determining the amount of slime on the surface of a material is applied (Schoenen and Schöler 1983; DVGW 1990). In the Netherlands, the biomass production potential (BPP) test has been developed, which is based on the biofilm formation potential test by including the amount of suspended biomass in the measurements (van der Kooij and Veenendaal 2001). The BPP value (pg ATP/cm^2) is defined as the average value of the sum of the concentrations of attached biomass and suspended biomass estimated after 8, 12 and 16 weeks of exposure. Typical BPP values range from less than about 100 pg ATP/cm^2 for unplasticized polyvinyl chloride to values above 10 000 pg ATP/cm^2 for certain plastic materials and rubber components (van der Kooij *et al.* 1999).

Reactive metal surfaces — i.e., corroding cast iron — also enhance microbial growth (LeChevallier *et al.* 1993a; Camper *et al.* 1996; Kerr *et al.* 1999), probably by adsorption of organic compounds on iron oxides (Camper *et al.* 1999).

11.4.2.2 Sediments and corrosion products

Sediments accumulating in distribution systems can serve as a food source for bacteria (Baylis 1930; Allen and Geldreich 1978; Allen *et al.* 1980; Martin *et al.* 1982). Detritus originating from biofilm sloughing may contribute to sediment accumulation, but particles present in treated water (e.g., algal cells) and corrosion products have also been observed in sediments (Ridgway and Olson 1981; Brazos and O'Connor 1996). In cast iron pipes, it is difficult to differentiate between sediments and corrosion products. Sediments and corrosion products protect microorganisms from the disinfectant (LeChevallier *et al*. 1990).

11.4.3 Temperature and hydraulic conditions

Water temperature, flow velocity (variations) and residence time have an impact on microbial activity. Biological activity increases about 100% when temperature increases by 10 ºC. A temperature of 15 ºC has been reported as critical for coliform growth (LeChevallier *et al.* 1996). Flow velocity (changes) affects supply of substrates and disinfectant, biofilm sloughing and sediment accumulation. An increasing residence time in chlorinated supplies results in a decreasing free chlorine concentration (Lu *et al.* 1995; Vasconcelos *et al.* 1997; Prévost *et al*. 1998). Locations with long residence time — e.g., peripheral parts of the distribution system and service reservoirs (Speh *et al*. 1976; LeChevallier *et al*. 1987; Prévost *et al.* 1997) — are vulnerable for regrowth because of

decreased disinfectant residual, the transportation of sediments and increase of water temperature in summer.

11.4.4 Models

A number of models have been developed to describe the relationships between water quality parameters, distribution system conditions and the extent of regrowth. Lu *et al.* (1995) described a mathematical model for transport of substrates and microorganisms in water pipes. The disinfectant consumption rate at the pipe wall plays a significant role in this model, and the chemical oxygen demand is used as the growth substrate parameter. Servais *et al.* (1995) developed the Sancho model for describing BDOC and biomass fluctuations in distribution systems. In this model, BDOC is divided into a fraction that is rapidly utilized and a fraction of complex substrates that are available only after enzymatic activity (hydrolysis). The model has been validated in practice in distribution systems. A third model, developed by Dukan *et al.* (1996), combines a hydraulic model (Piccolo) with a water quality model, including BDOC, chlorine residual and bacteria. From this model, a BDOC value of 0.25 mg/litre and a temperature of 16 ºC were derived as threshold values above which problems can be expected. These values are in agreement with observations in practice. It is not clear to what extent these models are predicting the quality changes in distribution systems, because many studies have shown that microbial activity depends on many variables (LeChevallier *et al.* 1996; van der Kooij 1999). The development and improvement of models are continuing (Huck and Gagnon 2002), with the aim to obtain a tool supporting optimal design and water quality management in distribution systems.

11.4.5 Biological stability

Biologically stable water does not promote the growth of microorganisms during its distribution due to a lack of growth substrates (Rittmann and Snoeyink 1984). Defining biological stability in terms of water quality parameters, however, is rather complicated, because microbial activities as described above are affected by a number of different conditions and the properties of the microorganisms. A low concentration of growth-promoting compounds in treated water is an important factor. Many types of heterotrophic bacteria are adapted to aquatic environments with very low concentrations of easily biodegradable compounds, such as amino acids, carboxylic acids and carbohydrates (van der Kooij and Hijnen 1981, 1984, 1985; van der Kooij *et al.*

1982a). Also, undesirable bacteria, such as *P. aeruginosa*, *Aeromonas* spp. and coliforms, multiply rapidly at substrate concentrations of a few micrograms per litre (van der Kooij *et al.* 1982b; van der Kooij and Hijnen 1988a, 1988b; Camper *et al.* 1991). Consequently, the concentrations of such compounds must be very low in treated water. Based on these findings and observations on the effect of water distribution on AOC concentrations, an AOC concentration of 10 µg carbon/litre has been derived as a reference value for biological stability (van der Kooij 1984, 1992; van der Kooij *et al*. 1989). AOC concentrations below this concentration hardly decrease during distribution in unchlorinated supplies, and HPC values (two days, 22 ºC) remain below 100 cfu/ml (Schellart 1986; van der Kooij 1992). From studies in the USA, it was concluded that coliform regrowth was significantly reduced in chlorinated supplies at AOC values below 50–100 µg carbon/litre (LeChevallier *et al.* 1991, 1996). Observations on changes of BDOC concentrations in the distribution system of Paris led to the conclusion that treated water with a BDOC value below 0.2 mg/litre has a high degree of biological stability (Servais *et al.* 1992; Dukan *et al.* 1996).

In groundwater supplies in the Netherlands, multiplication of *Aeromonas* was observed at AOC concentrations below 10 µg carbon/litre and HPC values (three days, 22 ºC) remaining below 100 cfu/ml. These observations demonstrated the complexity of defining the biological stability of water. For these water types, a clear relationship was observed between the BFR value and the 90th-percentile values of *Aeromonas* concentrations (cfu/100 ml). The risk of exceeding a 90th-percentile value of 200 cfu/ml was less than 20% at BFR values below 10 pg ATP/cm^2 per day (van der Kooij *et al.* 1999). Consequently, biological stability assessment in the Netherlands is based on determining the AOC concentration as a measure for the concentration of potentially available compounds, and the BFR value is an indication of the rate at which these compounds (and possibly also compounds not included in the AOC test) can cause biofilm accumulation. Still, this combination of parameters does not completely describe biological (in)stability in distribution systems because of the effects of materials, corrosion processes and sediment accumulation.

In some situations, at relatively high concentrations of humic compounds, the availability of phosphorus was found to be growth limiting instead of the energy source. A sensitive method has been developed to assess the concentration of available phosphorus (Lehtola *et al.* 1999; Miettinen *et al.* 1999).

Testing of materials for biological stability is also needed, and methods are available for this purpose in several European countries (see above). At present, in the framework of developing a European Acceptance Scheme for products in contact with treated water, investigations are conducted to harmonize test methods.

11.4.6 Suite of tools

Microbial activity in distribution systems depends on complex processes. Controlling microbial activity requires knowledge about these processes and tools to elucidate water quality parameters and distribution system conditions. These tools include:

- methods for assessment of the biological stability of treated water;
- methods for assessment of the biological stability of materials in contact with treated water; and
- models for describing the effects of water quality parameters and distribution system conditions on microbial activity.

11.5 CONTROLLING MICROBIAL ACTIVITY

11.5.1 General

Controlling (limiting) microbial activity in distribution systems is needed to prevent water quality deterioration resulting in non-compliance with regulations, consumer complaints, disease or engineering problems. Microbial activity in the distribution system largely depends on the introduction of energy sources. As has been described above, such compounds may originate from treated water and from the materials in contact with treated water. Accumulated sediments also promote growth. The following approaches can be used for controlling (limiting) microbial activity:

- distribution of biologically stable drinking-water in a system with non-reactive, biologically stable materials;
- maintaining a disinfectant residual in the entire distribution system;
- distribution of treated water with a low disinfectant residual and a relatively high level of biological stability; and
- optimization of the distribution system to prevent stagnation and sediment accumulation.

11.5.2 Biological stability

11.5.2.1 Water treatment

Biologically stable water can be achieved by applying an appropriate water treatment, which includes biological processes. In surface water treatment in the Netherlands, one or several of the following biological processes are applied:

storage in open reservoirs, soil/dune passage, granular activated carbon filtration, rapid sand filtration and sand filtration. These processes are used in combination with physical and chemical treatment processes such as coagulation/sedimentation and oxidation/disinfection (ozone, chlorine) to obtain multiple barriers against microorganisms, pollutants and biodegradable compounds (Kruithof 2001). Thus, achieving biological stability in surface water treatment is only one objective, and the design and dimensions of water treatment are to a large extent determined by the microbial safety and the removal of undesirable chemical compounds.

Biological filtration processes are effective in AOC and BDOC removal. Significant reductions up to 80% can be obtained within about 10 min contact time (van der Kooij 1984, 1987; Zhang and Huck 1996; Carlson and Amy 1998). When ozone is applied in water treatment, usually two filtration stages are needed to reduce the AOC concentration to a level of about 10 µg carbon/litre (van der Kooij 1984). This second filtration stage is also important for the removal of biomass and particles (e.g., carbon fines; Morin *et al.* 1996) as produced in the first filtration stage. The presence of chlorine in the influent of filter beds should be prevented, because the disinfectant hampers biological activity. Coagulation/sedimentation processes can also result in a considerable AOC reduction (van der Kooij 1984), but Volk *et al.* (2000) did not observe an AOC reduction despite 30–38% BDOC removal.

Aerobic groundwater abstracted from sandy soils has a high degree of biological stability as the result of extended biological processes in the aquifer. Anaerobic groundwater usually contains higher concentrations of organic compounds as well as ammonia and methane. The AOC concentration of anaerobic groundwater treated with aeration and one or two filtration steps is usually below 10 µg carbon/litre, and the low AOC/DOC ratio (about 1 µg AOC/mg DOC) suggests that organic carbon has a high degree of biostability (van der Kooij 1992). In such supplies, HPC values remain below 100 cfu/ml, but *Aeromonas* regrowth has been observed (Havelaar *et al.* 1990), and relatively high BFR values have been observed in treated water (van der Kooij 1999). Biostability was improved by cleaning (or replacing) filter material and/or intensifying aeration. These measures resulted in better removals of methane and ammonia, but also gave lower concentrations of iron and manganese in the filtrate (Reijnen *et al.* 1993).

A new development in water treatment is the application of membrane processes. In 2000, a surface water treatment plant including ultrafiltration and reverse osmosis was installed in the Netherlands. Treated water had a high degree of biostability (Kruithof 2001). However, the effects of membrane filtration processes are not yet clear. Microbial activity decreased in an experimental pipe loop supplied with nanofiltered water (Sibille *et al.* 1997), but

other reports suggest that nanofiltration removes BDOC but not AOC (Escobar and Randall 1999).

11.5.2.2 Materials

Selection of appropriate materials is important to maintain biostability in drinking-water distribution systems. This requires a systematic approach based on reliable test methods and criteria. Much information is available about effects of materials on microbial growth (Schoenen and Schöler 1983; Colbourne 1985; van der Kooij and Veenendaal 2001).

11.5.3 Disinfection

Maintenance of high pressures in the mains and prevention of cross-connections are crucial measures for ingress prevention. Maintaining a disinfectant residual, aimed at further ensuring the microbiological quality of water in the distribution system by protecting against microbial contamination and preventing regrowth, is common practice in most water supplies in North America and Europe (Trussell 1999). The discovery of trihalomethane (THM) formation by chlorination (Rook 1974) has caused much debate, and in a number of European countries the use of chlorine in water treatment and distribution is restricted as much as possible (van der Kooij *et al.* 1999; Kruithof 2001). In situations where treated water is not stable, adding a disinfectant to treated water is the only option to limit regrowth, but this approach has a number of limitations and drawbacks, which are listed below.

11.5.3.1 Chlorine

Chlorine is an effective disinfectant against viruses and bacteria, but to a lesser extent against protozoa. Payment (1999) demonstrated that disinfectant concentrations as used in distribution systems had only a limited effect on pathogens. Free chlorine concentrations up to 0.3 mg/litre must be maintained to prevent regrowth and formation of biofilms (Geldreich *et al.* 1972; Speh *et al.* 1976). This approach has the following limitations:

- Chlorine is a highly reactive compound, which forms undesirable side products (THMs) for which maximum values are defined in legislation — e.g., 200 µg/litre for chloroform, 100 µg/litre for bromoform and dibromochloromethane, and 60 µg/litre for bromodichloromethane, recommended by WHO (1996); 100 µg/litre in Europe (European Union 1998); and 25 µg/litre in the Netherlands (VROM 2001).

- Low concentrations of chlorine affect the taste and odour of drinking-water, causing consumers to complain or to use alternative sources (Burttschell *et al.* 1959; Bryan *et al.* 1973).
- Chlorination increases the AOC concentration in water, probably by oxidation of large organic molecules (van der Kooij 1984, 1987).
- The chlorine residual rapidly declines in the distribution system. Usually after about a 10-h residence time, the concentration has dropped below 0.1 mg/litre. Pipe material, in particular cast iron, plays an important role in chlorine reduction (Lu *et al.* 1995; Vasconcelos *et al.* 1997; Prévost *et al.* 1998). Chlorine also enhances the corrosion process.
- Low concentrations of chlorine are not effective in biofilms and sediments (LeChevallier *et al*. 1988a, 1988b, 1990; Herson *et al.* 1991), explaining why coliforms may be observed in the presence of a free chlorine residual (Wierenga 1985; LeChevallier *et al.* 1996).
- Certain microorganisms can survive or multiply in the presence of low concentrations of chlorine. As a consequence, chlorination is causing a shift in the microbial community (LeChevallier *et al.* 1980; Ridgway and Olson 1982). Norton and LeChevallier (2000) observed that chlorination caused a shift to Gram-positive bacteria. Gräf and Bauer (1973) isolated a chlorine-resistant *Corynebacterium* from tap water. Also, mycobacteria are relatively resistant to disinfectants (Carson *et al.* 1978; Taylor *et al.* 2000). Nagy and Olson (1982) found more filamentous fungi in chlorinated than in unchlorinated supplies. The hygienic consequences of these shifts are not clear.

These limitations show that chlorine is not the ideal method to limit regrowth in distribution systems. However, the required technology is simple and cheap, and maintaining a chlorine residual throughout the distribution system is an essential safety measure when distribution system integrity cannot be assured.

11.5.3.2 Monochloramine

Monochloramine is used on a large scale for distribution system residual maintenance and has replaced free chlorine residuals in many supplies in the USA and also in a few supplies in Europe. Monochloramine is less reactive than chlorine, and its application has a number of advantages, including less THM production, limited effect on taste and odour, greater stability in the distribution system and relative effectiveness against biofilms (LeChevallier *et al.* 1988b, 1990). Distribution systems receiving water with monochloramine had lower

coliform-positive samples than distribution systems with chlorinated water (Neden *et al.* 1992). LeChevallier *et al.* (1996) demonstrated that coliform counts in distribution systems were 35 times higher in chlorinated than in chloraminated water. A remarkable achievement of using monochloramine is the reduction in cases of legionellosis compared with chlorinated supplies, which has been explained by the effect of monochloramine on biofilms (Kool *et al.* 1999). However, using monochloramine has a number of drawbacks, including formation of nitrite (Wolfe *et al.* 1990; Skadsen 1993) and reaction with elastomers. Furthermore, monochloramine is toxic to humans, which limits its maximum concentration in water, and is also toxic to fish (Bull and Kopfler 1991). Finally, monochloramine is less effective than chlorine against suspended microorganisms, and application may also result in a shift in the microbial community (see above).

The change from chlorine to chloramine in many supplies indicates that monochloramine has certain advantages over chlorine. However, when compared with systems maintaining quality without disinfectant, the use of monochloramine is not attractive.

11.5.4 Distribution system configuration and maintenance

Reduction of microbial activity can also be achieved by measures in the distribution system. Such measures include preventive actions and corrective activities. Improved system design for maintaining water quality during distribution aims at reducing residence time and stagnation and the use of non-corrosive materials. Conditioning of the water to limit corrosion also appears to be effective in regrowth prevention (LeChevallier *et al.* 1993a). Corrective measures such as cleaning by flushing or pigging have only a limited effect, because these techniques are difficult to apply in transmission mains and trunk lines (LeChevallier *et al.* 1987).

11.5.5 Multiple barriers against microbial activity in distribution systems

Microbial activity in the distribution system is affected by many factors. Therefore, controlling microbial activity can be achieved only with a combination of measures (multiple barriers). Removal of biodegradable compounds from the water is of major importance, but a systematic approach in eliminating or preventing growth-promoting conditions in the distribution system is also essential. When biostability is not achieved, maintaining a disinfectant residual is necessary to prevent water quality deterioration. The

level of disinfectant needed to control microbial activity may be related to the degree of instability, but local conditions (water composition, size of distribution system, water temperature) will also have a large impact. Consequently, a tailor-made solution requires a systematic analysis of the potential hazards to define appropriate control measures and critical control points. This approach should be part of a water safety plan that covers all aspects of drinking-water safety.

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Biodegradable Compounds and Biofilm Formation in Water Treatment and Distribution

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ABSTRACT

In the past decade surveys and case studies have been conducted in several European countries and in Northern America to assess the growth-promoting properties of treated water and its impact on water quality during distribution. BDOC and AOC methods, which are based on different principles, were most commonly applied in these studies. Median values of the AOC and BDOC concentrations in treated water in the USA were about 100 and 300 μg C/L, respectively. In the Netherlands the median AOC value was less than 10 μg C/L. Coliform regrowth in chlorinated water supplies in the USA is limited at AOC values below 100 μg C/L. In the Netherlands, regrowth of heterotrophic bacteria in unchlorinated supplies is limited at AOC values below 10 μg C/L, but regrowth of aeromonads can occur at such low concentrations. Biofilm Formation Rate (BFR) values of treated water with an AOC concentration below 10 μg C/L, as assessed with a biofilm monitor, ranged from less than 1 pg ATP/cm^2•d in slow sand filtrates to values greater than 50 pg ATP/cm^2•d in a number of ground water supplies. Regrowth of *Aeromonas* was limited at BFR values below 10 pg ATP/cm^2•d. Based on these observations it was concluded that the combination of AOC and BFR methods is needed for the assessment of biological stability. AOC and BFR tests are also useful for determining the biofouling potential of antiscalants and water used for membrane systems.

INTRODUCTION

Organic compounds in treated water

In the past 25 years the number of quality criteria for drinking water in (inter) national legislation has increased considerably. Main reasons for this development are : (i), increasing pollution of the raw water sources with man-made contaminants (*e.g.* pesticides) and pathogenic micro-organisms ; (ii), improved analytical tech-

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niques and (iii), the increase of knowledge about undesirable properties of substances and micro-organisms. Concentrations of dissolved organic compounds in treated water range from less than 0.5 mg/L to about 7.5 mg/L in the Netherlands (Vewin, 2000), and up to about 10 mg/L in treated water in the USA (Allgeier *et al.*, 1998). Most organic compounds present in treated water are of natural origin and not directly harmful to the consumer. However limiting the total concentration of organic compounds in treated water is desirable for a number of reasons, including : (i), the colour of the water ; (ii), the reaction with disinfectants resulting in the formation of chlorinated organic compounds (Rook, 1974) and (ii), the presence of biodegradable compounds. In the USA TOC values below 2 mg/L in ground water supplies and below 4 mg/L in surface water supplies avoid the ICR treatment study requirement (Allgeier *et al.*, 1998). EU legislation related to the quality of water intended for human consumption prescribes that the total concentration of organic compounds should not show abnormal changes and the oxidisability (determined with potassium permanganate) of treated water should be less than 5 mg O_2/L (EU, 1998). Biodegradable organic compounds are a variable fraction of the total concentration of organic compounds, but no specific criterion for the concentration of these substances seems to be included in (inter) national legislation.

Biodegradable compounds in water treatment and distribution

Achieving water quality objectives generally requires the application of multiple barriers, which also have an impact on the concentration of biodegradable compounds. Physico-chemical processes, including coagulation/sedimentation and adsorption processes (in granular activated carbon filters) reduce the total concentration of organic compounds, including biodegradable compounds. Membrane filtration processes (nanofiltration and reverse osmosis) also can remove organic compounds. Biological activity in treatment processes such as soil passage, storage in open reservoirs and filtration (sand, granular activated carbon) generally reduces the concentrations of biodegradable compounds. However, under certain conditions biologically activity may hamper treatment processes, *e.g.* clogging of filter beds and biofouling of membranes. Biological activity in the distribution system may impair water quality. Examples include : (i), the multiplication of coliform bacteria ; (ii), increase of colony counts (iii), effects on taste and colour ; (iv), the presence of invertebrates in distribution systems. These problems had been observed in the first half of the 20th century (Heymann, 1928 ; Baylis, 1930), but gained much more attention in the second half of the century when new treatment processes were installed and water quality criteria became more stringent. A new problem was the multiplication of opportunistic pathogens (*Legionella, Mycobacterium*) in plumbing systems (Tobin *et al.*, 1980 ; Wadowsky *et al.*, 1982 ; Fischeder *et al.*, 1991).

Controlling regrowth problems is a water quality objective in drinking water supply. Two different approaches options are used to achieve this goal : (i), distribution of drinking water with a disinfectant residual and (ii), distributing drinking water with a high degree of biological stability (Rittmann and Snoeyink, 1984). Even in the presence of a disinfectant residual it is important to limit the concentra-

tion of biodegradable compounds in drinking water. Consequently, the presence of biodegradable organic compounds in water during treatment and distribution and the related problems have been subject of many studies. These studies included the development of methods for determining the concentration of biodegradable organic compounds or the potential of water to produce microbial biomass. This paper gives a short overview of the developments related to the assessment and significance of biodegradable compounds in water treatment and distribution.

DETECTION METHODS

Shortly after the introduction of the Koch method for determining the concentration of bacteria in treated water it was observed that storage of water samples in many cases resulted in a dramatic increase of the heterotrophic plate counts (Frankland and Frankland, 1894). These observations made clear that (certain types of) bacteria were able to multiply at trace concentrations of organic compounds. Studies on this phenomenon were complicated by the use of cotton-wool stoppers in the test bottles and the fact that colony counts represent only a fraction of the microbial community present in the water. In the Netherlands Beijerinck (1891) suggested to assess the concentration of biodegradable organic compounds in treated water by determining the growth of a selected pure culture in a sterilised sample of the water to be tested. He also pointed at the importance of preventing the introduction of volatile biodegradable compounds into the test water, but test results do not seem available in the scientific literature.

At the beginning of the 20th century Heymann (1928) in the Netherlands developed an alternative method for determining the concentration of biodegradable compounds in (treated) water. He applied biological filtration using sand from a well-functioning slow sand filter and determined the reduction of the concentration of organic compounds (measured as potassium permanganate demand) after several passages, each with a retention time of one day. Heymann (1928) reported that the fraction of biodegradable organic compounds amounted up to 60% in surface water before treatment (Fig. 1) and amounted about 15 to 45% of dune water after rapid sand filtration followed by slow sand filtration. Figure 1 shows that addition of chlorine (0.6 mg/L) increased (about 30%) the concentration of biodegradable organic compounds in water in which biofiltration did not further reduce the concentration of organic compounds. This observation seems to be the first experimental demonstration of this side effect of chlorination.

In about the same period Baylis (1930) reported the increase of counts of coliform bacteria in drinking water distribution systems and demonstrated that biodegradable compounds resulting in sediment formation were the main cause of this problem. About 40 years later a series of reports appeared in the international literature about regrowth problems in distribution systems, in a number of cases related to the use of ozone in water treatment (Geldreich *et al.*, 1972; Dietlicher, 1970). The use of ozone and granular activated carbon filtration in water treatment also stimulated

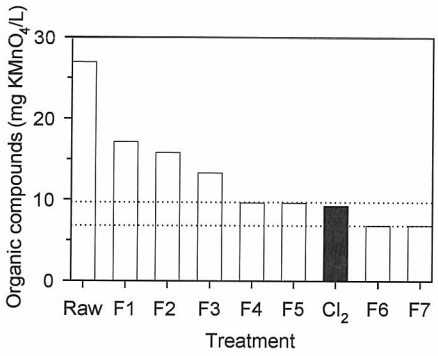

Figure 1. The effect of passages through a sand filter (F) on the concentration of organic compounds (measured as $KMnO_4$ demand) in lake water. Cl_2 indicates the effect of chlorination (0.6 mg/L) on the concentration of organic compounds. F6 and F7 are filter bed passages following chlorination. Based on data from Heymann (1928).

Table 1. Classification of methods developed for determining the microbial growth potential or the concentration of biodegradable organic compounds in treated water.

Key parameter	Mode	Method (units)*	References
Dissolved organic carbon	Batch	BDOC (mg C/L)	Joret and Levy (1986); Servais *et al.* (1987)
Dissolved organic carbon	Flow through	BDOC (mg C/L)	Lucena *et al.* (1990); Ribas *et al.* (1991)
Biomass	Batch	AOC (μg C/L)	van der Kooij *et al.* (1982); van der Kooij (1992); Kaplan *et al.* (1993); LeChevallier *et al.* (1993)
Biomass	Flow through	BFR (pg ATP/cm^2•d)	van der Kooij *et al.* (1995a)

* BDOC: biodegradable dissolved organic carbon; AOC: assimilable organic carbon; BFR: biofilm formation rate (expressed as amount of adenosine triphosphate per cm^2 of exposed surface and per day).

research on the development of methods for determining the concentration of growth-promoting substances in treated water in European countries and in Northern America. The developed methods can be distinguished based on the principle of either using the production of biomass as the key parameter, or by using the reduction of the concentration of dissolved organic compounds (Table 1).

Apart from principal differences between methods, differences also exist between methods with identical names. The BDOC method as developed by Joret and Levy (1986) determines the decrease of the DOC concentration of water samples incubated for a few days with sand from a biological filter. The BDOC method as developed by Servais *et al.* (1987) determines the DOC decrease in water as caused by the indigenous microbial community after an incubation period of 30 days. Volk *et al.* (1994) reported that the BDOC values as obtained with suspended indigenous bacteria are about 60% of the values obtained with samples incubated with sand. The BDOC method based on the flow through system resembles the method as

developed by Heymann (1928) and has the advantage of giving a rapid result. However, installation of a column on site and adaptation of the microbial community are preconditions for proper test results (Kaplan and Newbold, 1995).

CONCENTRATION RANGES AND CRITERIA

The tests mentioned in Table 1 have been applied in many case studies and in a number of surveys aiming at obtaining information about the effects of water treatment on the concentration of biodegradable organic compounds and the relationship with regrowth problems in the distribution system, respectively. Table 2 shows that the median BDOC value in treated water, as observed in surveys in the USA, is about 0.2 to 0.3 mg/L. Similar values have been observed in case studies in France and Canada (Joret and Levy, 1986 ; LeCapellier *et al.*, 1993 ; Kerneis *et al.*, 1995 ; Mathieu *et al.*, 1995 ; Prévost *et al.*, 1998). The BDOC values were 0.4 to 52.8% of DOC (Kaplan *et al.*, 1994) and 5 to 21% of DOC (Volk and LeChevallier, 2000), respectively.

The median value of AOC concentrations in treated water in the USA and in Finland was about 90 to 130 μg C/L (Table 2). Volk and LeChevallier (2000) reported that AOC concentrations were 2.4 to 44% of the DOC values and the AOC concentrations as reported by Miettinen *et al.* (1999) were about 10% of the DOC value. The AOC values of treated water in the Netherlands were much lower than those reported for the USA and Finland, despite the relatively high DOC values (van der Kooij, 1992). These low AOC concentrations which were 0.15% to 1.35% of DOC value, may be explained by (i), a more extensive treatment, including biological filtration ; (ii), no addition of a disinfectant which increases the AOC concentration, and also (iii), differences between the applied AOC methods.

Based on studies relating BDOC values and AOC values to regrowth problems, threshold or reference values have been proposed for biologically stable drinking water :

1) a BDOC value between 100 to 200 μg C/L was derived from the decrease of BDOC in the distribution systems (Servais *et al.*, 1995) although some heterotrophic growth has been observed at even lower levels (Sibille *et al.*, 1997).
2) a threshold AOC value of 100 μg C/L has been suggested to limit coliform

Table 2. Ranges of concentrations of DOC, BDOC and AOC in treated water as observed in a few surveys. Median values are given between brackets.

Country	Systems[#]	DOC (mg C/L)	BDOC (mg C/L)	AOC (μg C/L)*	Reference
Netherlands	20	0.3-8.6 (3.3)	ND**	1.1- 57 (8.1)	van der Kooij (1992)
USA	79	0.2-4.3 (2.0)	0.01-0.97 (0.24)	18-322 (110)	Kaplan *et al.* (1994)
Finland	24	0.6-5.0 (2.7)	ND	45-315 (130)	Miettinen *et al.* (1999)
USA	31	0.6-4.5 (2.0)	0.03-1.03 (0.32)	14-491 (94)	Volk and LeChevallier, 2000

[#] number of supplies included in the investigation ; * acetate-C equivalents/L ; ** ND, not determined.

regrowth in distribution systems with a disinfectant residual (Volk and LeChevallier, 2000 ; LeChevallier *et al.*, 1996).

3) HPC values remained below 100 CFU/ml in unchlorinated supplies at AOC values below 10 μg C/L. At this value AOC uptake in the distribution system was very limited (van der Kooij, 1992).

As has been mentioned by Volk and LeChevallier (2000), BDOC and AOC methods determine different types of biodegradable compounds. The AOC values include the concentration of easily assimilable organic compounds such as amino acids and carboxylic acids that are favourable growth substrates for heterotrophic bacteria, even at concentrations as low as a few micrograms per litre. BDOC includes organic compounds that are utilised by a consortium of microorganisms present at a high biomass concentration. The presence of relatively high BDOC concentrations in tap water at locations far from the treatment plant (Mathieu *et al.*, 1995) suggests that not all BDOC is rapidly utilised in the distribution system. Effects of temperature, seize of the distribution system and the use of a disinfectant residual all affect the relationship between regrowth and BDOC or AOC, thus making it complicated to define the biological stability of treated water.

BIOFILM FORMATION

Biofilm formation rate

In the Netherlands, an additional tool was developed to assess the growth promoting properties of drinking water, because it was found that even at AOC concentrations below 10 μg C/L some regrowth of aeromonads could occur in unchlorinated supplies. A biofilm monitor was developed which enabled the assessment of the biofilm formation rate (BFR) value of water (van der Kooij *et al.*, 1995a). This biofilm monitor consists of a vertically placed glass column (length : 60 cm ; ϕ, 2.5 cm) containing glass cylinders on top of each other. One or two cylinders are periodically sampled from the column. Biomass is removed from these cylinders with ultrasonic treatment and subsequently the concentration is determined using adenosine triphosphate (ATP) analysis. Typical BFR values as observed in treated water in the Netherlands range from less than 1 pg ATP/cm^2•d (slow sand filtrate, aerobic ground water) to a maximum of 100 pg ATP/cm^2•d for a poorly functioning ground water treatment plant using anaerobic ground water (with ammonia and methane) as the source. The biofilm monitor system had been calibrated with acetate added to treated water. A concentration of 10 μg acetate-C/L gave a BFR value of 360 pg ATP/cm^2•d and a BFR value of 35 pg ATP/cm^2•d corresponds with 1 μg C/L of easily available carbon compounds (van der Kooij *et al.*, 1995b). These observations demonstrate that very low concentrations of AOC may affect biofilm formation. From the observed BFR values it can be derived that in most cases the concentration of rapidly available compounds is less than 1 μg C/L in most supplies, and therefore less than 10% of the AOC concentration.

A clear relationship has been observed between the 90 percentile value for aer-

omonads in the distribution systems of unchlorinated ground water supplies and the BFR value of water leaving the treatment plant. From this relationship it was calculated that the risk of exceeding the guideline value for *Aeromonas* (200 CFU/100 ml) is less than 20% at BFR values below 10 pg ATP/cm²•d (van der Kooij *et al.*, 1999). These observations have resulted in a two dimensional approach for assessing and defining biological stability, *viz.* determination of AOC which gives the concentration of available compounds, and the BFR value which give information about the rate of uptake.

Materials in contact with treated water

Materials in contact with treated water may stimulate microbial growth by releasing biodegradable compounds. Tests have been developed to assess the growth promoting properties of such materials. In the Netherlands, the Biomass Production Potential (BPP) test has been developed for this purpose (van der Kooij and Veenendaal, 2001). This test is based on assessing the concentration of active biomass (as ATP) in water and on pieces of a material to be tested during incubation in biologically stable drinking water (slow sand filtrate). The BPP value is expressed as pg ATP/cm² and can be compared with biofilm concentrations on surfaces in distribution systems (van der Kooij *et al.*, 1999).

Biofouling of membrane systems

Biofouling is seriously hampering the use of nanofiltration (NF) and Reverse Osmosis (RO) membranes in water treatment. The biofilm concentration in a membrane elements from a number of installations was determined applying autopsy followed by ATP analysis. The observations revealed that biofilm concentrations on the membranes (including spacer) ranged from less that 100 pg ATP/cm² to more than 10,000 pg ATP/cm² (Fig. 2). An increase of the pressure drop was generally observed at biofilm concentrations above 1,000 pg ATP/cm²•d (Vrouwenvelder *et al.*, 2000).

The rate of biofouling depends on the growth-promoting properties (AOC, BFR) of the feed water, which in turn depend on the composition of the raw water and the

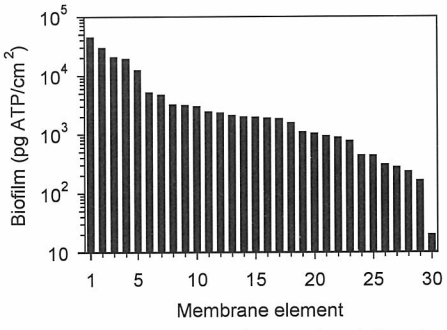

Figure 2. Biofilm concentrations (expressed as pg of adenosinetriphosphate/cm²) as observed in NF and RO membrane elements taken from a number of pilot plants and installations in practice.

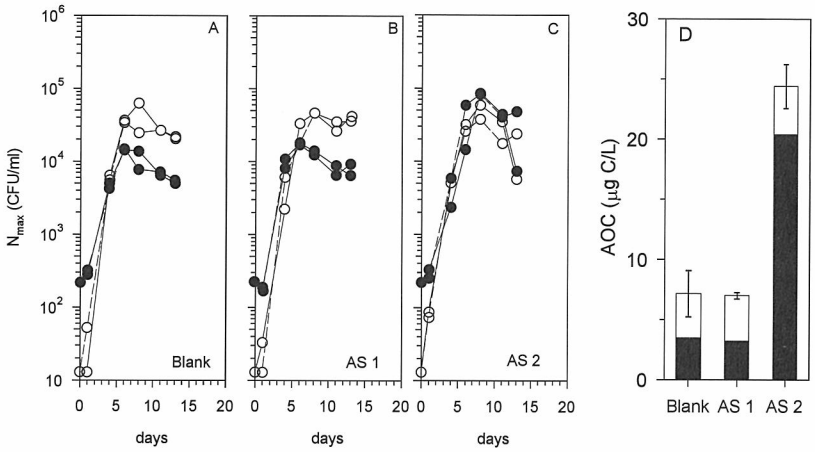

Figure 3. Effect of addition (5 mg/L) of an antiscalant on the AOC concentration of slow sand filtrate. A: growth curves of strain *Pseudomonas fluorescens* strain P17 (l) and Spirillum species strain NOX (⁻) in slow sand filtrate (blank) ; B: slow sand filtrate with antiscalant #1 ; C: antiscalant #2 ; D: AOC concentrations calculated from the maximum colony counts of the strains, using the yield values for acetate. Black bar : contribution of strain P17 to AOC ; open bar : strain NOX.

applied water treatment. Addition of an antiscalant can also affect biofouling when the added compound stimulates microbial growth. For this reason, the growth-promoting properties of a series of antiscalants have been determined (Vrouwenvelder *et al.*, 2000) by applying AOC analysis and BFR measurements. Figure 3 shows that the AOC test can be used to determine the growth potential of an antiscalant.

AOC and BFR measurements can also be used to investigate treatment processes. Effects of ozonation and GAC filtration on AOC concentrations have been described in many reports.

CONCLUSIONS

1) The concentration of biodegradable compounds in treated water has been a concern for more than a century ;
2) A number of methods have been developed to determine the concentration of biodegradable compounds or the growth promoting properties of treated water. These methods are based on different principles (decrease of organic carbon or increase of biomass), or differ in the applied test conditions (sample volume, biomass parameter, static or dynamic), respectively.
3) Data have been collected about the properties of treated water regarding the growth-promoting properties, but the effects of other water quality parameters (*e.g.* temperature, presence of disinfectant residual) and distribution system properties (retention time, materials) on biological processes in distribution systems make it difficult to define the biological stability of treated water. This

may explain why such a parameter is not (yet) included in legislation regarding the quality of treated water.

4) The assessment of the biostability of treated water in the Netherlands is based on applying the AOC test and the BFR test. The AOC test gives information about the concentration of easily available organic carbon compounds, and the BFR test gives information about the rate of uptake of these compounds in the biofilm.
5) Certain materials in contact with drinking water in the distribution system may release biodegradable compounds into the water, and promote the growth of microorganisms. For this reason a test method (Biomass Production Potential, BPP) has been developed which is based on determining the concentration of biomass (as ATP) in water and on the material (biofilm) which is incubated in biologically-stable water (van der Kooij and Veenendaal, 2001).
6) Assessment of the concentrations of active biomass in the situations mentioned gives a framework for comparison and evaluation of results. This approach has been designated as the Unified Biofilm Approach (van der Kooij *et al.*, 1999)

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Tools for fouling diagnosis of NF and RO membranes and assessment of the fouling potential of feed water

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Abstract

A set of coherent tools for (i) analysing the fouling of NF and RO membranes and (ii) determining the fouling potential of feed water is presented in this paper. These tools — developed for diagnosis, prediction, prevention and control of fouling — are applied in practice and have proven their value in controlling fouling. A database obtained with the described tools at plants with and without operational problems is available for the interpretation of observations in pilot plants and in practice.

Keywords: Tools; Fouling; Biofouling; Scaling; Particulate fouling; Diagnosis; Membranes; Drinking water; Autopsy

1. Introduction

Operational problems in membrane installations for nanofiltration (NF) and reverse osmosis (RO) used in water treatment can be caused by a variety of fouling types. Fouling mechanisms of NF and RO membranes include biofouling, organic fouling, inorganic fouling (including scaling) and particulate fouling [1]. Fouling will lead to higher operational costs: higher energy demand, increase of cleanings and reduced life-time of the membrane elements. An effective control of fouling requires a good diagnosis of the foulant present. This diagnosis is preferentially based on the complete picture (Fig. 1) of the type(s) of fouling.

The research of Kiwa is focused on developing methods for diagnosis, prediction, prevention and control of fouling. A set of coherent tools has been developed for (i) determining the fouling potential

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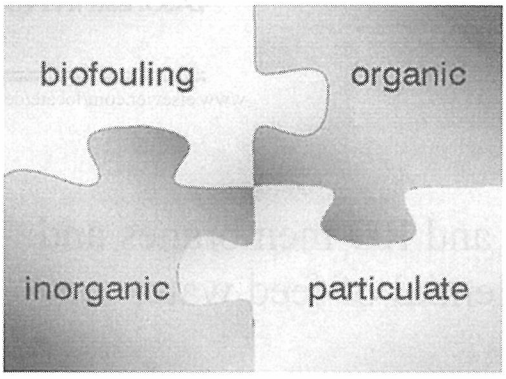

Fig. 1. Complete picture is needed for a reliable diagnosis of the fouling.

of feed water and (ii) analysing the fouling of NF and RO membranes. An overview of the tools — which are applied in practice and have proven their value — is shown in Table 1. These tools will be illustrated in more detail in this paper.

2. Integral diagnosis (autopsy)

Diagnosis of the type(s) and extent of fouling is the first step in controlling fouling in order to maintain plant performance. Studies at (pilot) plants showed that operational problems can be caused by other types of fouling than expected for and that dosed chemicals can pose a risk for (bio)fouling. Therefore, a systematic approach based on the application of an autopsy of membrane elements followed by analysis has been developed, which enables an integral diagnosis of the type(s) and extent of fouling [2]. The autopsy can be performed on-site or in a laboratory (Fig. 2 shows an unfolded membrane element). Analysis includes both biological parameters for biomass quantification (ATP), biomass characterisation, microscopic observations and chemical parameters for determining the presence of inorganic compounds (ICP-MS). This approach gives complete and conclusive information (within 8 h) about the nature and extent of fouling of the membrane filtration plant.

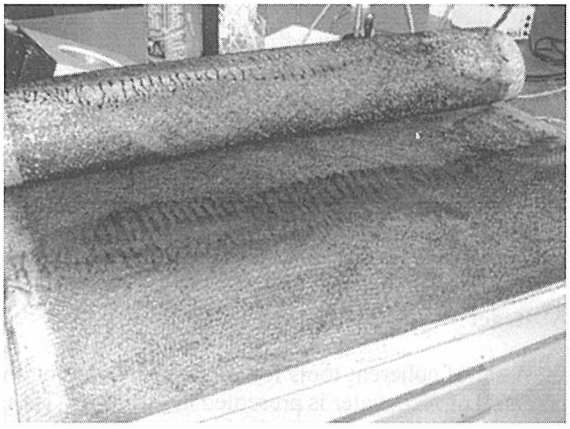

Fig. 2. Unfolded RO-membrane element from a plant with severe fouling within 7 months after start-up of the plant. Is the diagnosis biofouling, (in)organic or particulate fouling?

Table 1
Overview of tools available for determining the fouling potential of feed water and fouling diagnosis of NF and RO membranes used in water treatment

Tool	Fouling diagnosis	Comment
Integral diagnosis (autopsy)	Biofouling, inorganic compounds and particles	Diagnosis of foulant in membrane elements
Biofilm monitor and AOC	Biofouling	Prediction and prevention of biofouling by determining the (growth) potential of water
SOCR	Biofouling	Non-destructive method for determining active biomass in Membrane systems
MFI-UF	Particulate	Particulate fouling potential of water
ScaleGuard	Scaling	Optimising recovery, acid dose and anti-scalant dose

The results of the autopsy — and operational parameters — compared with the database with results of autopsies related to plant performance (NPD, MTC), chemicals dosed (e.g. scale inhibitor) showed whether fouling is present and elucidates the type and extent of fouling present. Diagnosis of the type of fouling enable directed and more effective actions for prevention and control of (bio)fouling.

Autopsies have been conducted in 52 NF and RO membrane elements (several brands) obtained from 17 different (pilot) plants. The various plants were supplied with water, which varied in: (i), type of source water (aerobic, anaerobic, ground water, surface water, tap water and seawater; (ii), pre-treatment; (iii), temperature (from 1 to 30°C, stable and fluctuating) and (iv), dosage of chemicals (e.g. scale inhibitor), respectively. Operational problems with certain plants include an increase of the NPD over 300% or a decrease of the MTC value of about 25% at the time of autopsy.

Quantitative relationships have been established between the pressure drop increase and biofilm development in the membrane modules as determined by the integral autopsy.

3. Biofouling potential of the water

The concentration of growth promoting substances in water can be assessed by the AOC-test [3] and the Biofilm Formation Rate (BFR) in a biofilm monitor [4]. Severe biofouling has been observed in cases where the feed water had BFR-values — temporarily — exceeding 100 pg ATP/cm^2.d. BFR-values lower than 1 pg ATP/cm^2.d, as can be achieved by extensive pre-treatment, viz. slow sand filtration, enabled stable operation for periods up to two years without cleaning [2].

The bacterial growth potential tests (AOC, BFR) showed that the feed water quality could be influenced by (seasonal) variations of water quality prior pre-treatment, which are not completely eliminated in the pre-treatment. Also, the dosage of — even one batch of impure — chemicals (flocculent, scale-inhibitor) to the feed water can cause serious biofouling, leading to early replacement of membranes [5,6]. Testing (AOC, BFR) of 14 commercially available scale-inhibitors based on polymers and dosage to single element units demonstrated that chemicals used to prevent scaling differ greatly in their ability to promote growth of micro-organisms and confirmed the experiences in practice [7].

Quantitative relationships have been established between the pressure drop increase and the microbial growth potential of the feed water and biofilm development in the membrane modules (integral autopsy).

4. The Specific Oxygen Consumption Rate (SOCR)

The Specific Oxygen Consumption Rate (SOCR) is a parameter for determining the presence of active biomass in membrane systems by measuring the oxygen demand [8]. The SOCR is determined with a (in situ) non-destructive method. Plant studies showed a low oxygen demand at a plant without operational problems and low biomass densities in the membrane elements (determined with a autopsy) and a high oxygen demand at another plant with operational problems and high biomass densities in the membrane elements (see Table 2).

At the RO-installation with severe biofouling (plant 2 in Table 2) cleanings were applied with industrial chemicals focused on the removal of biofouling. The cleanings had a moderate and temporarily effect on the NPD-value.

After the cleaning again the SOCR-value was determined and an autopsy of a membrane element was performed. The time between the SOCR and autopsy before and after the cleaning was about 48 h. The SOCR-analysis and the autopsy — both determined after the cleaning — showed the presence of active biomass in the RO-installation indicating that the cleaning was not very effective in the inactivation and removal of the biomass

Table 2
Plant performance, diagnosis of fouling by autopsy and the SOCR of two plants

Plant	Operational characteristics	Diagnosis of autopsy	SOCR (mg $O_2/m^2.h$)
1	Stable, no indications of fouling	No fouling	Low (< 0.05)
2	Severe fouling (NPD increase 300%)	Severe biofouling	High (3.2)

present in the membrane elements before the cleaning (Fig. 3). The SOCR-analysis determined 22 d after the cleaning showed a SOCR-value similar to the value before the cleaning. This indicates that rapid growth of microorganisms in the membrane elements occurred. The fouling was apparently not controlled by the cleaning applied.

The SOCR — which is in the development stage — can be used as an early (on-line) warning system for biofouling.

5. MFI-UF

The MFI-UF is an adapted method of the existing MFI-test [9]. The membrane fouling index is measured with an ultrafiltration membrane in order to measure even the smallest particles. In contrast to the SDI there is a linear correlation with the concentration of particles. The MFI-UF is in the validation stage. The correlation between fouling in full-scale plants and this parameter is investigated by Kiwa in cooperation with other research institutes (TZW, AWWARF, CRC).

Fig. 4 shows the effect of pre-treatment processes on the MFI-UF-value.

6. Scaleguard

The ScaleGuard is a continuous on-line monitor — with a single spiral wound membrane element — which detects scaling in an early stage when fed with the concentrate of a pilot plant or full scale plant [10]. Scaling is observed in the ScaleGuard before it occurs in the full-scale installation. The ScaleGuard has been used to optimise the recovery of six full-scale plants and is also applied for optimisation of the dosage of acids and anti-scalants.

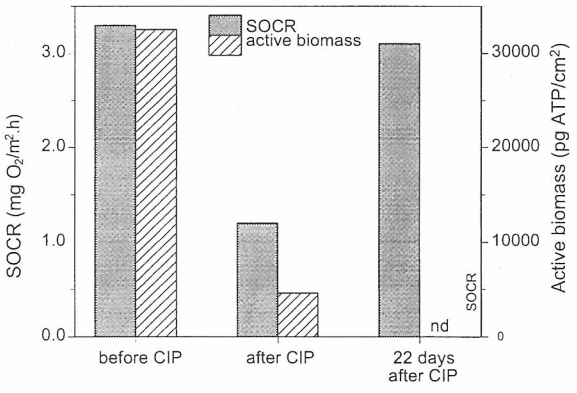

Fig. 3. SOCR and ATP concentrations obtained at a severely fouled RO-membrane installation before and after cleaning in place (CIP). The SOCR was determined in situ and ATP was determined by autopsy on a membrane element taken from the installation.

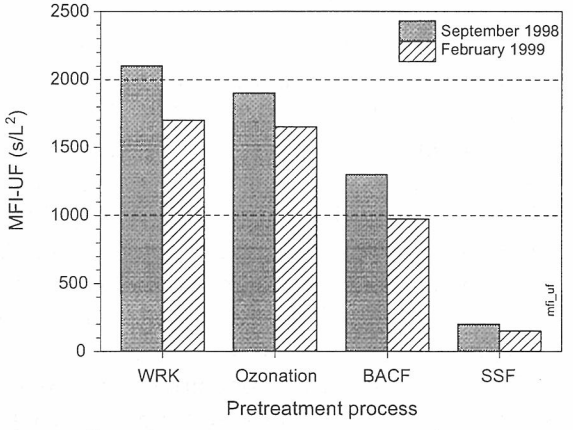

Fig. 4. Effect of pre-treatment processes on the MFI-UF at the River Rhine RO pilot plant which applies conventionally pre-treated water (WRK-I) followed by ozonation, biologically activated carbon filtration (BACF) and slow sand filtration (SSF).

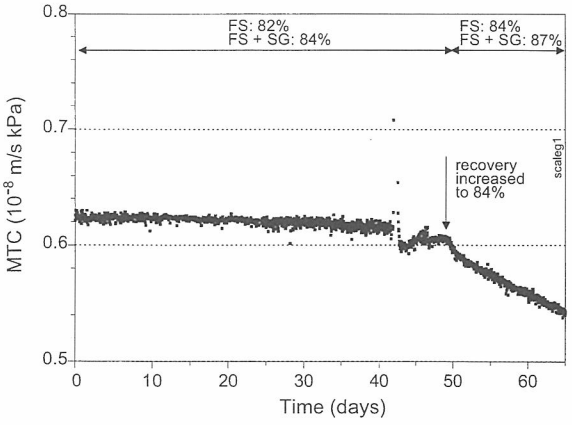

Fig. 5. MTC-data in the ScaleGuard at a recovery of the full-scale plant including the ScaleGuard (FS+SG) at 84% and 87%, respectively. The recovery of the FS without the ScaleGuard (FS) was 82% and 84%, respectively.

Fig. 5 shows the MTC-data of the ScaleGuard in time at a nanofiltration plant. At a recovery of 82% of the full scale installation (FS) the recovery of the FS including the ScaleGuard (FS +SG) was 84%. Increasing the recovery of the FS to 84% led to an increase of the recovery of the FS+SG up to 87%. In the first period a slight decrease of the MTC can be observed. Because the recovery in the SG is about 2% higher than the recovery of the full scale, no scaling is expected in the last element of the full scale. After increasing the recovery to 87% (FS+SG) the MTC-decrease is much faster indicating that scaling occurred.

7. Evaluation

A set of coherent tools is available for (i) determining the fouling potential of feed water and (ii) analysing the fouling of NF and RO membranes. The tools presented can be used to (i) assess the cause of fouling, (ii) further define criteria for feed water to predict and minimise the risk of fouling and (iii) evaluate cleaning strategies. Appropriate use of these tools can reduce the operational costs of membrane plants.

A database obtained with the described tools from analysis of feed water and membrane elements from plants with and without operational problems is available for the interpretation of observations in pilot plants and in practice.

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ASSIMILABLE ORGANIC CARBON (AOC) IN DRINKING WATER.

See BIODEGRADABLE DISSOLVED ORGANIC CARBON IN DRINKING WATER

ASSIMILABLE ORGANIC CARBON (AOC) IN TREATED WATER: DETERMINATION AND SIGNIFICANCE

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CONTROLLING MICROBIOLOGICAL WATER QUALITY

The quality of drinking water at the consumer's tap depends on a chain of processes, namely, the effects

of water treatment on the raw water, the effects of distribution on the composition of treated water, and the effects of the household plumbing system, respectively. Water quality criteria related to health are defined in national and international regulations and the water industry has the obligation to provide a safe drinking water. Microbiological safety is of major importance because ingestion of pathogens can have a direct impact on the health of the consumer. For this reason, multiple barrier approaches are used in water treatment in combination with frequent monitoring of indicator bacteria in treated water and pathogens (protozoa and viruses) in raw water, respectively. A multiple barrier approach is also used to ensure the safety of drinking water in the distribution system (1). Barriers against contamination of drinking water in the distribution system include (*1*) a constant high pressure in the mains, (*2*) cross-connection prevention measures, (*3*) safety procedures for activities in the distribution system, (*4*) reliable materials and construction techniques. Also, appropriate water quality monitoring is important to check the effectiveness of these preventive measures. Maintaining a disinfectant residual in drinking water during distribution is considered as an additional safety factor (2), but in a few European countries it is allowed to distribute drinking water without disinfectant residual (3–5). The main disadvantages of maintaining a disinfectant residual are the effect on taste and odor of the water, and/or the formation of disinfection by-products, including trihalomethanes with toxic properties (6–9).

A main function of the disinfectant residual is the prevention of multiplication of microorganisms in drinking water distribution ("regrowth"). Controlling regrowth is important to the water industry for the following main reasons (*1*) multiplication of coliform bacteria results in noncompliance with regulations (10), (*2*) the multiplication of opportunistic pathogenic bacteria, including species of *Legionella*, *Mycobacterium*, *Pseudomonas, Aeromonas*, and *Flavobacterium* poses a potential health threat to the consumer (11–15), (*3*) heterotrophic plate count values may exceed those defined in national legislation and may cause problems in the food industry, (*4*) microbial activity and biomass may affect turbidity, color, taste, and odor of the water (16), (*5*) microbiological processes may accelerate corrosion (Microbiologically Induced Corrosion, MIC) of pipe materials (17,18), (*6*) invertebrates multiplying on bacterial biomass may cause consumer complaints (19,20).

In the absence of a disinfectant residual regrowth is controlled by distributing biologically stable water, as obtained by a far-reaching removal of biodegradable compounds from water during treatment (21). Assessment of the degree of biological (in)stability requires a method for determining the concentration of growth-promoting compounds in water or the microbial growth potential. This entry describes the assimilable organic carbon (AOC) method and its significance for defining biological stability.

REGROWTH PROCESSES AND GROWTH KINETICS

Regrowth is a complex phenomenon affected by many variables. Key processes in regrowth are the uptake of biodegradable compounds by microorganisms, formation of biofilms on the exposed surface detachment of (micro-)organisms and dead biomass from the pipe wall and accumulation of sediments, respectively. The presence of biodegradable compounds is a major driving force in these regrowth processes but other environmental conditions (e.g., temperature, system hydraulics, and retention time) and physicochemical processes (adsorption, oxidation/reduction, and coagulation/sedimentation) also have significant effects on the extent to which microorganisms multiply. When a disinfectant is added to water before distribution, the situation is even more complex because the concentration of disinfectant generally changes in time and differs among various locations in the system (22).

Bacteria present in the biofilm are released into the water and may be detected in routine monitoring of the microbiological quality of drinking water. Most bacteria predominating in regrowth processes do not seem to constitute a direct hygienic or aesthetic problem (23,24), but their ability to produce biomass under the prevailing conditions is a main cause of the enables Parts of the biofilm sloughing from the surface may settle in pipes at low flow conditions and contribute to the formation of sediments. Biofilms and sediments of decaying biomass are niches for undesirable bacteria including coliforms (25), aeromonads (12), *Legionella* spp. (13,15), *Mycobacterium* spp. (11,14), but also fungi and yeasts may be present (26). Certain invertebrates use biofilms and sediments as a food source (19,20). (See also this Encyclopedia Invertebrates and protozoan in drinking-water distribution systems, Van Lieverloo et al.).

The rate and extent of multiplication of microorganisms in water depend on the concentration and the composition of the available food sources. These food sources provide energy and the elements needed for the biomass synthesis. Heterotrophic bacteria utilize organic compounds as a source of energy and carbon. In addition, certain inorganic compounds are required, in particular nitrogen and phosphorus, and a number of other elements at much lower amounts, for example, sulfur. The need for carbon, nitrogen, and phosphorus can be derived from the gross composition of microbial biomass, $C_5H_7NO_2P_{1/30}$, and the proportion (50%) of organic carbon used for dissimilation (27). Hence, organic compounds serving as a source of energy and carbon are needed in much larger amounts than the inorganic nutrients nitrogen and phosphorus (C : N : P = 100 : 10 : 1). Nitrate, a common compound in drinking water, is a good nitrogen source for many aquatic bacteria, although certain types (e.g., *Aeromonas* spp.) require ammonia-nitrogen for growth (28). Phosphate is also a common compound in the aquatic habitat, and it is only needed in very low quantities relative to the carbon source. Hence, the availability of suitable organic compounds will be the growth-limiting parameter for heterotrophic bacteria in most water types. Some studies suggest that phosphate may be growth limiting in certain types of groundwater, which contain a relatively high concentration of organic compounds (29).

The amount of biomass produced on a certain amount of substrate depends on the yield (Y) values of the bacteria for the present compound(s). Generally, 1 mg of substrate

carbon yields 1 mg of biomass (dry weight) during aerobic growth (27). Expressing the yield value as number of bacteria (colony forming units, CFU) is more appropriate for describing regrowth processes. Typical yield values for compounds serving as favorable growth substrates for heterotrophic bacteria (low molecular organic compounds) of 4×10^6 to 2×10^7 CFU have been reported (see later). Hence, utilization of 1 µg of C/L theoretically corresponds with a maximum colony count value of 10^4 CFU/mL. The substrate concentration also affects the rate of growth. This relationship is described by the Monod equation (30):

$$V = V_{max} \times S/(K_s + S) \quad (1),$$

where: V = the growth rate (doublings/hour) in the exponential growth phase at substrate concentration S; V_{max} = maximum growth rate (h^{-1}); K_s = the substrate saturation constant, is the concentration of S at which $V = 1/2V_{max}$. An example of such a relationship is presented in Figure 1, which clearly demonstrates that the increase of the growth rate is less than proportional to the increase of the substrate concentration. However, at very low values for S, that is, $S \ll K_s$ then:

$$V = V_{max}/K_s \times S \quad (2)$$

and the growth rate is linearly related with the substrate concentration S and also depending on the values for V_{max} en K_s, which are constants for specific combinations of organism and substrate. The quotient V_{max}/K_s ($h^{-1} \ldots \mu g^{-1} \ldots$ L, the substrate affinity constant is numerically identical to the growth rate calculated for a substrate concentration of 1 µg C/L.

Bacteria representing the indigenous bacterial flora of drinking water have K_s values for easily degradable low molecular weight compounds as low as a few micrograms of C/L. Relatively low K_s values have been also been observed for the high molecular weight compounds amylopectin and amylose (Table 1). *Escherichia coli*, coliforms, and also *P. aeruginosa* have higher K_s values than the typical aquatic bacteria for identical substrates. Still, the K_s values of 13 to 72 µg of glucose-C/L (31,32), and 50 to 150 µg of yeast extract/L (38) as reported for *E. coli* are also relatively low. Despite these kinetics, *E. coli* and *P. aeruginosa* do not belong to the indigenous bacterial community of drinking water. This may be due to the relatively slow growth of these bacteria at temperatures below 15 °C (34) and the very low concentrations of easily available compounds in treated water.

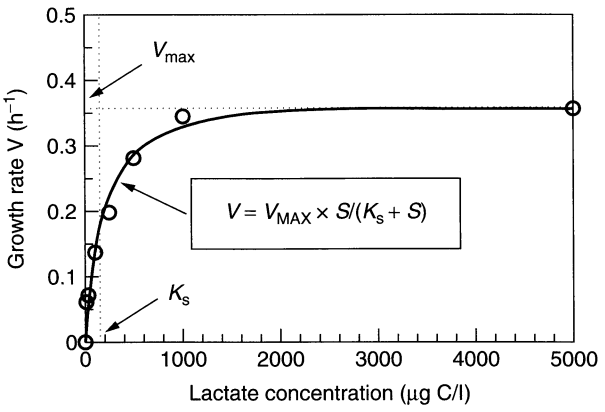

Figure 1. The growth rate of an *Escherichia coli* strain (isolated from river water) at various concentrations of lactate in autoclaved slow sand filtrate (+PO_4^{3-}-P and NH_4^+-N) incubated at 25 °C. The constants of the Monod relationship (K_s and V_{max}) are given in Table 1.

As a result of maintenance energy requirements, a certain minimum substrate concentration (S_{min}) is required for survival without growth (35). Typical maintenance rates for bacteria due to endogenous substrate utilization range from 0.005 to 0.02 h^{-1} (36), but a much higher value (0.3 h^{-1} at 37 °C) has been reported for *E. coli* (31). The values for the substrate affinity of a number of bacteria presented in Table 1, are below the value of maintenance rate. Consequently, these bacteria are not able to grow at a concentration of 1 µg of C/L of the specified compounds. In practice the situation is more complicated because in most situations, several compounds will be present. Most of the bacteria described in Table 1 are able to utilize a number of substrates simultaneously when present at an individual compound concentration of 1 µg C/L. A mixture of amino acids stimulated the multiplication of *A. hydrophila* at an individual compound concentration as low as 0.05 µg C/L (28).

The growth kinetics as presented in Table 1 demonstrate that low concentrations of easily available organic compounds can cause a rapid and significant growth of bacteria present in treated water. For this reason, the method for assessing the regrowth potential of treated water as developed in the Netherlands was mainly directed at determining the concentration of such compounds.

Assessment of the Microbial Growth Potential of Treated Water

Already at the end of the nineteenth century, studies were conducted on bacterial multiplication in treated water (44). It was observed that the number of culturable bacteria was much higher in stored samples of a high-quality deep-well water with a low concentration of organic compounds (up to 5×10^5 CFU/mL) than in river water samples (4,300 CFU/mL). The assumption was made that the well water obtained from the chalk contained a relatively high concentration of biodegradable compounds because of the absence of bacteria in this water directly after abstraction. On the other hand, it was known at that time that the relatively strong growth in treated water stored in flasks with a cotton was related to compounds present in the air. Also, the difference in growth between well water and river water was most probably the result of the multiplication of a few types of bacteria contributing to the HPC values in well water, whereas many of the bacteria present in river water were not able to grow on a solid medium. These observations show that determining the growth potential using colony counts of the indigenous bacterial community has a number of limitations. In the Netherlands, Beijerinck (45) suggested in 1891 that the growth promoting properties of drinking water be studied by inoculating selected pure cultures in sterilized

Table 1. Growth Kinetics of Bacteria Isolated from Drinking Water*

Organism	Compound	Temp. (°C)	K_s (μg C/L)	V_{max} (h^{-1})	V_{max}/K_s (h^{-1}).μg^{-1}.L	Reference
Aeromonas hydrophila	Acetate	15	11	0.15	0.013	28
Aeromonas hydrophila	Glucose	15	16	0.28	0.018	28
Aeromonas hydrophila	Amylose	15	93	0.26	0.0028	37
Aeromonas hydrophila	Oleate	15	2.1	0.23	0.109	28
Citrobacter freundii	Glucose	15	95	0.17	0.0018	38
Enterobacter sp.	Glucose	15	60	0.21	0.004	38
Escherichia. coli	Lactate	25	142	0.37	0.0026	This paper
Flavobacterium sp.	Glucose	15	3.3	0.21	0.063	39
Flavobacterium sp.	Glucose	15	109	0.15	0.001	40
Flavobacterium sp.	Maltose	15	23.7	0.37	0.016	40
Flavobacterium sp.	Maltopentaose	15	5.7	0.44	0.077	40
Flavobacterium sp.	Amylose	15	26	0.50	0.020	40
Flavobacterium sp.	Amylopectin	15	11	0.48	0.044	40
Klebsiella pneumoniae	Maltose	15	51	0.49	0.0096	41
Klebsiella pneumoniae	Maltopentaose	15	92	0.41	0.0045	41
Pseudomonas fluorescens	Acetate	15	4.	0.18	0.045	42
Pseudomonas fluorescens	Glucose	15	57	0.22	0.004	42
Pseudomonas aeruginosa	Acetate	15	28	0.09	0.003	34
Spirillum sp.	Oxalate	15		0.24	0.016	43

Note: * growth measurements conducted in slow sand filtrate

samples of the water that was to be tested. He also pointed out the necessity to prevent contamination by volatile compounds. However, no results were presented and it seems that this method did not attract much attention. In 1928 a completely different approach was reported by Heymann (46) who determined the effect of a series of passages of water through sand columns on the concentration of organic compounds, measured as permanganate value. However, this method also did not gain wide application.

Interest in the regrowth phenomenon strongly increased in the Netherlands in the 1970s as a result of the introduction of ozonation and granular activated carbon (GAC) filtration in water treatment for the removal of persistent organic pollutants. In 1978 a method for determining the heterotrophic growth potential of treated water was described, which was based on determining the maximum level of growth of a selected pure culture in samples of water collected and contained in thoroughly cleaned glass-stoppered Erlenmeyer flasks (47,48). Subsequently, this method, which was designated as the AOC method, was improved by including a bacterial strain with the ability to utilize certain carboxylic acids, as produced by ozonation (49). Regrowth phenomena gained also attention in other European countries and in the United States, and a series of methods for assessing the growth potential of drinking water have been developed (Table 2).

Table 2. Characteristics of Methods for Assessing the Microbial Growth Potential of Treated Water

Method*	Mode	Organisms	Parameter	Reference
AOC	Batch	Pure culture	CFU	43,47,48,53
AOC	Batch	Indigenous bacteria	ATP	54
AOC	Batch	Pure cultures	ATP	55
BGP	Batch	Indigenous bacteria	Turbidity	56
BDOC	Batch	Indigenous bacteria	DOC	57,58,59
BDOC	Column	Indigenous bacteria	DOC	60,62
BFR	Column	Indigenous bacteria	ATP	5,51,52,62

*AOC, easily Assimilable Organic Carbon; BGP, Bacterial Growth Potential; BDOC, Biodegradable Dissolved Organic Carbon; BFR, Biofilm Formation Rate.

Batch tests are most commonly used because such test can be conducted under controlled conditions in the laboratory. These tests give information about the growth potential but the test conditions do not reflect the processes occurring in the distribution system. Another limitation of the AOC and BDOC tests is that the growth potential of treated water may also be affected by the presence of inorganic compounds (e.g., ammonia and sulfides) and methane, which are not included in these tests. A variety of techniques and devices is available to simulate the biofilm formation as occurring in distribution systems, for example, Rototorque system (49), Robbins device, coupon test (50), and a biofilm monitor (51,52). A combination of growth tests, determining the (effects of) concentrations of rapidly and more slowly available compounds, as well as chemical analysis (e.g., for ammonia and methane), may be needed to assess the biostability of treated water. In the Netherlands biostability assessment of water is conducted by using the AOC test and the BFR test (5).

AOC METHOD

Principle of the Test

As explained earlier, easily biodegradable organic compounds may cause significant growth at a level of a few micrograms per liter. Therefore, a test was developed aiming at determining low concentration of these compounds, which were designated as assimilable organic carbon (AOC), derived from earlier descriptions where the terminology "assimilable compounds" was used (46). Assessment of the concentration of easily assimilable

organic carbon (AOC) is based on growth measurements of a mixed culture of two select bacterial strains in a sample of pasteurized water collected and contained in a thoroughly cleaned glass-stoppered Erlenmeyer flask. On the basis of the maximum colony counts of these organisms, the original concentration of substrates is calculated using the yield values of the bacteria for acetate. Hence, the AOC concentration is expressed as μg of acetate-carbon equivalents/L.

Test Strains

The strains used in the AOC test are *P. fluorescens* strain P17, which is capable of utilizing a wide range of low molecular weight compounds at very low concentrations (42) and a *Spirillum* sp. strain NOX, which only utilizes carboxylic acids (43). The general properties of these organisms are described in Table 3, together with another organism specialized in the utilization of carbohydrates (63).

P. fluorescens strain P17 originates from tap water and belongs to one of the most commonly occurring biotypes of the fluorescent pseudomonads in drinking water (64). Growth measurements conducted in drinking water supplemented with mixtures of compounds revealed that the organism can utilize most or all amino acids, a number of carboxylic acids, some carbohydrates and a number of aromatic acids, at a concentration of 1 μg of C/L when present as a mixture (Fig. 2a; 42). In addition, the organism requires a simple nitrogen source and multiplies rapidly on agar media. For these reasons, strain P17 was selected for determining the concentration of easily AOC in (drinking) water (48). The yield factor of strain P17 for acetate-carbon (4.1×10^6 CFU/μg carbon) is used for calculating the AOC concentration from the maximum colony counts (N_{max}, CFU/mL) obtained in the water types tested.

Spirillum species strain NOX had been isolated from slow sand filtrate enriched with 25 μg C/L of oxalate, glyoxylate and formate, respectively (43). The strain is specialized in the utilization of carboxylic acids, including formate, oxalate, glycollate and glyoxylate, which are not used by strain P17. Growth tests conducted with mixtures of substrates with an individual compound concentration of 1 μg of C/L clearly revealed that only carboxylic acids were utilized at this low concentration (Fig. 2b). As a result of its preference for carboxylic acids, the N_{max} value of strain NOX gives information about the concentration of carboxylic acids in the water tested. Yield factors have been determined for acetate and for oxalate, respectively. The yield (CFU/μg of carbon) of strain NOX for acetate is about four times higher than for oxalate-carbon. This difference can be explained by the low energy content of oxalate (27). The organism is used in a mixed culture strain P17 for determining the concentration of easily assimilable organic carbon (AOC) in (drinking) water.

Flavobacterium species strain S12 was obtained from slow sand filtrate incubated at 15 °C after enrichment with 100 μg of starch-C/L (63). The organism is specialized in utilizing carbohydrates, including amylose, amylopectin, maltose, and maltodextrins, which are not used by strain P17 nor by strain NOX. Growth tests conducted with mixtures of substrates with an individual compound concentration of 1 μg of C/L clearly revealed that only certain carbohydrates were utilized at this concentration. N_{max} values of strain S12 therefore give information about the concentration of maltose- and maltodextrin-like compounds in the water tested. Yield factors of strain S12 for starch, maltose, and maltodextrins range from 2.0×10^7 to 2.3×10^7 CFU/μg C (40). Concentrations of

Table 3. Properties of *P. fluorescens* Strain P17, Spirillum sp. Strain NOX, and Flavobacterium sp. Strain S12, Respectively (42,43,63)

Characteristic	*P. fluorescens* strain P17	*Spirillum sp.** strain NOX	*Flavobacterium* sp. strain S12
Origin	Tap water	Slow sand filtrate	Slow sand filtrate
Shape	Rod	Curved rod	Rod
Motility	+	+	−
Gram stain	−	−	−
Oxidase	+	+	+
N-sources	NO_3/NH_4	NO_3/NH_4	NO_3/NH_4
Max. growth temperature (°C)	30	30	30
Arginine dihydrolase	+	+	−
Denitrification	+ (N_2)	−	−
O/F test with glucose	+/−	−/−	−/−
Hydrolysis of:			
– Gelatin	+	−	−
– Tween-80	+	−	−
– Starch	−	−	+
– Chitin	−	−	−
Preferred substrates	Versatile organism	Carboxylic acids	Carbohydrates

*a recent 16SRNA sequencing analysis indicated that the organism is related to the genus *Ultramicrobium* (unpublished observation).

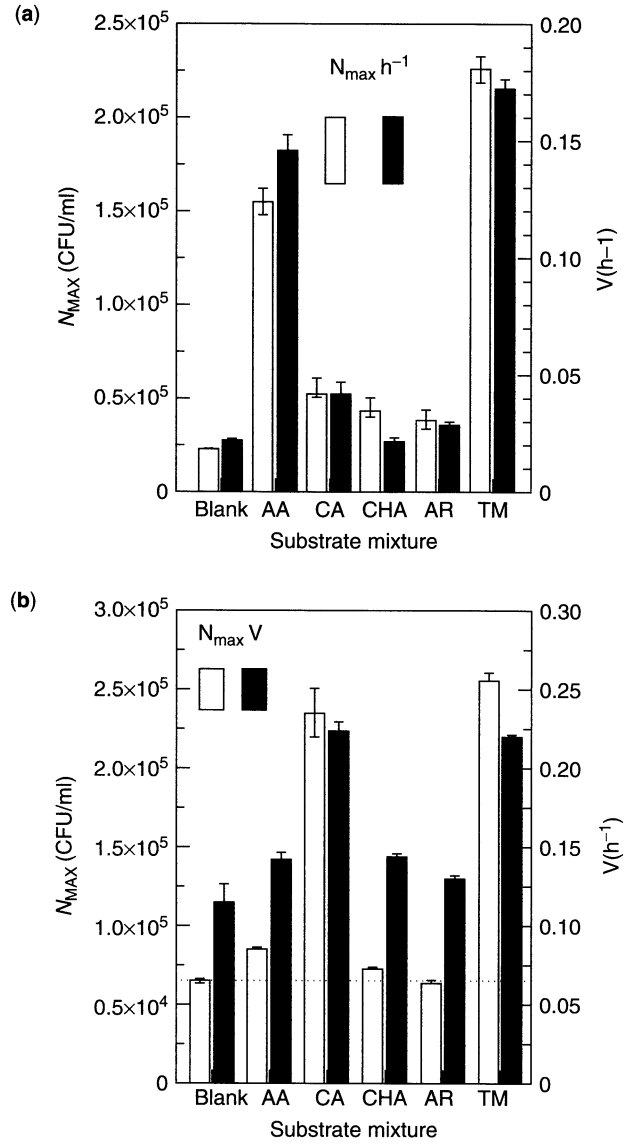

Figure 2. (**a**) Maximum colony counts and growth rates of *P. fluorescens* strain P17 in the presence of mixtures of compounds at an individual compound concentration of 1 µg C/L. Blank: slow sand filtrate without added substrate; AA, 19 amino acids; CA, 14 carboxylic acids; CHA, 6 carbohydrates/alcohols; AR, 7 aromatic acids; TM, total mixture (46 µg C/l). Error bars indicate duplicate measurements. Data adapted from Fig. 3. (**b**) Maximum colony counts and growth rates of *Spirillum* sp. strain NOX in the presence of mixtures of compounds at an individual compound concentration of 1 µg *C/L*. Error bars indicate duplicate measurements.

carbohydrates available for strain S12 in drinking water usually are very low (<1 µg of C/L) and therefore, the organism is not included in the test for determining the concentration of easily assimilable organic carbon (AOC) in (drinking) water. In certain water types however, a significant concentration of carbohydrates may be present, for example, when algal growth occurred in the raw water and/or when a starch-derived coagulant aid is used in water treatment (40).

Other strains can be used for growth measurements to determine the utilization of present or added substrates or to determine the growth potential of the water for a specific organism. Typical examples of such strains include *Aeromonas* species (28), representatives of the coliform group, including *E. coli* (65,66; Fig. 1) and also *P. aeruginosa* (43).

Test Conditions

Sampling and transportation of the samples and sample treatment are essential to obtain an accurate AOC value. For this reason, representative samples of the water to be investigated are collected in duplicate in thoroughly cleaned (including heating at 550 °C for four hours) Erlenmeyer flasks. In these flasks the water samples are transported, pasteurized, and incubated, respectively. Hence, contact with surfaces and air is restricted as much as possible. Pasteurization aiming at inactivating the indigenous bacterial community as present in the water samples is conducted by placing the flasks in a water bath at 90 °C. The flasks are removed from the water bath when water temperature has reached 60 °C and subsequently are placed in an incubator at 60 °C for 30 minutes. After cooling with cold tap water, the test strains are added to

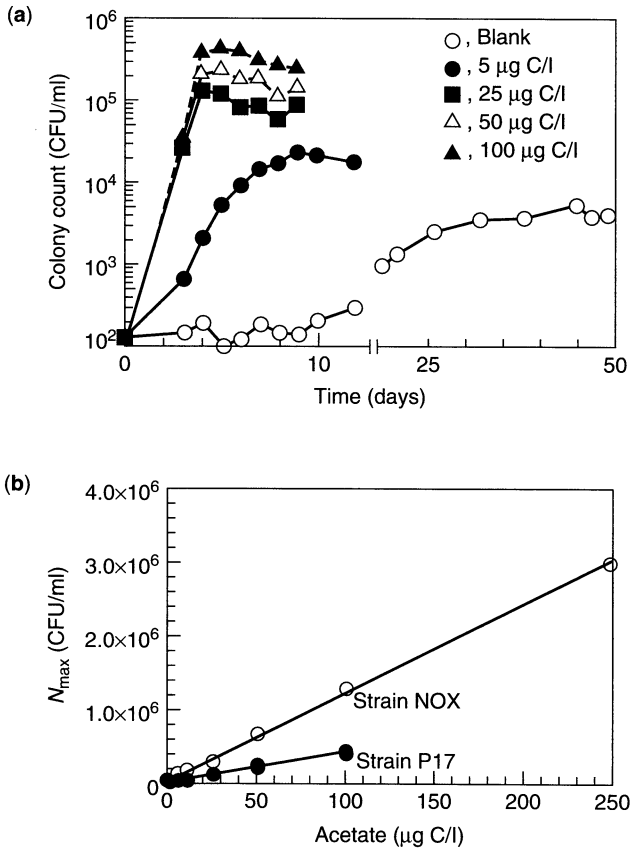

Figure 3. (**a**) Growth curves of *P. fluorescens* strain P17 in pasteurized slow sand filtrate (DOC = 2.3 mg/L) supplemented with different acetate-C concentrations (at 15 °C); (**b**) calibration curves of strain P17 and strain NOX for acetate. Yield values are presented in Table 4.

the flasks. Cultures of the test strains grown in tap water at an initial concentration of 1 mg of acetate-C/L, in which the maximum colony count had been reached are used as inoculum. The inoculum gives is about 50 to 500 CFU/mL in the pasteurized samples. The flasks are incubated at 15 ± 1 °C in the dark, without shaking.

Membrane filters have been used to remove the indigenous bacteria from the samples. However, organic compounds can release from: (*1*) the filters, (*2*) the equipment used for membrane filtration, and (*3*) the flasks in which the filtered water is collected. Furthermore, membrane filtration does not always result in sterile samples because of the presence of very small bacterial cells (ultramicrobes). Hence, application of membrane filters may give unreliable results. Also, autoclaving is not appropriate because it causes a strong increase (three- to fourfold) in the AOC concentration, depending on the water type (unpublished results).

In certain samples, detoxification of the water is required, for example, in the presence of a disinfectant residual, or in the presence of copper. Growth measurements with thiosulphate, used to neutralize a disinfectant residual showed an AOC increase of about 5 μg C/L at a concentration of 10 mg/L. EDTA and NTA do not affect the AOC concentration as determined with strains P17 and NOX (unpublished results). The use of NTA is preferred because this compound is more easily biodegradable in the environment than EDTA.

Growth Measurements and Calibration Curves

The growth curve is determined by periodic colony counts for which purpose water volumes of 0.05 mL are spread in triplicate over the surface of agar medium plates, followed by incubation at 25 °C for 2 (P17) to 3 (NOX) days. A broth agar medium (Lab Lemco Agar, Oxoid) is used routinely, but the R_2A medium 67 has the same recovery for strain P17 and strain NOX (data not shown). Colonies of strain P17 are larger than those of strain NOX and produce a fluorescent pigment on R_2A medium.

The yield (Y) values of strain P17 and strain NOX for acetate are needed to calculate the AOC concentration from the maximum colony counts. Typical growth curves of strain P17 in slow sand filtrate supplemented with different acetate concentrations are presented in Figure 3. N_{max} values were reached after five to seven days at acetate concentrations greater than or equal to ≥10 μg C/L, and after about 10 to 14 days at an initial acetate-carbon concentration of 5 μg C/L. Similar experiments have been conducted with strain NOX 43. The relationship between N_{max} values and the acetate concentrations is used for calculating the yield values (Fig. 3b) At acetate-carbon concentrations above 100 μg C/L, the N_{max} value of strain P17 remains below the level expected on the basis of the values observed at lower concentrations (data not shown). This is probably because the cells are larger when grown at higher substrate concentrations. This phenomenon has not been observed with strain NOX. Yield values of a number of test strains are given in Table 4.

AOC concentrations are determined using a mixed inoculum of strain P17 and strain NOX. The AOC concentration is determined from the N_{max} values of each of the strains, using their Y value for acetate (Fig. 4). Next to the AOC concentration, information also is obtained about the AOC composition because strain NOX can only grow on carboxylic acids (40).

Figure 5 shows the contributions of strain NOX and strain P17 to the AOC concentration when grown simultaneously in pasteurized slow sand filtrate supplemented with yeast extract. Growth of strain NOX was not enhanced by the addition of yeast extract. The Y value of strain P17 for yeast extract is 0.35 μg AOC/μ g YE.

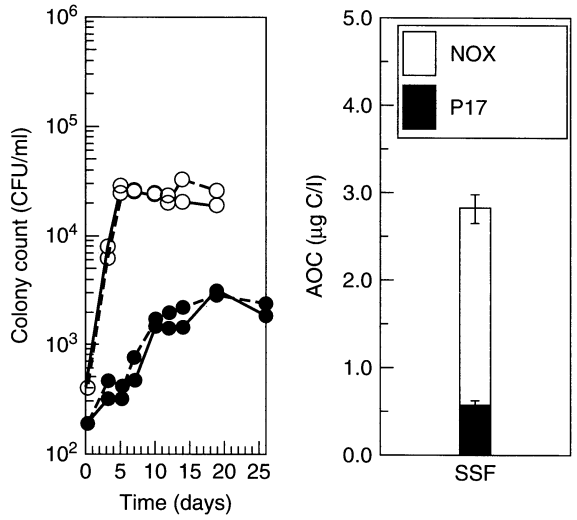

Figure 4. Growth curves of test strains P17 and NOX in slow sand filtrate (SSF) and the AOC value calculated from the N_{max} values of the strains. In these samples the concentration of AOC compounds utilized by strain P17 was less than 1 μg C/L.

Table 4. Yield Values of a Number of Bacterial Strains Used in Growth Experiments (28,40,42,43,48)

Organism	Y value (CFU/μg C) Acetate	Oxalate	Glucose	Starch	Lactate
P. fluorescens strain P17	4.1×10^6	No growth	Nd*	No growth	Nd*
Spirillum sp. strain NOX	1.18×10^7	2.9×10^6	No growth	No growth	Nd*
Flavobacterium strain S12	No growth	No growth	1.8×10^7	2.2×10^7	No growth
A. hydrophila strain M800	6.8×10^6	No growth	8.0×10^6	Nd*	Nd
Escherichia coli strain 8872**	Nd*	Nd	4.5×10^6	Nd*	6×10^6

Note: *nd, not determined; **tested at 25°C, see Fig. 1.

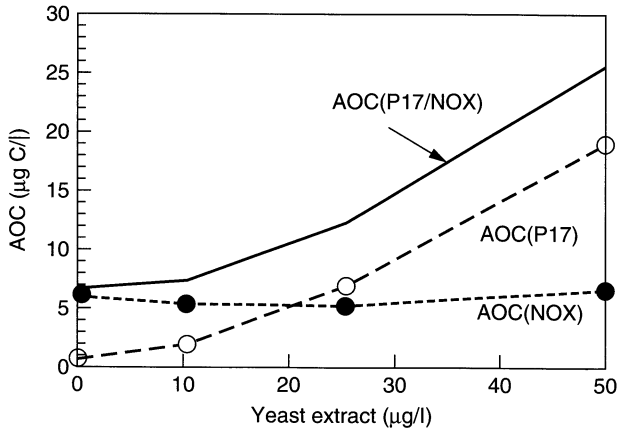

Figure 5. AOC concentrations at various concentrations of yeast extract added to slow sand filtrate. AOC-P17 is the AOC concentration derived from the N_{max} values of strain P17 grown in the presence of strain NOX; AOC-NOX is the AOC concentration calculated from the N_{max} values of strain NOX. AOC-P17/NOX is the total AOC concentration.

Effect of Separate Growth of Test Strains

Both strain P17 and strain NOX are able to utilize carboxylic acids. In a mixed culture, there is competition for these substrates. When grown separately, compounds available to the two strains are used by both strains. Figure 6 shows the AOC concentrations determined in two different water types by the individual strains and by growth as a mixture, respectively. In both water types, the AOC concentration mainly consists of compounds available to strain NOX (carboxylic acids). From these results, it can be calculated that 45 to 66 % of the compounds potentially available to strain P17 were utilized by strain NOX when grown as a mixed culture. In addition, a small fraction of the compounds available to strain NOX is utilized by strain P17 when grown as a mixed culture.

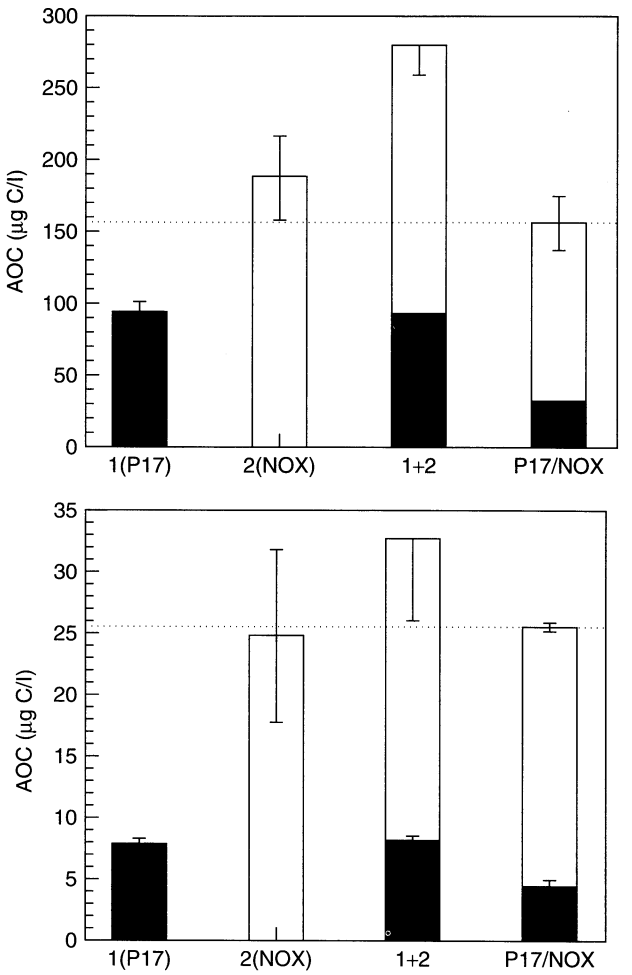

Figure 6. Effect of separate growth of the test strains in two water types. (**a**) water after ozonation; (**b**) ozonated water after GAC filtration. 1(P17), strain P17 alone; 2(NOX) strain NOX alone; 1 + 2, AOC calculated from 1 and 2; P17/NOX, strains P17 and NOX grown as mixed culture. Error bars give standard deviations.

Reproducibility and Repeatability of AOC Determinations

A large number of AOC determinations have been conducted since the development of the method. In all cases, the test was done in duplicate sample flasks. Hence, information is available about the reproducibility of the test. Figure 7 shows the Relative Standard Deviations (RSD, %) of a total of 430 AOC tests, conducted within a one-year period, most of which with an AOC value below 25 µg C/L. The STD values were below 10% for 60% of all samples tested. In 10% of the samples STD values were greater than 35% (Fig. 7b). The repeatability of the test is shown by the constant yield values of the test strains for acetate and the constant AOC values obtained for one particular slow sand filtrate over a period of more than 15 years (with values <5 µg C/L) (results not shown).

Comparison of Described AOC Procedures

The AOC test as described earlier, has been adapted by a number of researchers to improve its use as a monitoring tool (55,60). A major adaptation is the use of small-capped containers, in combination with a single measurement in a container (in triplicate) after a defined incubation period. Table 5 compares the conditions of the AOC test as described in standard methods (6g) with the AOC test that was described earlier. A further adaptation to the procedure described in standard methods is the use of ATP for determining the growth of the test strains. With the ATP analysis, the AOC test can be conducted within two to four days (55).

AOC CONCENTRATIONS IN WATER

AOC Concentrations in Raw and in Treated Water

The AOC test had been developed for assessing the biostability of treated water. However, it is also used in raw water and in water in various treatment stages. A problem observed with the use of the AOC test in raw water is the presence of spore-forming bacteria

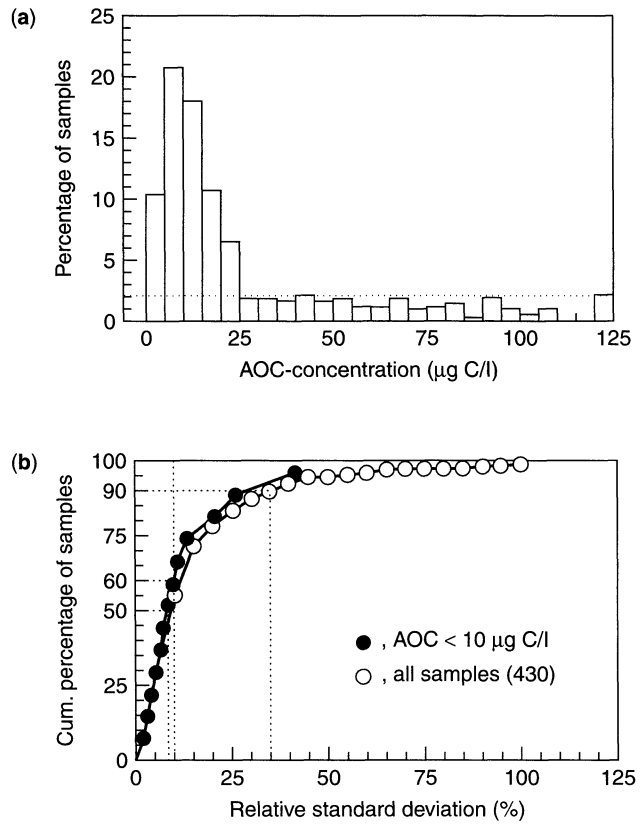

Figure 7. (**a**) AOC concentrations as measured in 430 samples and (**b**) the relative standard deviation (RSD) of the AOC concentrations (relative to the average value of duplicate measurements).

or other types of bacteria surviving the pasteurization procedure. These organisms hamper the AOC test either by growing on the plates and/or by growing in the water samples and thus consuming an unknown part of the substrates potentially available to the test strains. Pasteurization at a higher temperature (e.g., 70 °C for one to two hours) can be applied to eliminate this problem.

Typical AOC concentrations in river (Meuse) water ranged from about 50 µg C/L to 400 µg C/L, depending on the season, with the highest concentrations in spring and in late summer (Fig. 8a). About 70 to 90% of these AOC concentrations was available to strain P17, indicating that the substrates available to strain P17 (e.g., amino acids, peptides, carbohydrates, etc.) were present in much higher concentrations than the carboxylic acids. The concentration of dissolved organic carbon (DOC) was 3.2 to 4.2 mg/L and the AOC concentrations were 1.5 to 10% of the DOC concentration.

In a survey conducted in 1986 to 1987 (53), AOC concentrations observed in drinking water in the Netherlands ranged from 1 to about 60 µg acetate-carbon equivalents per liter (Fig. 8b). The highest values were observed in surface water supplied with ozonation a treatment step. In all types of treated water the fraction available to strain NOX was the largest proportion of the AOC concentration, indicating that carboxylic acids were the predominating compounds. The AOC concentration utilized by strain P17 was less than 1 µg C/L in most samples of treated water. The data presented in Figure 8b and many observations conducted since then clearly demonstrated that the AOC concentrations in treated groundwater water in the Netherlands usually are below 10 µg of C/L. Also, in surface water supplies with slow sand filtration as the final treatment step such low AOC concentrations were found.

AOC concentrations in treated water are only a small fraction (<1.7%) of the DOC concentration (Fig. 9). The lowest AOC/DOC ratios have been observed in groundwater supplies, in which AOC concentrations usually were below 10 µg of C/L. Minimum values for the AOC/DOC quotient were all close to 0.1%.

AOC values in treated water observed in the Netherlands are low in comparison to values reported in other countries. Several surveys conducted in the United States revealed that AOC concentrations in treated water

Table 5. Comparison of AOC Procedures

Test Characteristic	AOC (Original Method)	AOC in Standard Methods (69)
Containers	Borosilicate glass Erlenmeyer (1 L); glass stoppered	Borosilicate glass vial (45 mL) with TFE-lined silicone septa
Sample volume	600 mL	40 mL
Surface/volume (cm^{-1})	0.63 (equivalent to pipe diameter of 6 cm)	1.5 (equivalent to pipe diameter of 2.7 cm)
Test strains	P17 and NOX	P17 and NOX, separate growth
Biomass parameter	Colony count	Colony count or ATP (55)
Sample treatment	30 minutes at 60°C	30 minutes at 70°C
Addition of chemicals	none	Thiosulphate (50 mg/L)
Incubation period	≥14 days	9 days
Calculation of AOC	N_{max} values in duplicate flasks	Average of colony counts in triplicate vials on days 7,8, and 9
Result (units)	µg of acetate-c equivalents/L	µg of acetate-C equivalents/L

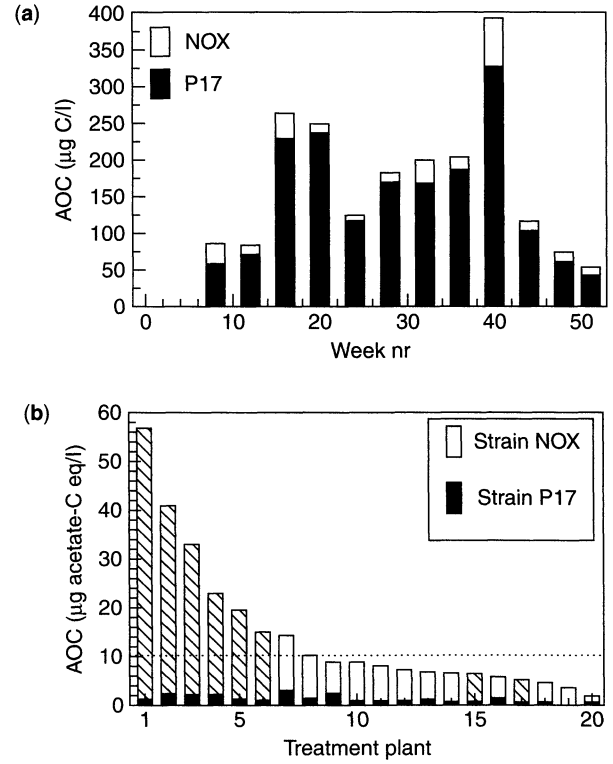

Figure 8. (**a**) AOC concentration in river water in a one year period. (**b**) AOC concentrations in treated water of 20 different treatment plants. Hatched bars represent treated surface water; open bars represent treated groundwater. Groundwater was treated without chemical disinfection. Nrs. 15 and 17 are slow sand filtrates. Adapted from D. Van der Kooij, *J. Am. Water Works Assoc.* **84**(2), 57–65 (1992).

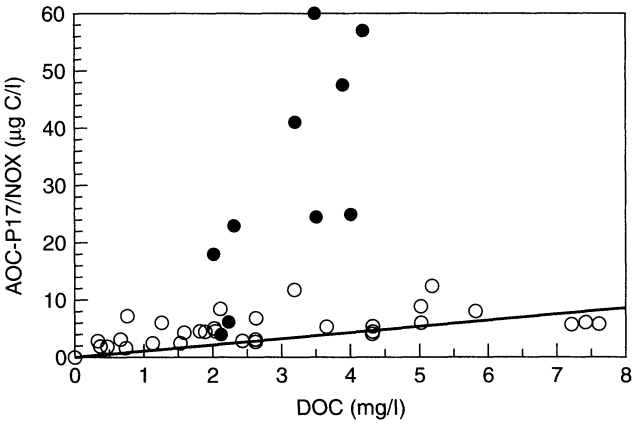

Figure 9. AOC concentrations in treated water as a function of the DOC concentration. Open circles: groundwater supplies; closed circles: surface water supplies. Line shows Lowest AOC/DOC ratios. Data (adapted) from D. Van der Kooij, *J. Am. Water Works Assoc.* 84(2), 57–65 (1992).

ranged from about 20 to 200 or more than 300, respectively (70,71). Median AOC values observed in these studies were about 100 µg C/L. In one of these surveys, the fraction of AOC available to strain P17 exceeded 50 µg C/L in a number of water types. Hence, AOC values in treated water in the United States are much higher than those in treated water in the Netherlands. These differences in AOC levels may be due to differences in water treatment (less biological treatment and addition of chlorine). Also, the AOC test as used in the United States is not identical to the one described in this report (Table 5), but a few comparisons between the test methods did not show large differences between the results (72). Still, the separate growth of the test strain will have an effect in certain water types (cf. Fig. 6).

Effects of Water Treatment

From the differences between the AOC concentration in raw river water and the AOC concentration in treated water, it can be concluded that surface water treatment as applied in the Netherlands can cause an AOC reduction of more than 90%. A variety of processes have different effects on the AOC concentration Fig. 10. The AOC concentration is reduced by biological processes in filter beds when these processes are applied properly and also during soil passage physicochemical processes such as coagulation/sedimentation and adsorption also can reduce the AOC concentration of water. On the other hand, oxidation processes clearly cause an increase of the AOC concentration. The effect of ozonation is related to the degradation of large molecules of natural organic compounds, such as fulvic and humic acids, into low molecular compounds, as has been demonstrated in studies, using advanced chemical analytical techniques. AOC concentrations increase with increasing ozone dosage (74,75,76).

The effect of biological filtration processes on the AOC concentration is of major importance in achieving a biologically stable drinking water. In the Netherlands such processes are included in all water treatment plants, either as rapid sand filtration, GAC filtration, slow sand filtration, or soil passage of surface water (river bank filtration, artificial dune recharge). Fig. 11 shows

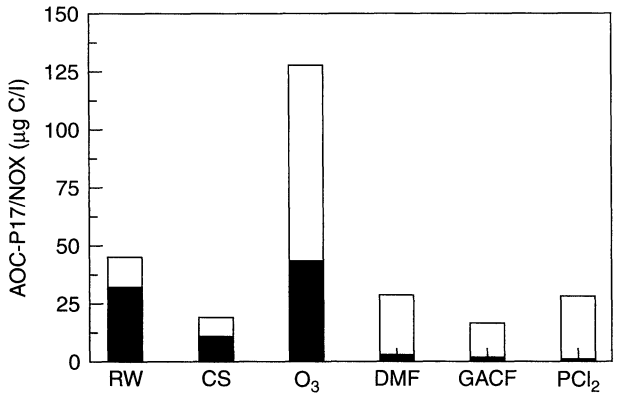

Figure 10. The effects of water treatment on the AOC concentration. RW, raw water river Meuse water after storage in open reservoirs; CS, coagulation/sedimentation; O_3, ozonation; DMF, dual media filtration; GACF, granular activated carbon filtration; pCl2, postchlorination. The water is distributed after postchlorination. Black bar represents the AOC fraction utilized by strain P17.

that the AOC reduction decreases with decreasing AOC concentration in the influent.

From the data shown in this figure, it can be calculated that the AOC reduction in this type of process is negligible at AOC concentrations below 6 μg of C/L. Similar observations have been reported by other investigators (77). Figure 11 also shows that the presence of a chlorine residual in the influent of rapid sand filters decreased the AOC removal, most probably by hampering the biological processes.

Membrane filtration processes have a huge potential in water treatment because of the variety of available membrane types, and the capacity of removing undesirable particles (including bacteria) and dissolved compounds without side-effects on water quality. Ultrafiltration cause an AOC removal up to 84% (from 25 to 3.8 μg C/L) in a surface water treatment, probably by removing particulate compounds of biological origin (78). Reverse osmosis is capable of reducing the AOC concentration to a level below 1 μg C/L. In this water, inorganic nutrients were added to conduct the AOC test (unpublished result 5).

Effects of Distribution

Observations on water sampled from a number of distribution systems have revealed that AOC concentrations may decline rapidly in the distribution system, depending on the initial AOC concentration (Fig. 12). Most likely, biofilm processes play an important role in AOC uptake (53,79). Figures 12 and 13 demonstrate that AOC reduction is very limited at values close to and below 10 μg C/L.

Effect of Storage of Water Samples on AOC and HPC Values

Water samples collected in thoroughly cleaned Erlenmeyer flasks were incubated without pasteurization to obtain information about the effect of the multiplication of the indigenous population on the AOC concentration. Figure 14 shows that the AOC concentration of river water (Fig. 9) collected from a storage reservoir initially followed an exponential decrease. After three days at an AOC concentration of about 10 μg C/L the rate of decrease

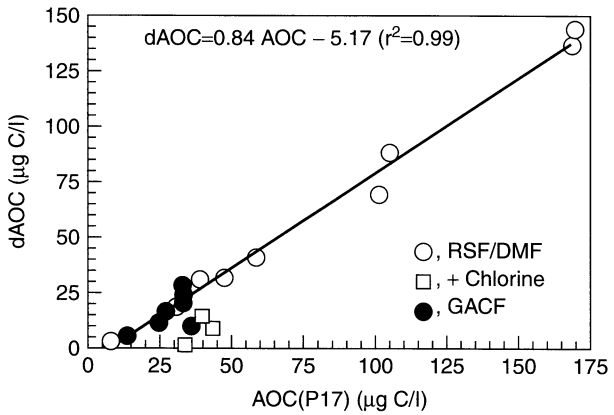

Figure 11. AOC reduction (dAOC) as achieved with biological filtration. RSF, rapid sand filtration; DMF, dual media (anthracite/sand filtration); GACF, granular activated carbon filtration. Adapted from Van der Kooij, 1984 (73).

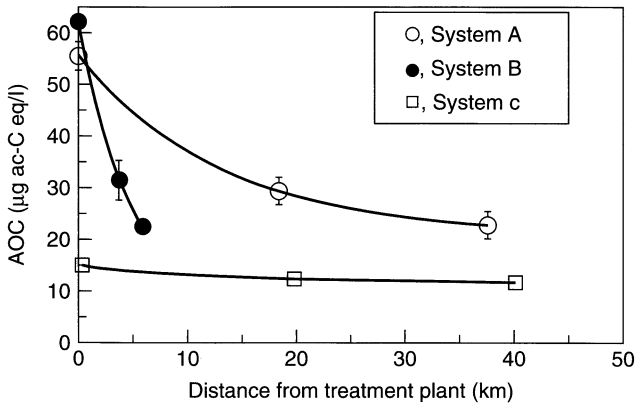

Figure 12. The effect of distribution on the AOC concentration in three different distribution systems. In all cases, ozonation was used as a treatment step. From: D. Van der Kooij, *J. Appl. Microbiol. Symp. Suppl.* **85**: 39S–44S (1999).

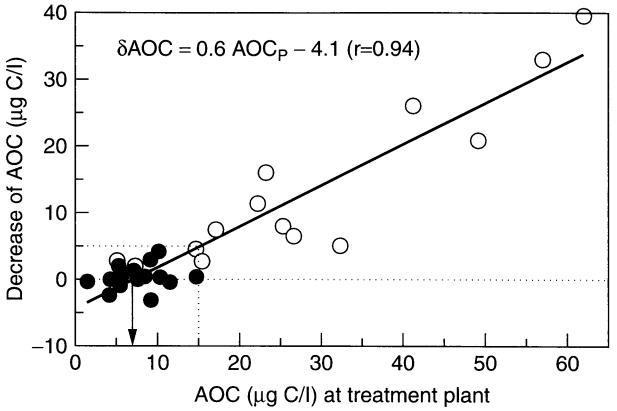

Figure 13. Maximum AOC decrease observed in distribution systems as a function of the AOC concentration of treated water. Open circles: surface water supplies; closed circles: groundwater supplies. Adapted from D. Van der Kooij, *J. Am. Water Works Assoc.* **84**(2), 57–65 (1992).

declined sharply and followed a linear function with time in the test period up to four weeks. In ozonated water, the AOC concentration rapidly declined to a value of 15 μg of C/L. Thereafter the decline became linear with time (Fig. 14(b)). In the stored river water, the fraction of AOC utilized by strain P17 constituted 72% of the initial AOC concentration. In ozonated water the fraction available to strain P17 initially was less than 10% of the total AOC concentration. Obviously, biomass compounds (amino acids, peptides, etc.) predominated in stored river water, whereas carboxylic acids were the major AOC fraction in ozonated water.

The following equations can be used to describe the observed AOC decrease:

Phase 1 (up to five days); exponential decrease with residual:

$$AOC_T = AOC_0 \times e^{-kT} + AOC_R, \quad (3)$$

Phase 2 (after about 5 days); linear decrease:

$$AOC_T = AOC_5 - Ux(T - 5) \quad (4)$$

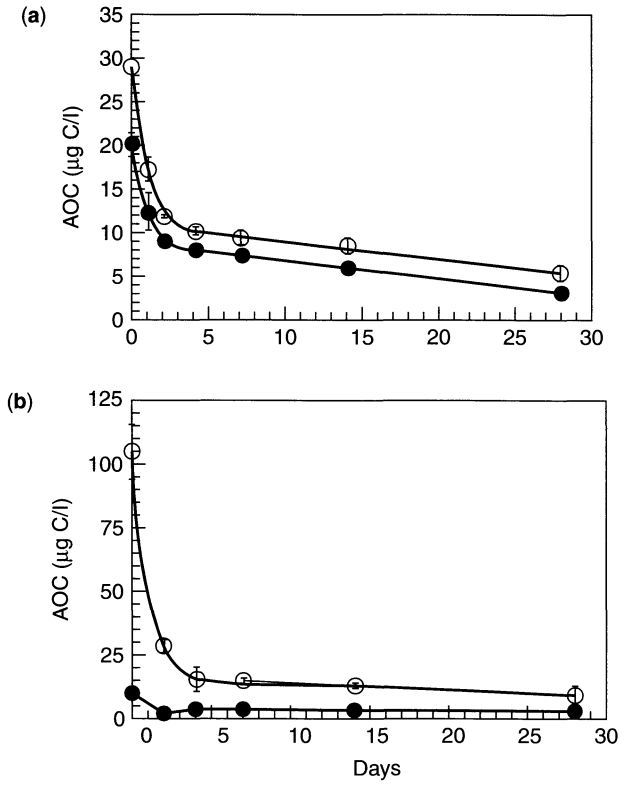

Figure 14. AOC decrease in water samples incubated at 15 °C in thoroughly cleaned Erlenmeyer flasks. (**a**) river water from an open storage reservoir; (**b**) water after ozonation. Symbols: ○, AOC P17/NOX; ●, Fraction of AOC available to strain P17. Error bars give standard deviation of the AOC value (duplicate flasks).

In these equations AOC_T is the AOC concentration at time T, AOC_0, the AOC concentration at the start (zero time), AOC_5 the AOC concentration at day five, and AOC_R, is the apparent residual AOC concentration, respectively. K is the decay constant (d^{-1}) in the exponential phase, and U is the uptake rate ($\mu g \ldots l^{-1} \ldots d^{-1}$) in the linear phase. The constants of the equations for the two water types are given in Table 6.

Simultaneously with the AOC decrease, the numbers of bacteria increase in the water samples during incubation. This increase can be determined with several techniques, including the heterotrophic plate count (HPC), the total direct (microscopic) count (TDC), or the concentration of adenosinetriphosphate (ATP). These techniques all have their specific advantages and limitations. The main limitations of these parameters include: (*1*) the HPC value of a mixed population of aquatic bacteria is always a (small) fraction of the total active population, and requires a long incubation period; (*2*) the TDC values include active and inactive cells, which may also differ greatly in size; (*3*) the ATP test is fast, but the ATP concentration declines rapidly once the maximum level has been attained. For a number of samples, maximum HPC values have been determined in relation to the initial AOC concentration. The results presented in Figure 15 clearly show that there is a good correlation between the AOC concentration and the maximum HPC values, with an average yield of 7.37×10^6 CFU/µg C. Obviously, the compounds serving as sources of carbon and energy for the indigenous population are those compounds used by the AOC test strains P17 and NOX. However, differences between the bacterial communities in the various water types result in different yield values and differences in the fraction contributing to the HPC value influence the N_{max} values.

BIOLOGICAL STABILITY

Comparison of AOC and BDOC

The AOC and BDOC methods are used to assess the biological (in)stability of treated water. Table 2 gives an indication of the difference between these two approaches. AOC values are a small fraction of the DOC concentration of river water and even lower (0.1 to 1%) in treated water. BDOC values may constitute a significant proportion (19 to 54%) of the DOC concentration (3.5 to 13.3 mg/L) of river water (80). Surveys revealed that BDOC concentrations of drinking water from 79 utilities in the United States ranged from 0.01 to 2.4 mg/L and were 0.4 to 52.8 % of the DOC concentration (70). In another study, BDOC values for treated water of 31 plants averaged by site ranged from 0.03 to 1 mg/L and constituted 5 to 21% of the DOC concentration (71). In both studies significant correlations were found between concentrations of AOC and BDOC, respectively. However, the correlation coefficients were

Table 6. Kinetics of the AOC Decrease in Samples (600 mL) of Two Water Types Stored at 15 °C in Thoroughly Cleaned Erlenmeyer Flasks

Parameter*	River Water from Open Storage Reservoir	Water After Ozonation
DOC (mg/L)	3.4	2.9
AOC_0 (at time zero) (µg C/L)	29.1 ± 6.1	105 ± 11
AOC_R (µg C/L)	9.6 ± 0.7	13.2 ± 0.8
K (day^{-1})	0.98 ± 0.11 ($r^2 = 0.99$)	0.90 ± 0.04 ($r^2 = 0.99$)
Uptake coefficient (µg C/L.day)	0.2 ± 0.01 ($r^2 = 0.99$)	0.27 ± 0.02 ($r^2 = 0.99$)

Note: *DOC, dissolved organic carbon; AOC_0 = AOC concentration at time zero; AOC_R = residual AOC concentration; K = exponential decay rate (d^{-1}).

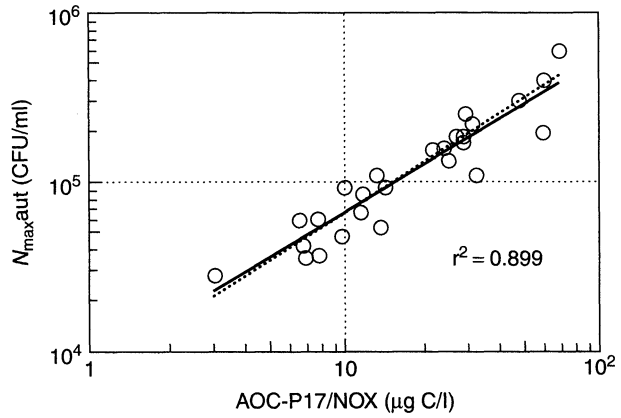

Figure 15. The relationship between the AOC concentration and the maximum colony count (N_{max}) of the autochthonous bacterial community in water samples stored at 15 °C in thoroughly cleaned Erlenmeyer flasks. HPC values were determined using diluted broth agar medium (spread plate method), incubated at 25 °C for 10 days.

relatively low, as a result of the wide range of AOC to BDOC ratios. On average, the AOC concentration was about 20 to 30% of the BDOC concentrations in treated water.

The main reasons for the difference between AOC and BDOC concentrations include:

- the use of an adapted microbial community in the BDOC test;
- the use of a large amount of biomass (bacteria attached to sand) in the BDOC test.

Also, the basis of the methods differ. The AOC concentration gives information about the production of biomass, which is the parameter of concern. The difference between the *Y* values for acetate and oxalate (cf Table 4) implies that the relationship between the amount of utilized organic carbon (BDOC) and biomass formation may differ between water types. In fact, using the *Y* values for oxalate in the AOC test gives much higher AOC values, some of which being close to the BDOC value (70).

From various studies a BDOC concentration of 0.1 to 0.2 mg/L has been derived as a reference value for biological stability (81,82).

The data about growth kinetics (Table 1), suggest that the concentrations of easily biodegradable compounds in treated water should be less than 1 µg of C/L to prevent multiplication. However, complete growth inhibition cannot be achieved and is also not required. The goal is to limit microbial growth to such an extent that water quality deterioration does not occur. Defining an acceptable level of growth is complicated, and may be different for different systems and seasons.

In the Netherlands an AOC value concentration of 10 µg of C/L has been derived as the reference value below which treated water has a very limited regrowth potential. This value is based on the following observations:

1. Samples collected from drinking water distribution systems revealed that AOC concentrations did not decrease at values close to or below 10 µg of C/L (cf. Figs. 12 and 13). Such values are observed in slow sand filtrate and in groundwater supplies in the Netherlands, where rapid sand filtration is the final treatment step;
2. AOC reduction in biological filtration (e.g., rapid sand filtration) is very limited at values below 10 µg C/l. (Fig. 11);
3. AOC concentrations in samples of stored water decline rapidly to a level of about 10 µg C/L (Fig. 14);
4. A statistically highly significant relationship was observed between the AOC concentration and the heterotrophic plate counts as determined on a nutrient poor medium (diluted-broth agar). At AOC values below 10 µg of C/L, the increase of these colony counts during distribution remained limited (53).
5. In a number of water types, the AOC/DOC ratio was as low as 1 µg of C/mg C. Values below this level have not been observed, suggesting that this level is indicative for organic compounds with a high degree of biostability. With DOC values below 10 ppm, this also gives AOC values below 10 µg of C/L.

For limiting regrowth of coliforms in chlorinated supplies AOC concentration of 50 to 100 µgC/l have been derived from extended studies, which indicated that many factors affected regrowth of these organisms (83).

Biofilm Formation Rate

Practical experience in the Netherlands show that the 90-percentile values of the heterotrophic plate counts (22 °C, 3 days incubation) remain below 100 CFU/mL in water types with AOC levels below 10 µg of C/L. In groundwater supplies this low AOC value is achieved with traditional treatment processes, that is, aeration followed by biological (rapid sand) filtration. In most supplies in the Netherlands the AOC level of 10 µg of C/L is attained, but defining biological stability was found to be more complicated. Even at AOC values below 10 µg C/L and at low heterotrophic plate counts, aeromonads were found to increase in numbers (expressed as CFU/100 mL) (5). Observations indicated that these organisms multiplied in biofilms and in sediment (28). Therefore, a biofilm monitor had been developed for determining the Biofilm Formation Rate (BFR) values of treated water. In this device the biofilm concentration on glass surfaces exposed to flowing water (0.2 m/s) increases as a linear function of time (51,52). Biofilm concentrations are determined by assessing the concentration of adenosinetriphosphate (ATP) with the luciferin-luciferase test. In the Netherlands the BFR values typically range from <1 pg ATP $cm^{-2}\,d^{-1}$ to a value greater that 100 pg ATP $cm^{-2}\,d^{-1}$ (5). Values below 1 pg ATP $cm^{-2}\,d^{-1}$ have been observed in slow sand filtrate and in drinking water prepared from aerobic groundwater. These water types, with AOC values clearly below 10 µg Cl^{-1} represent drinking water with the highest degree of biostability. Higher BFR values have been

found in drinking water prepared from anaerobic groundwater, but also these water types had AOC values below 10 μg C/L. Dosage of acetate at a concentration of 10 μg of C/L to a biofilm monitor gave a BFR value of about 360 pg ATP/cm^{-2} d^{-1}. This and other observations showed that a concentration of 1 μg of acetate-C/L causes a BFR value of 35 pg ATP/cm^{-2} d^{-1} (84). A significant relationship has been observed between the level of regrowth of aeromonads in groundwater supplies and the BFR value of the water leaving the treatment facility. At a BFR value of 10 pg ATP cm^{-2} d^{-1} the risk of exceeding the guideline value for *Aeromonas* (90-percentile value of 200 CFU 100 mL^{-1}) is 20% (5).

Based on these observations, the definition of biostability has been extended and a two dimensional approach is used. Hence, AOC concentrations below 10 μg of C/L in combination with BFR values below 10 pg ATP/cm^{-2} d^{-1} represent treated water with a high degree of biostability. Only in a few water types (slow sand filtrate and aerobic groundwater) lower values can be achieved with biological processes.

Materials in Contact with Treated Water

Materials in contact with drinking water can also affect bio(in)stability by releasing biodegradable compounds into treated water. For this reason methods have been developed to test the growth promoting properties of materials. In the United Kingdom the MDOD (mean dissolved oxygen difference) test is used (85), and in Germany a test based on measuring slime production is used (86,87). In the Netherlands the Biofilm Formation Potential (BFP) test is applied, which determines the biofilm concentration (pg ATP/cm^2) on the material as a function of time in a batch test in slow sand filtrate (88). Typical BFP values are below 10 pg ATP/cm^2 for glass and 20 to 50 pg ATP/cm^2 for PVC, whereas values between 500 to 3,000 have been observed for polyethylene materials. Such BFP values can directly be compared with biofilm concentration values as observed on the surface of pipe segments collected from the distribution system. In addition, BFR values as observed in the biofilm monitor also give information about the biofilm concentration, which can be attained on the pipe wall in contact with the water tested. In this way a simple framework is obtained, which enables the assessment and evaluation of the biostability of both water and materials with a consistent approach, the Unified Biofilm Approach.

CONCLUSION

- The AOC method described above has proven to give highly reproducible results. Contamination of the water with biodegradable compounds during sampling, transportation, handling and analysis is a main concern, with glassware and air as potential sources. The described procedures aim at preventing such contamination.
- The two test strains are not capable of utlizing all biodegradable compounds present in a water type. The compounds, which are utilized by the test strains are ubiquitous in water and low AOC concentrations in treated water demonstrate that easily available compounds have been removed.
- AOC concentrations in treated water in the Netherlands are below 10 μg C/l in the groundwater supplies and in most supplies as the result of the application of multiple biological processes in water treatment. The much higher AOC values (median: 100 μg C/l) as reported for treated water in the USA may be ascribed to the absence of biological filtration and the effect of the disinfectant on the AOC concentration;
- A reference AOC value of 10 μg C/l has been derived from effects of biological filtration on the AOC concentration, the AOC decline in distribution systems and the relationship between AOC and HPC values, respectively in unchlorinated supplies. AOC values below 100 or 50 μg C/l have been suggested for limiting coliform regrowth in chlorinated supplies in the USA.
- Even at AOC values below 10 μg C/l, certain microorganisms may multiply in the distribution system. Assessment of the Biofilm Formation Rate in combination with AOC values gives more complete information about the biological stability of treated wate;
- Materials of pipes and reservoirs may release biodegradable compounds into treated water. Assessment of the growth-promoting properties contributes to the selection of appropriate materials.

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ASTROVIRUSES

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INVERTEBRATES AND PROTOZOA (FREE-LIVING) IN DRINKING WATER DISTRIBUTION SYSTEMS

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"Just a glass of water! You see a glass in front of you, filled with pure water from a water supply. It is without taste and odor; it is clear; there is nothing remarkable in sight. Nonetheless, this glass contains a world of wonders (...). Although your eye presumably sees nothing but pure water, it is the residence of a large number of animals that are invisible to the naked eye, to which the glass is a large pool, a vast lake, a sea (...). However, drink it without concern: the little creatures are completely harmless and will, taken in by your body, soon have lost their lives."

These translated parts of a chapter of the *Reading Book for Primary Schools*, published in 1899 in The Netherlands (1), present a view on the significance of free-living invertebrates and protozoans in drinking water on health, that most likely is still valid for drinking water in developed countries in temperate zones. It, however, also emphasizes the unease that was and still is stirred in most drinking water consumers when confronted with the knowledge that "animals," although microscopically small, are emerging from their taps. When invertebrates emerge in a clearly visible form, it is not surprising that these animals usually evoke downright disgust. Man has learned to associate the presence of worms, insect larvae, and crustaceans in food and water with a diminished wholesomeness and, since the discovery of microorganisms as possible origins of diseases, with diminished hygiene. In many domestic situations, this association is justified and the presence of protozoans and invertebrates in drinking water in developing countries may be far from harmless.

The presence of invertebrates, being present in all natural freshwater habitats, is to be expected in most, if not all drinking water distribution systems in the world. Drinking water and the distribution systems by which it is supplied are not sterile, nor should they be. Adequate source water treatment and hygiene during operations will prevent contamination with pathogens and will limit multiplication of (micro)organisms in distribution systems. Free-living invertebrates should however be considered a natural part of the biocoenosis (i.e., an association of organisms forming a community) in drinking water supply systems. This contribution will discuss the significance of the presence of invertebrates and, briefly, free-living protozoans in drinking water and water supply systems, preceded by a summary of the significance of their parasitic relatives. The fact that monitoring of invertebrates is apparently not required by law anywhere is the probable cause of the relative scarcity of knowledge available on the subject. An overview of the most important taxa is presented, followed by an evaluation of ecological processes (immigration, survival, feeding, growth, and reproduction) and an impression of the abundance of invertebrates found in drinking water and mains. Finally, control strategies and benchmark values are presented.

SIGNIFICANCE

Parasitic Invertebrates and Protozoans

The WHO (2) assumes that pathogenic invertebrates only may be present in drinking water in Asia, Africa, and South America and only when contaminated surface water or groundwater is not treated prior to (distribution and) consumption. *Dracunculus medinensis* (Nematoda) and *Schistosoma* spp. (Platyhelminthes) are mentioned as the most important pathogens; other worms are considered of lesser or very little importance for drinking water. An infection by the guineaworm *D. medinensis* is considered the only disease transmitted exclusively by drinking water. The larvae reach the stomach of many millions of people via their intermediate hosts, some cyclopoid Copepoda [mostly *Mesocyclops* spp. (3)], and 5 to 10 million people are taken ill each year. The infection usually is not lethal, but the adult female can reach lengths of more than 50 cm in limbs (mostly the legs) and causes painful ulcers (from within). Cercaria (i.e., one of the larval forms) of *Schistosoma* spp. in most cases enter the human body via the skin during bathing or washing of clothes in contaminated water, snails being the intermediate hosts. Although infections may occur by the distribution of untreated surface water, most infections occur in wells and ponds. An estimated 200 million people are infected yearly by schistosomiasis and some die from this intestinal or kidney infection (2,4). In temperate zones as well, invertebrate parasites are known to be (possibly) waterborne, such as *Ascaris lumbricoides* (Nematoda) and nonhuman parasitic Schistosomatidae (Platyhelminthes). Outbreaks via drinking water have not been reported (5). However, the presence of parasitic Nematoda and other invertebrates in drinking water from surface water supplies may be a transmission route for plant diseases (6).

As for parasitic protozoans, the possibility of contamination of finished water with pathogenic protozoans such as *Entamoeba histolytica* has long been acknowledged, as well as the multiplication in drinking water distribution systems of free-living protozoans such as *Naegleria fowleri* and *Acanthamoeba* spp. that may be pathogenic under certain conditions (5,7). For a large part of the twentieth century, water supply utilities in most developed countries relied on the capacity of disinfectants—in most cases

chlorine, chlorine derivatives, or ozone—to inactivate pathogens in surface water treatment. The importance of multibarrier treatment to remove chlorine-resistant (oö)cysts of the pathogenic protozoans *Cryptosporidium* spp. and *Giardia* spp. from surface water and their high resistance to disinfectants became painfully clear in both small and large epidemics caused by their presence in drinking water at the end of the twentieth century in the United States and the United Kingdom (5,8–11). Other pathogenic protozoans (*Toxoplasma*, *Cyclospora*, *Microsporidium*) are possibly important for drinking water hygiene as well and are presently under scrutiny (5,11).

Invertebrate and Protozoan Hosts for Pathogens

As invertebrates may harbor pathogenic viruses, bacteria, and protozoans in and on their body, mostly in their digestive tracks, invertebrates resistant to disinfection may take these pathogens with them while penetrating drinking water distribution systems through treatment plants. Nematoda, in particular, have been under scrutiny and are still suspect, although their role in pathogen entry into drinking water has never been fully established. It has been suggested that small crustaceans and fly larvae could also affect microbiological safety by harboring pathogenic microorganisms in or on their body (12). No pathogenic bacteria or fecal coliforms could however be found associated with individuals isolated from drinking water (13), although midge fly larvae (Tipulidae) have recently been associated with the number of total coliforms in finished water of a groundwater treatment plant in Germany (14). Some protozoans have been shown to protect pathogenic bacteria from chlorination (15). *Legionella* bacteria may multiply in free-living amebae in distribution systems and also be protect by them from chlorination (16,17); *Acanthamoeba* spp. have been known to harbor *Mycobacterium* spp. and viruses, accentuating the public health importance of these organisms (5).

The significance of intermediate hosts in distribution systems as possible vectors for invertebrate parasites in temperate zones also has been suggested (18). Theoretically, the penetration of parasitic worms or their intermediate hosts into the distribution system cannot be excluded, for example, in the case of mains bursts and other situations in which the integrity of the system is impaired. The intermediate hosts may be infected with parasites and may survive in the distribution system. A small percentage of surface water Asellidae (Crustacea, Isopoda), for example, can be infected with the larvae of Acanthocephala, a group of parasitic worms that infect the digestive tract of vertebrates (19,20). Freshwater snails can be intermediate hosts of blood flukes (Schistosomatidae, Platyhelminthes) causing "swimmer's itch," and Copepoda may be intermediate hosts of parasites such as *Gnathostoma spinigerum* (Nematoda) and *Spirometra mansonoides* (Platyhelminthes) (5). Although finished water of treatment plants is microbiologically safe, chances that an infected intermediate host (*1*) penetrates into the distribution systems, (*2*) survives, (*3*) emerges from a consumer tap, and (*4*) can pass on the parasite to a human will have to be considered negligible.

Aesthetic Significance and Consumer Complaints

An anonymous pamphlet distributed in London in 1827 pointed out the presence of a large variety and quantity of invertebrates, protozoans, and algae in the Thames river water distributed for domestic use. The pamphlet drew public attention to the inlet being situated within three yards of one of the largest city sewer outlets (21). After the start of slow sand filtration in London in 1829 (21), an introduction of this technique throughout Europe followed, although many cities (partly) continued to be supplied with untreated surface water long after. The quality of the water in distribution systems subsequently improved greatly in Europe and the rest of the industrialized world in the nineteenth and twentieth century, causing a distinct reduction of drinking water-related outbreaks of microbiological diseases. Simultaneously, major changes took place in the invertebrate communities in distribution systems. However, at the same time, consumer expectations regarding water quality rose. The sudden infestations of invertebrates emerging from taps and the resulting consumer complaints have driven water utilities to despair. Presently, consumer complaints concerning invertebrates under normal conditions probably are not abundant in most distribution systems in developed countries, but there is little or no literature to corroborate this assumption. In The Netherlands (population 16 million), consumer complaints regarding invertebrates currently are estimated to be limited to an average of 10 each year, although there is no comprehensive overview available. In Antwerp, Belgium, complaints about Asellidae and Gastropoda emerging from taps were approximately 25 to 30 each year before major air-scouring mains started in 1975, reducing complaints to maximally one per year since 1985 (22). During a symposium on consumer complaints in 1965, the London Metropolitan Water Board disclosed that 160 (3%) of the complaints in the period between 1957 and 1964 concerned "crustaceans, worms, insects and so forth" (23). The literature shows that most problems elsewhere are also caused by these three groups of invertebrates.

Invertebrate taxa that also have caused complaints are Gastropoda (snails) (22,24–27), Cladocera (water fleas) (27), Copepoda (copepods) (26,27), Hirudinea (leeches) (27,28) and Gammaridae (freshwater shrimps) (27,29,30). Not just invertebrates but their fecal pellets (31), body parts, and degradation products as well can lead to consumer complaints. The death of large numbers of invertebrates used to cause taste and odor problems in the early days of water supply, especially when oxygen concentrations dropped. Large numbers of invertebrates present in distribution systems normally are noticed because individuals are likely to emerge from taps, but sessile invertebrates such as Bivalvia (mussels) (28), Ectoprocta (bryozoans) (32) and Porifera (sponges) (32,33) were only detected when they caused taste and odor problems or clogged pipes. Their presence is usually limited to untreated surface water because these sessile filter feeders depend on relatively high densities of POM (particulate organic matter) in the water. Mussel larvae (Bivalvia), for

instance, may penetrate surface water treatments, but levels of POM in drinking water are usually too low to sustain mussel growth in present-day distribution systems (34).

The other effects of large numbers of invertebrates can be the clogging of service lines and water meters. Clogging by Asellidae has been reported in The Netherlands. Furthermore, freshwater snails and mussels will easily cause clogging owing to their hard shells (22,24–26,35). Especially in the early years of distribution systems, when water treatment was absent or rudimentary, mains constrictions and clogging were more common, for example, by Bivalvia (36), Ectoprocta (32), and Porifera (33).

Invertebrates not originating from the distribution system can be a nuisance for water utilities as well: spiders and adult insects seeking refuge in the neck of taps will emerge when water is used. Particles may also be mistaken for invertebrates. Convincing consumers of the excellent hygienic quality of their drinking water in these cases usually has been proven difficult.

Microbiological Deterioration of Water Quality

A detectable transmission of pathogenic microorganisms into drinking water by invertebrates has never been reported. However, protozoans and bacteria may multiply on the remains of invertebrates [e.g., ciliates (37,38).] and in or on their fecal pellets [*Aeromonas* bacteria in pellets of midge fly larvae (39)]. These substrates presumably contribute to heterotrophic plate counts in sediments [as high as 6.10^8/g DW (40) and 5.10^9/g DW (41)] and plate counts of *Aeromonas* spp. (30 °C) in sediments [as high as 5.10^9/g DW (41)]. Many heterotrophic bacteria will grow on easily assimilable low-molecular compounds from the water, forming a biofilm on the sediment particles, much in the same way as it is formed on all water-exposed surfaces in distribution systems. *Aeromonas* bacteria however are also able to use high-molecular substrates present in dead (micro)organisms such as proteins, fats, and complex carbohydrates (42), for example, chitin from the exoskeletons of insects (unpublished personal observations by D. van der Kooij). As the bacteria were hardly found in the fresh biofilms (43), it is likely that multiplication of *Aeromonas* in distribution systems in The Netherlands predominantly occurs in sediments, using dead microorganisms and invertebrates as part of their food supply. Total coliforms have been found in sediments from distribution systems as well, with averages ranging from 10 to 1.10^5/g DW. Samples were collected via hydrants and were not handled aseptically and therefore contamination of the samples could not be ruled out (41). In conclusion, it is likely that invertebrates lower the overall density of bacteria and protozoans in distribution systems by feeding on them, therefore increasing the conversion rate of organic carbon to carbon dioxide and hence limiting the total food supply for microorganisms. Invertebrates however also change the composition of the microorganism community in distribution systems by their presence (stimulating symbiotic microorganisms) and by converting their food into fecal pellets and eventually in molting cuticles and dead bodies, changing the substrates available for microorganisms. It is likely that these changes benefit coliforms and (opportunistic) pathogens.

DRINKING WATER FAUNA

Figure 1 shows pictures of a protozoan and invertebrate taxa commonly found in drinking water distribution systems, giving an impression of their morphology. Protozoans, Rotifera, and Tardigrada are very small and are not known to have caused consumer complaints or specific problems for water utilities. Hydrachnellae have been encountered in distribution systems in Japan, probably contributing to the colonization of swimming pools (44), but have not been known to be a nuisance. Ostracoda normally are not ubiquitous and have a high density owing to their hard shell and will not be flushed out easily via consumer taps, probably explaining the lack of consumer complaints about these organisms. Collembola (springtails, 0.5 to 5 mm, not shown in the figure) are insects sometimes found in samples of sediment flushed out of mains via hydrants. Although these organisms most likely originate from the dry part of hydrants, contaminating the samples during sampling, they are sometimes quantified in samples as possible members of drinking water fauna.

Protozoans

A wide variety of free-living protozoans are present in surface water and groundwater. It is very likely that many species that are able to feed on bacteria from biofilms also multiply in water supply systems. Many species have been found in treatment works (47), finished water (reservoirs) (48,49) and drinking water distribution systems (37,45,46). In finished water reservoirs and distribution systems they have been found to multiply on the cadavers of invertebrates (37,38). Free-living species of the Mastigophora (flagellates) (37,49), Sarcodina (amebae) (37,49), and Ciliophora (ciliates) (37,38,47) have been reported.

Nematoda

Nematoda (nematodes or roundworms) are eel-like worms (Fig. 1) that usually range in length from 0.5 to 2.5 mm. Although able to swim, nematoda usually glide through biofilms. Free-living species prey on microorganisms and small invertebrates, generally reproducing sexually via eggs. Upon investigating tap water for the presence of amebae in Ohio (U.S.A.) in 1955, Chang and coworkers (50) detected the nematoda (*Diplogaster nudicapitatus*[1]) in their samples. A few years earlier, the presence of the nematoda (*Trilobus gracilis*) was also established in finished water and tap water in Norwich (U.K.) (51), and even long before that, nematoda were reported to be common in drinking water (52). Although the abundance of the organisms in Norwich led to investigations on control methods, the (free-living) organisms were not considered a possible health threat (51). Chang and collaborators however suggested the possibility of drinking water contamination by pathogenic bacteria, ingested by

[1] It should be noted that in many cases the names of species and genera of organisms referred to in literature have been changed since publication. In this contribution, the names originally used have been presented.

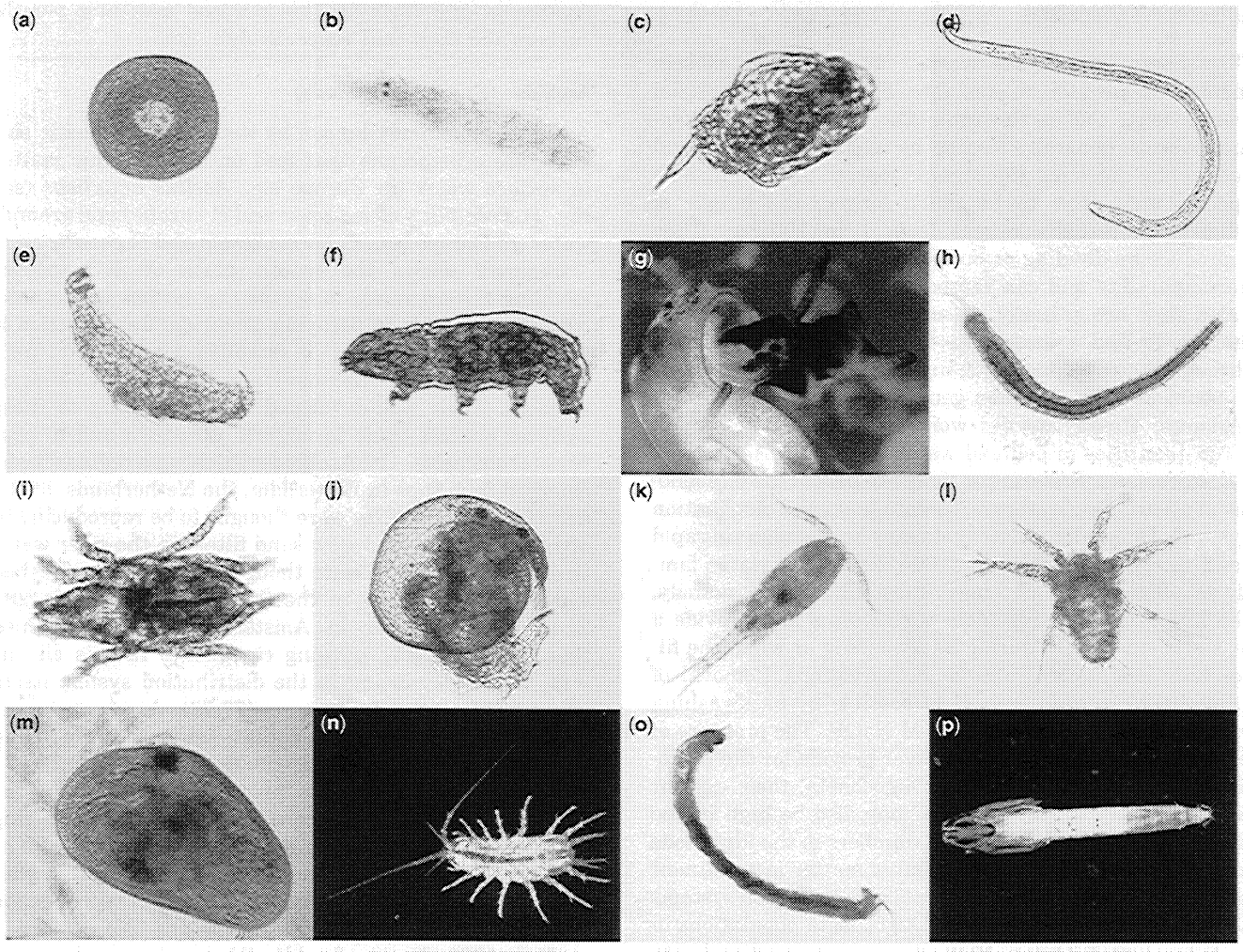

Figure 1. Examples of a protozoan and invertebrate taxa commonly found in samples from drinking water distribution systems (**a**) Testacea (shelled amoebae, c. 25–400 μm); (**b**) Turbellaria (flatworms, c. 0.5–20 mm); (**c**) Rotifera (0.1 to 1 mm); (**d**) Nematoda (roundworms, 0.5 to 2.5 mm); (**e**) Gastrotricha (0.1–1 mm); (**f**) Tardigrada (0.1–1 mm); (**h**) Oligochaeta (common worms, 0.5–100 mm); (**g**) Gastropoda (snails, 1 to 10 mm); (**i**) Hydrachnellae (water mites, 0.3–1 mm); (**j**) Cladocera (water fleas, 0.5–2 mm); (**m**) Ostracoda (0.5 –2 mm); (**k**) Copepoda (0.5–3 mm); (**l**) larvae of Copepoda (nauplius larvae, 0.2–0.5 mm), (**n**) Asellidae (aquatic sow bugs, 1 to 12 mm), (**o**) larva of Chironomidae (midge flies, 4 to 30 mm); (**p**) pharate adult of Chironomidae (c. 4 mm). Dimensions are common diameters or lengths in distribution systems, excluding extremities (from J. H. M. van Lieverloo, *J. Am. Water Works Assoc.*, (submitted).

nematoda and thus protected against chlorination during surface water treatment. They found many species during a survey of nematoda in the water supplies in United States (53). Pathogens (*Shigella*, *Salmonella*, and Coxsackie virus) were found to be able to survive several days in some chlorine-resistant species (*Cheilobus quadrilabiatus* and *D. nudicapitatus*) (54). The latter results were later corroborated by research on *Salmonella* survival in *Pristionchus lheritieri* (55). Chang and collaborators however could not prove the infectiveness of nematoda on mice (56) and concluded that under normal conditions the chances of pathogens actually being transmitted into drinking water distribution systems by nematoda must be considered very remote (57). This conclusion however did not lessen interest and ever since, investigations on nematoda removal in treatment plants and presence in drinking water have been reported predominantly in the United States (58,59,60), but also in Canada (61,62), Germany (63), India (64), Italy (65), and Poland (66). In Italy, no pathogenic bacteria could be isolated from nematoda collected in both raw and finished water of a surface water treatment plant (67). In the Netherlands, many water utilities monitor finished water of surface water treatment plants for nematoda, among other invertebrates (68). Numbers of more than 1,000 per m^3 are considered very high for finished water of surface water treatment plants. For groundwater treatment plants, numbers of 100 per m^3 or more are very high (45).

Oligochaeta (Annelida)

Oligochaeta (oligochaete worms) form a group of segmented freshwater worms (see Fig. 1) and terrestrial worms (e.g., the earthworm), also known as the common worms, generally feeding by ingesting detritus and sediments, digesting the organic matter. Although some species may grow to be much longer, the length of Oligochaeta usually ranges from 1 to 100 mm. They reproduce by dividing or budding, but the organisms are hermaphrodite and can reproduce sexually as well, provided they are sexually mature and densities are high enough to find a mate. The organisms usually burrow through these sediments; many species however are able to swim as well. Common genera such as *Nais*, *Tubifex* (Aristotle already noticed worms resembling *Tubifex* in large quantities in polluted water wells (21)), *Aeolosoma*, *Chaetogaster* or *Stylaria* probably are part of the biocoenosis in all drinking water treatment plants and distribution systems. Generally, Oligochaeta may be abundant in rapid sand filters and GAC filters (69–72). A freshwater family of Oligochaeta that may infest filter beds, namely, the Lumbriculidae, resemble earthworms and provide a less attractive sight during backwashing or when the filter beds are at rest (personal observation). Removal of Oligochaeta from filter beds is possible by backwashing with 5% NaCl (70) or with 0.01 M NaOH. The presence of biofilm as food supply will however provide for the reproduction of the few remaining Oligochaeta, their eggs or new immigrants. The numbers may also be high in the distribution system, even when numbers in the filter beds are low. It is likely that the organic matter in treatment plant filter beds and the sediments in distribution systems provide the food to sustain the Oligochaeta populations in water supplies. In the Netherlands, major Oligochaeta infestations have occurred in water supplies in 1939 (73) and 1964 (74), causing many complaints and much negative publicity. Little is known about the causes and control of the first infestation, other than that it instigated the installation of the Biological Research Commission, leading to many decades of (micro)biological research since. Limiting detritus in distribution systems to cut food supply was considered the most effective long-term control measure. The second Oligochaeta infestation was the result of overloading the 90-year old river water treatment plant in Rotterdam, which was only relieved when in 1966 the new plant increased the city's water supply capacity. Meanwhile, chlorine concentrations were increased by dosing chloramine, starting at 2 mg/L Cl_2, causing disintegration of the worms. After the infestation in the distribution system subsided, numbers were controlled with chloramine doses of 1 mg/L Cl_2. Similar problems and solutions have been reported by utilities in the United Kingdom and the United States (52,75). Maintaining concentrations of 0.2 mg/L free chlorine is considered a long-term control measure for Oligochaeta (27). Short-term control measures include high chlorine concentrations and mechanical cleaning methods, for example, flushing. The long-term effects of periodic control methods are limited (76) because of reproduction of worms that survive in dead ends (e.g., mains couplings) or those that cling to mains surfaces (e.g., *Aeolosoma* and *Nais* for this purpose use their mouth-suckers and their tails respectively).

Asellidae (Crustacea, Isopoda)

Asellidae, commonly known as water lice or aquatic sow bugs, are crustaceans that feed on dead organic matter in surface water (in Europe e.g., *Asellus aquaticus* (see Fig. 1), *Proasellus meridianus* and *P. coxalis*) and groundwater (in Europe e.g., *P. hermallensis* and *P. cavaticus).*

Figure 2 shows the mouth parts of *A. aquaticus*, enabling the organism to scrape the surface of (organic) matter, preferably in decaying condition. Reproduction is sexual, limiting chances to establish a population from low initial densities. Locomotion is limited to walking on surfaces. Their well-developed ability to cling to these surfaces enables them to migrate upstream and to pass vertical obstacles. The Asellidae which, on a regular basis, emerged from taps in Rotterdam, the Netherlands, at the end of the 19th century were thought to be reproducing in abandoned uncovered slow sand filters in the river water treatment plant and were thought to enter the finished water through cracks in the finished water canals (29). Some 30 years later in Amsterdam it was assumed that the organisms, causing complaints in this city as well, were reproducing in the distribution system itself, using biofilm as a food source (77,78). A recent survey in the Netherlands showed that Asellidae were found in 35 of 36 investigated distribution systems (45,76,87). Most consumer complaints about invertebrates in the Netherlands presently concern Asellidae and the clogging of water meters by these organisms has been reported. The number of complaints in the United Kingdom are higher in the summer because many adults die after reproduction and are swept more easily from the mains (27).

Since 1930, dozens of publications and notes described attempts to remove Asellidae from water supplies in Germany (28,79–81), the United Kingdom (30,82–86), Belgium (22), the Netherlands (78), and France (79). The most effective method to remove the organisms, albeit temporarily, proved to be flushing of the distribution system after treatment with the botanical insecticide

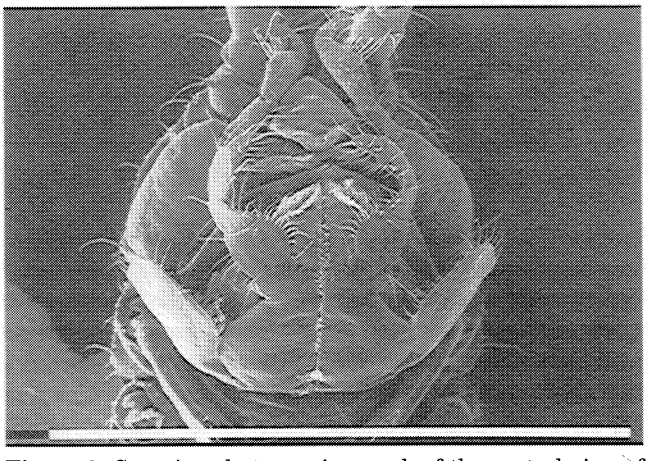

Figure 2. Scanning electron micrograph of the ventral view of the head showing the mouthparts of *Asellus aquaticus* (Crustacea: Isopoda), scale line = 1 mm; from (45).

pyrethrum (78,80,82) or the synthetic variant, permethrin (pyrethroids). This latter method is still applied in the United Kingdom (27) although under strict conditions to prevent consumer exposure to the risk of poisoning for kidney patients that apply extracorporeal dialysis at home and also commercial and domestic fish collections. The use of chlorine or other disinfectants to control Asellidae is very difficult (27) because the organisms are very resistant and it is difficult to maintain a high disinfectant residual in all parts of the distribution systems. In Antwerp (Belgium) and Hamburg (Germany), periodical air scouring of the distribution system proved to be very effective (22,81). Air scouring is advised in the United Kingdom as well (as is swabbing), especially because sediments and other invertebrates are removed better than during flushing with permethrin, preventing an increase of other species numbers that frequently follows a pyrethroids dosing exercise (18,27). Experiences in the Netherlands indicate that flushing the distribution system may be more cost effective than intensive methods such as swabbing and air scouring (76,88). Limiting biofilm formation and sediment accumulation in mains is thought to be the most effective long-term control measure.

Chironomidae (Insecta, Diptera)

Of the insects, the larvae of the nonbiting Chironomidae midge flies (Fig. 1) are most commonly found in distribution systems. Midge fly larvae usually range in length between 4 and 30 mm. Most likely, the species that live in drinking water distribution systems feed on organic material in sediments, which is also used by some species to build a case glued to surfaces. Outside this sediment case, they can swim in an undulating manner, much like the nonburrowing members of the same family. Especially in the first part of the twentieth century and also more recently (89,90), utilities in the United States and elsewhere were confronted with the infiltration of distribution systems by midge fly larvae via uncovered finished water reservoirs or poorly screened air inlets in treatment plants and reservoirs. In some cases, these infestations led to larvae emerging from consumer taps (6,89,91,92). The covering of reservoirs and the screening of ventilation ducts in most cases proved an effective remedy, but other control methods were used as well, including the method of adding DDT into the reservoir that is currently strongly discouraged (93). Because most insects can only reproduce sexually and adults depend on the presence of air for their oxygen supply, preventing immigration usually is effective to control the presence of insect larvae in distribution systems.

Some midge fly larvae however may procreate in the distribution system itself. This was discovered in 1937 in Germany (94) and in 1973 in Essex (England) (95). The water supplies were not able to control the organisms by closing all entrances for adult midge flies. The organisms eventually were identified as *Paratanytarsus* sp., a parthenogenetic genus that can reproduce eggs asexually in the so-called pharate adult stage before the adult stage (Fig. 3). Flushing after treatment with the insecticides pyrethrin or permethrin has proven to be an effective method (27,94,96), as it is for controlling

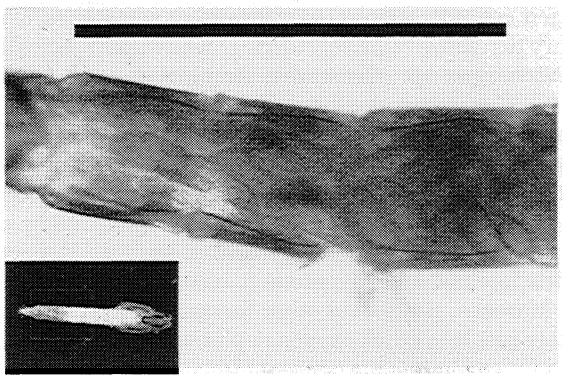

Figure 3. Pupal cuticle, containing discharged eggs, at the abdominal end of a pharate adult female of an unidentified parthenogenetic midge fly (Diptera: Chironomidae) collected from a drinking water mains in the Netherlands. Insert shows the habitus of the female, scale line = 1 mm; from J. H. M. van Lieverloo, et al., *J.—Am. Water Works Assoc.* (submitted).

Asellidae. Recently, a food-grade coagulant has been found effective in controlling the parthenogenetic species *Paratanytarsus grimmii* (97) as was shock chloramination for normal midge fly larvae (90).

Vertebrates

Eels and sticklebacks were common in early distribution systems supplying untreated surface water (98,99). Still, however, incidents of vertebrates such as a small eel clogging a water meter (in 1983) and a frog clogging a mainscock (early 1990s) have been reported in the Netherlands. These organisms must have entered the mains at one point, possibly after a mains break. Although it is not likely that these organisms survive in the mains for a long period, owing to the scarcity of food, a whole colony of eels has recently been pigged out of a potable water mains in the United States (100). Evidently, public reports on these incidents are rare.

ECOLOGY

Immigration from Groundwater

Although less conspicuous than in surface water, groundwater biocoenoses may harbor an abundance of invertebrates that are visible to the naked eye. The composition of the biocoenosis highly depends on soil structure and hydrology, because evidently the biocoenosis is interstitial (i.e., between the grains). Coarse sediments and cracks in rocky bottoms will facilitate the passage of surface water into groundwater supplies, especially via shallow wells. Soils with finer grains and coarse deep layers that are covered by fine-grained layers only harbor invertebrate species that are confined to groundwater and caves. Large sand formations usually are relatively poor in organic matter, resulting in the presence of oxygen. Even at concentrations of 0.2 mg/O_2L, invertebrates may be ubiquitously present, although it is not known whether the organisms are concentrated in pockets with higher

oxygen concentrations (101). Straining sometimes is necessary to remove relatively large groundwater species such as *Niphargus* sp. (Crustacea, Amphipoda) and *Proasellus* sp. (Crustacea, Asellidae) from groundwater obtained from coarse sediment layers (unpublished observations in groundwater from horizontal wells at 18-m depth, by the author and J. Notenboom). Finer sediments operate as giant slow sand filters and therefore allow only low numbers of small invertebrates such as Copepoda, Hydrachnellae, and Nematoda to pass into the water supply. Marshes, peats, and other formations rich in organic matter result in quick oxygen depletion, and therefore few or no invertebrates are present (101).

Immigration from Surface Water

Earlier, the abundance and diversity of the organisms in distribution systems carrying untreated surface water depended largely on the biocoenosis in the source system. Many species however cannot survive in distribution systems because of physical conditions, such as the lack of light and air, and the colonization of the system is limited by biological conditions, such as predation or unsuitable food. The constant supply of invertebrates from the source however will result in the presence of a large variety of surface water species, as was the case in many large cities in the nineteenth century and the start of the twentieth century, for example, in Breslau, Poland (102), London, United Kingdom (103); Boston, United States (33); Hamburg, Germany (98,99); and Paris, France (36). The microbiological quality of the water, although underestimated for a long time, was however of more concern than the zoological quality. Regrettably, this is still the case in large parts of developing countries. From the start of central water supply in the Netherlands (e.g., in 1853 in Amsterdam and in 1873 in Rotterdam), slow sand filters were used for surface water treatment (104). Presently, in most surface water treatment plants, protozoan and invertebrate removal is part of algae removal and is usually considered of minor importance. Microstrainers probably form the only treatment process applied specifically to remove invertebrates, especially those straining the filtrates of rapid gravity filters and GAC-filters. However, small motile invertebrates such as nematoda (59,60,62,64,65) and Rotifera (38,105) are hardly removed by some surface water treatment plants using coagulation and sedimentation followed by rapid gravity filtration, with or without chlorination. Nematoda are known to be resistant to high chlorine concentrations (106), but chlorination or ozonation before rapid gravity filtration improves the removal of invertebrates (27,38,107). Planktonic species however are better removed in treatment than benthic species (107). Furthermore, slow sand filters are known to remove invertebrates better than rapid gravity filters (27,107,108), and the backwash rate of rapid gravity filters controls invertebrate removal (109). In a survey of 17 finished water reservoirs in Germany, sediments in reservoirs of surface water supplies were found to harbor high numbers and a wide variety of planktonic species next to some benthic species, whereas the sediments in reservoirs of groundwater supplies contained only low numbers of Rotifera and nematoda. Furthermore, the numbers of Rotifera and larvae of Copepoda in one of the supplies dramatically decreased with distance from the treatment plant (110,111), probably owing to diminished food supply. Similar results were obtained in Switzerland (112). All these results indicate that penetration of invertebrates through surface water treatment plants is rather common. In finished water of at least 64% of 55 surface water treatment plants in the United States, invertebrates (predominantly nematoda) and free-living protozoans were found. Not all of these however were also found in the influent, indicating that part of the organisms may have originated from multiplication in the treatment plant itself (48).

Multiplication in Treatment Plants and Finished Water Reservoirs

Protozoans (47,49) and invertebrates may enter distribution systems by elution from water treatment systems and finished water reservoirs in which they multiply. Before slow sand filters and finished water reservoirs were covered, contamination of these systems led to infestation of the distribution systems supplied. Unscreened or poorly screened ventilation ducts still can be important immigration routes, especially for flying insects such as midge flies and for crawling invertebrates. Contamination may also occur via cracks in walls and roofs, although coliform counts will rise as a result of contaminated water seeping through, instigating inspection of structural integrity. For Asellidae, even backwash water outlets are possible routes to enter treatment plants from backwash water reservoirs or surface waters (79). Nowadays, in most cases, penetration into a treatment plant predominantly occurs from the water source, and the filter biocoenosis would seem most likely to resemble the benthic interstitial biocoenosis in (oxygenous) surface water sediments or groundwater systems. In surface water treatment plants, filter beds are expected to be harboring surface water species (21). The biocoenosis of the slow sand filters in Bremen (Germany), predominantly harboring groundwater species, resembles that of the banks of the supplying river (113). In Zürich (Switzerland), the surface water species *Canthocamptus* sp. (Copepoda, Harpacticoida) dominates the slow sand filters and the finished water reservoirs. Although the organism is not regularly found in the raw water, it is present in the sediments of the source water lake (114). However, in some plants in Germany, a limited variety of taxa have been found in the slow sand filters, predominantly consisting of groundwater species of Nematoda, Oligochaeta, and harpacticoid Copepoda, although Turbellaria, cyclopoid Copepoda, Cladocera, Asellidae, and Gammaridae have been found as well. For the slow sand filters in the water supplies using water from the river Ruhr (Germany), a colonization with groundwater species from below is likely because the filters are used to supplement the groundwater that is collected for drinking water production (115–117). GAC-filters have also been found harboring invertebrates (63,71,72,118). Filter grain size and backwashing procedures prove to be very crucial for filter colonization and invertebrate numbers in

filtrates. Removal of invertebrates from filtrates by straining with, for example, 30-μm mesh microstrainers is often necessary (68).

Immigration by Intrusion into Distribution Systems

When distribution system integrity fails (e.g., during mains breaks), organisms may inadvertently enter the mains, even when the water is still running (e.g., Asellidae). Rough surfaces, dead ends, and blind spaces in mains junctions prevent the invertebrates from being flushed out after repair. Holland (83) ascribed the presence of Asellidae in the Coventry (United Kingdom) distribution system, first detected in a water meter in 1942, to contamination of the system after many mains were broken during heavy bombardments in 1940 and 1941. Other possible immigration routes are as follows:

- Back-siphonage of wastewater or surface water via unprotected connections;
- Cross connections (18);
- Contamination during mains construction and repair (18).
- Locking-in of soil and dirt by unprofessional use of underground hydrants (i.e., opening the hydrant valve before opening the top-piece).

Chances of colonization due to (temporarily) diminished integrity of the distribution systems probably are much lower than the chances of immigration via surface water and groundwater treatment plants, but research data to corroborate this assumption are not available.

Survival and Settlement

Survival and settlement of invertebrates that penetrated into a distribution system depends on physical, chemical, and biological factors. Although air pockets may remain in mains after air scouring, normally air is absent and adult insects are bereft of oxygen (their larvae breathe through their skin or use gills). Most planktonic organisms will not withstand the current in distribution systems, although velocities are usually very low, and will be flushed out via consumer connections. Most Monogononta (Rotifera), for instance, are planktonic surface water species and their presence in distribution systems will depend entirely on their ability to pass surface water treatment. As far as chemical composition is concerned, normal concentrations of chlorine and chloramine in water leaving treatment plants are not very effective against most invertebrates found in distribution systems (27), but do limit the survival of some invertebrates. For instance, 1 to 2 mg/L of free chlorine will limit settlement of freely swimming mussel larvae (Bivalvia) (27). Considering drinking water standards in most countries, toxicity of other compounds will probably not be a determinative factor. Salinity, acidity, or alkalinity may however be too low to support some freshwater organisms. Facultative parasites, pathogenic microorganisms, and especially predators may have a large impact on chances for colonization of the distribution system by an invertebrate species. Competition for food however is most likely to determine long-term chances of successful colonization.

Feeding, Growth, and Reproduction

Availability of sufficient and suitable food is most likely to determine the abundance of invertebrates in distribution systems. Planktonic species (most Rotifera, Cladocera, and Copepoda) and sessile filter feeders [Porifera (sponges), Coelenterata (polyps), Bivalvia (mussels), and Ectoprocta (bryozoans)] depend on relatively high densities of algae or POM (particulate organic matter) in the water. Removal from surface water by coagulation and sedimentation, especially if followed by filtration, usually suffices to limit growth of these taxa in distribution systems. A study of invertebrates in sediments in finished water reservoirs in Germany revealed a significant decrease of Rotifera and larvae of Copepoda during distribution. The numbers of two planktonic surface water species of Rotifera (*Notholca caudata* and *Triarthra longiseta*) clearly decreased, whereas the numbers of benthic Rotifera slightly increased (111).

In most distribution systems, concentrations of dissolved compounds present in finished water from treatment plants are most likely to determine the abundance of bacteria, protozoans, and invertebrates. On all water-exposed surfaces in the distribution systems, benthic bacteria form the basis of a biofilm, a slime layer colored yellow, brown, or black by suspended particles that attach to it. Probably most of the bacteria are heterotrophic, using organic matter both as an energy source and as a source of base materials. Additionally, incomplete removal of ammonium, nitrite, and methane in treatment plants using anoxygenous groundwater will lead to growth of nitrifying bacteria and methane-oxidizing bacteria in distribution system biofilms as well. In these biofilms, the bacteria form the living food for protozoans and invertebrates (Nematoda, benthic Rotifera, Gastrotricha, Tardigrada, Turbellaria, Gastropoda, Ostracoda, benthic Copepoda, benthic Cladocera, juvenile Hydrachnellae, and Chironomidae larvae) that graze on these bacteria and protozoans. Large invertebrate species will mainly be found feeding on the biofilms at the bottom of mains and the biofilm-covered sediment particles accumulating there. These sediments are formed by (*1*) precipitation of particles suspended in finished water, (*2*) insoluble compounds formed in mixed water types, (*3*) parts of mains materials, and (*4*) sloughed-off biofilms, increasing the surface area available for bacteria and protozoans to grow on. Oligochaeta burrowing in these sediments ingest whole sediment particles and Asellidae scrape off biofilm from surfaces, meanwhile ingesting rust, bitumen, and asbestos fibers, as shown by the composition of fecal pellets of Asellidae ubiquitously found in sediments collected from mains (41). Small invertebrates such as Rotifera and small nematoda are attached to the biofilm while feeding and are likely to be ingested by biofilm grazers and sediment feeders. Planktonic Copepoda are found whirling up particles to feed on while skimming the sediment surface. Predators of many taxa may thrive on other invertebrates, provided the abundance of prey is sufficient and predators are not visually orientated. Figure 4 schematically summarizes the hypothesis

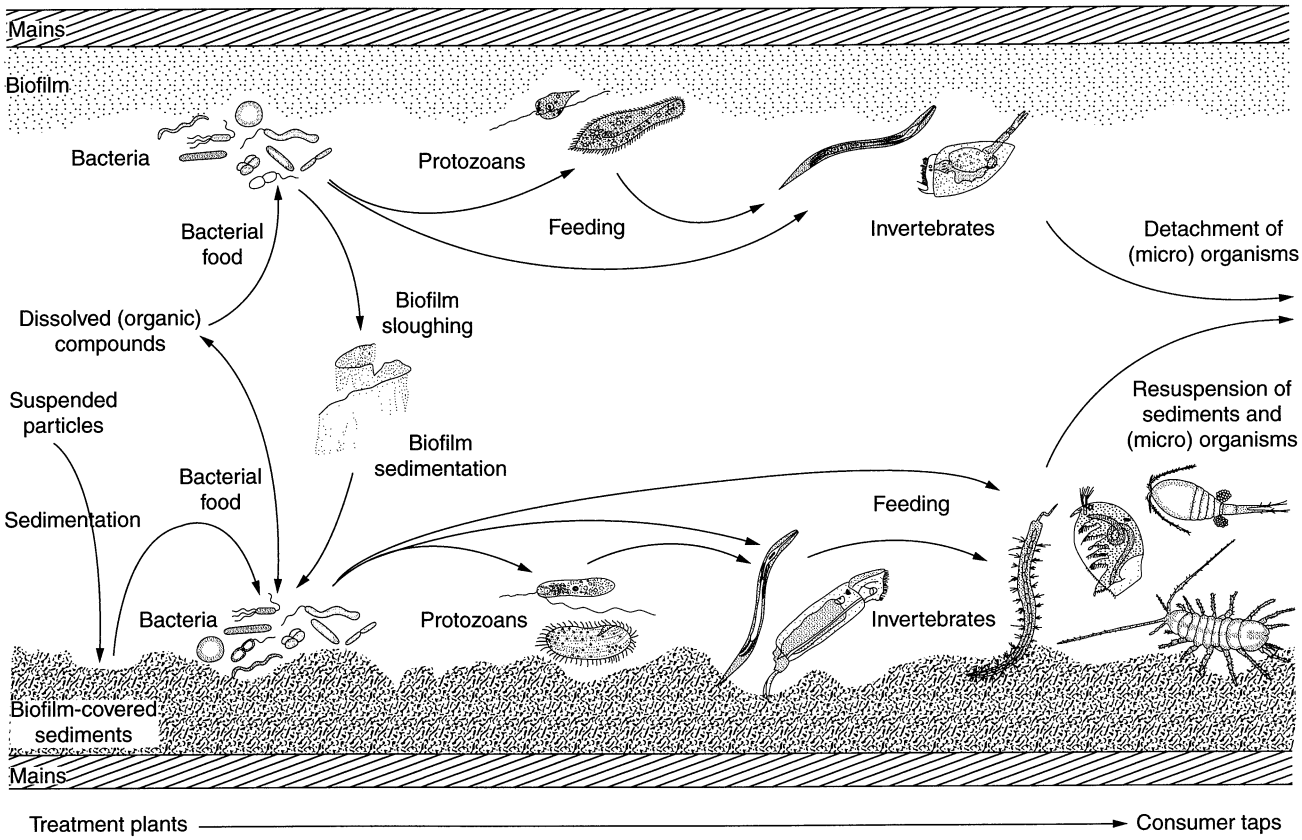

Figure 4. Schematic depiction of the hypothesis on food supply and feeding in drinking water distribution system biocoenoses; from J. H. M. van Lieverloo et al., *J.—Am. Water Works Assoc.* (submitted).

of food supply and feeding processes in the distribution systems (45).

Those species that are successfully competing for food in distribution systems will also depend on reproduction for establishing and maintaining a population. Although protozoans and most drinking water invertebrates can reproduce asexually, some taxa, for example, Copepoda and Asellidae, can only reproduce sexually, causing their chances of colonizing a distribution systems to depend on the initial population density, that is, the chances of mating. The abundance of relatively large benthic invertebrates in distribution systems such as Asellidae and Oligochaeta, compared with the low abundance of most of these organisms in finished water from treatment plants, could be seen as an indication that reproduction in the distribution system is largely determining abundance for these taxa. It is however very difficult to quantify immigration of benthic invertebrates walking and sliding into distribution systems, considering the size of trunk mains carrying the finished water of treatment plants.

ABUNDANCE

Methods to Assess Invertebrate Abundance

Freshwater zooplankton is usually collected by filtering water through plankton nets. Benthic invertebrates usually are scooped from the bottom or are filtered from sediment samples. Owing to the closed character of distribution systems, filtering of water emerging from the system is the most practical and cost-effective sampling technique. Planktonic invertebrates and suspended benthic invertebrates are collected by filtering finished water from treatment plants and by filtering drinking water from consumer taps, using plankton nets or filters, usually ranging between 10- and 45-μm mesh. In the Netherlands, 10-μm mesh filters are most commonly used, for it is known that large percentages, especially of protozoans, Nematoda, and Rotifera will pass even these filters. Benthos is collected by flushing or air scouring the mains via hydrants and filtering the flushing water with plankton nets, usually ranging between 30- and 500-μm mesh. Figure 5 shows a special sampling top-piece that was developed in the Netherlands over a period of 50 years for collecting invertebrates from mains. Of most taxa, an average of 50% or more is flushed from the mains; Oligochaeta and Chironomidae are flushed out less effectively however. On an average, only of Nematoda, Turbellaria, and larvae of Copepoda, less than 50% of the individuals is retained in the 100-μm mesh filters. Presently, extra 30-μm mesh filters are used to improve sampling efficacy (119). The invertebrates in the samples are identified and enumerated using an inverted microscope (drinking water samples) or a stereomicroscope (flushing-water samples).

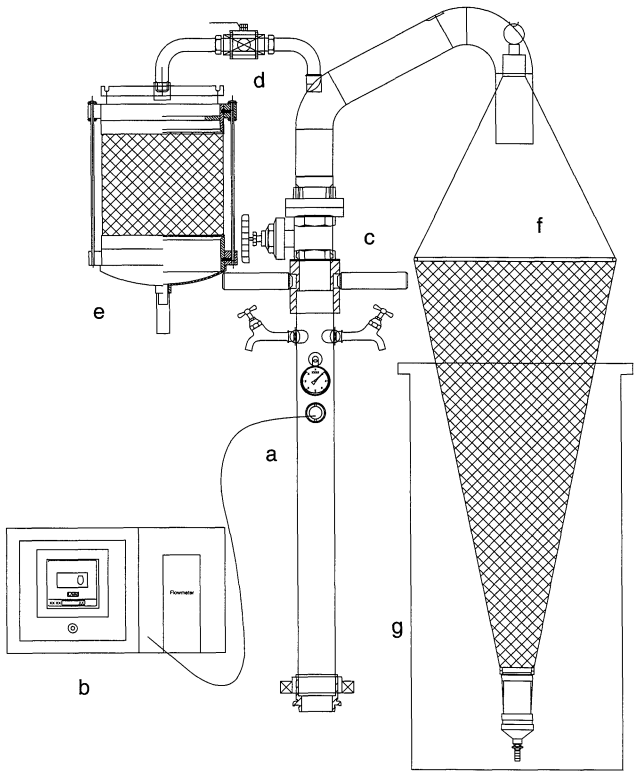

Figure 5. Drawing of the device for sampling flushing water, consisting of a top-piece for underground hydrants and filters a = Georg Fischer MK515 paddle wheel flow sensor in an aluminum plug; *b* = Georg Fischer F86 flow and volume indicator; *c* = slide valve; *d* = ball valve; *e* = 100-μm mesh plankton gauze filter containing a removable 500 μm mesh plankton gauze filter; *f* = 500-μm mesh plankton net; *g* = filtrate vessel with overflow. Not shown is the 30-μm mesh plankton gauze filter presently used to filter the filtrate of the 100-μm mesh filter. (From J. H. M. van Lieverloo et al., *Water Res.* (submitted).

Invertebrate and Protozoan Abundance in Drinking Water

Although water utilities may be aware of the presence of invertebrates in their distribution systems, not many periodically assess the size and composition of the community, most likely because it is not obligatory. If they do so, this information usually is not published (120). Results of some surveys, mostly targeting Nematoda, have been published (53,61,111,121,122) along with results of periodical monitoring by water utilities (22,58,68,114,116,123). In Figure 6, mean numbers of invertebrate taxa in finished water from treatment plants, drinking water from consumer taps and flushing water from mains are presented. The figure shows that data from literature (period 1948–1996), data from six utilities in the Netherlands using surface water (period 1964–1995), and those from a recent survey of 36 distribution systems in the Netherlands (period 1993–1995) (45,76,124) match surprisingly well for most of the invertebrate taxa.

Cladocera and Copepoda dominate the mains biocoenosis in numbers, mean numbers mounting to several hundreds per cubic meter of flushing water, whereas these and other visible invertebrates are hardly found in finished water from treatment plants and in drinking water from consumer taps. Most organisms under normal flow conditions apparently are not easily whirled up from the bottom of the mains. Owing to their size, Asellidae and Oligochaeta dominate the mains biocoenosis in biomass, forming approximately 86% and 12% respectively of total invertebrate biomass in the Netherlands (based on estimated biomass per individual organism) (45). Numbers in finished water from treatment plants and drinking water from consumer taps are dominated by protozoans, Rotifera, Nematoda, and Copepoda. Numbers of protozoans, Rotifera and Nematoda probably also are very abundant in the mains, but on account of their limited size, their numbers usually are not accurately (Nematoda) determined in water flushed from mains.

CONTROL

In 1964, the Biological Research Commission in the Netherlands, consisting of biologists and operators of research institutes and water utilities, formulated the following recommendations for invertebrate control: (*1*) limit assimilable organic matter in drinking water by intensive water treatment, (*2*) prevent mains contamination during mains construction and repair (and caused by cross connections) and (*3*) systematically flush distribution systems (18). In 1982, this view was still endorsed by the industry, adding the obvious necessity to adequately remove invertebrates in treatment plants (68). The balance of the intensity of all these control measures should depend on cost-benefit evaluations that include benefits for other variables (e.g., microbiology, color), environmental costs (e.g., wasting flushing water), and inconvenience to communities (e.g., pressure drops and colored water during flushing).

Limitation of Immigration

When finished water is microbiologically safe, immigration of invertebrates is not likely to play a major role in the abundance of invertebrates in distribution systems and drinking water. The increased awareness of possible contamination of finished water with the very small (oo)cysts of *Giardia* spp. and *Cryptosporidium* spp. will enhance the quality of many treatment plants throughout the world, probably diminishing the possibilities of a contamination by invertebrates even further. Furthermore, awareness of the risks of intrusion of microorganisms into distribution systems is growing (127), which could lead to the limitation of intrusion of invertebrates as well.

Periodical Removal from Distribution Systems

Flushing of distribution systems, either only with water or by injecting air (air scouring) or by introducing plastic swabs (swabbing), is only effective temporarily because the organisms that were not removed will be able to reestablish the population, using sediments and biofilms as food. If distribution systems are flushed periodically, abundance and species diversity will diminish and some species will be flushed out entirely. Many benthic species such as Asellidae however are used to periodic increases of water flows (128) and will be able to withstand high water

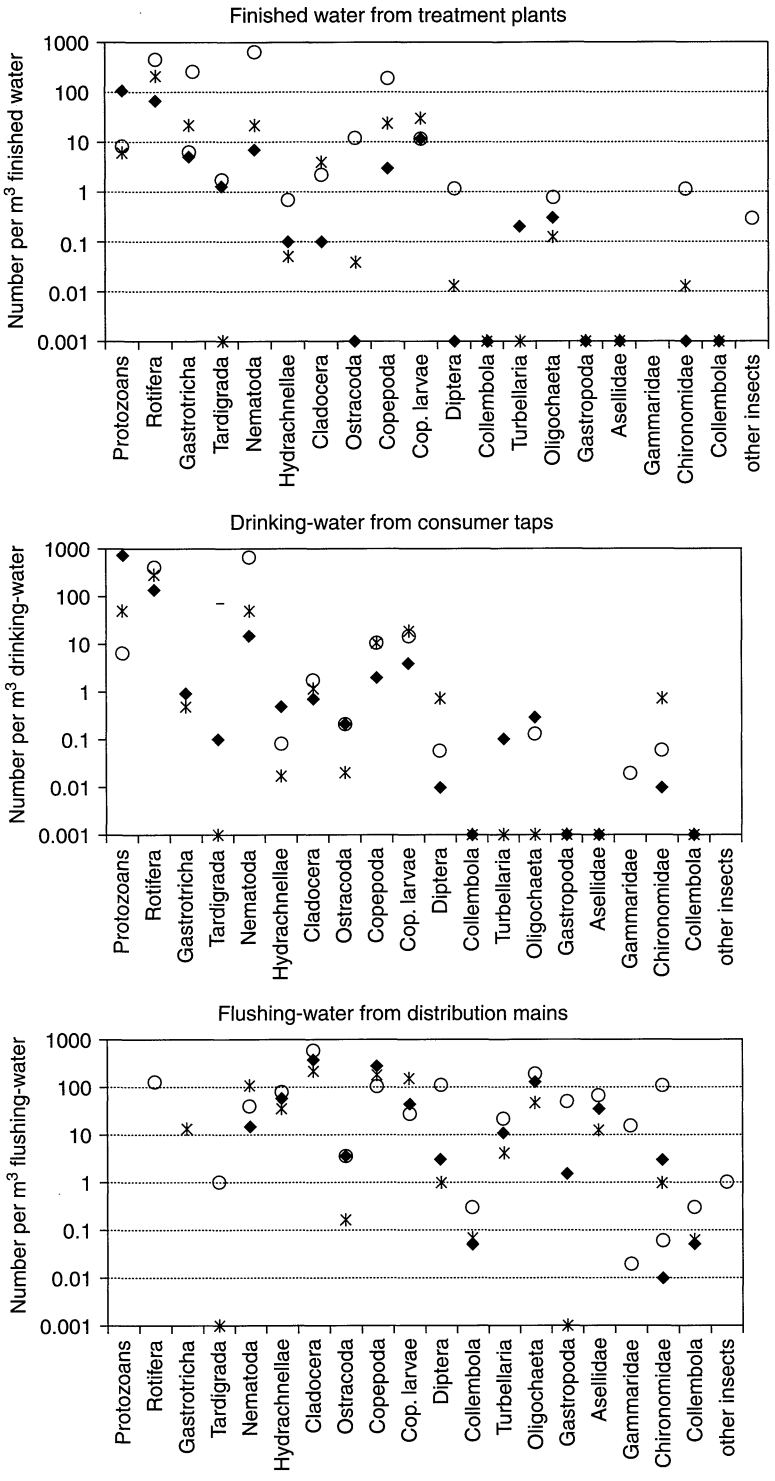

Figure 6. Abundance of protozoans and invertebrates in water supplies. ○ Literature data: arithmetic mean of means provided, calculated, or estimated per reference (1948–1996) (22,50,58,59,61,65, 68,78,95,105,107,111,114,116,121–123,125,126). * Data from six utilities in the Netherlands predominantly using surface water, one report per utility (1964–1995): mean of means provided per report. ♦ Averages from a survey in distribution systems of 36 treatment plants in the Netherlands (1993–1995) (45,76,124). Means of invertebrates taxa that were looked for, but were not found, are presented as 0.001 per 1,000 L.

velocities (83). In seven experiments studying sampling accuracy in the Netherlands, only 42% (SD 15%) of living Asellidae and 83% (SD 29%) of dead Asellidae were sampled from mains at 1,0 m/s (119). Another study in the Netherlands showed that levels of invertebrates (predominantly Asellidae and Oligochaeta), sediments, and bacteria in flushing water from hydrants were back to previous levels within nine months after systematically cleaning parts of distribution systems. Only fine sediment volumes (retained in 30- to 100-μm mesh filters) remained significantly lower for more than 15 months. Although the immediate effects of intensive techniques such as air scouring or pigging were larger than the effects of flushing, there were no long-term differences, probably

because of reaccumulation of sediments from trunk mains (not cleaned) and from the treatment plants (76,88,124). In Antwerp, Belgium, good results were reported in removing Asellidae and Gastropoda only after five years of annually air scouring all mains generally distributing water at low flows, that is, dead ends, mains with drastically changing diameters and shuttle zones. Consumer complaints about invertebrates dropped from 25 per year (1975) to below 1 per year (since 1985) (22). In Hamburg, Germany, and in the United Kingdom, air scouring is used successfully as well (27,81). The cleaning frequency required is likely to depend on the rate of sediment accumulation and biofilm formation in the distribution system. If only mechanical methods are used, multiplication of invertebrates is usually only limited by cleaning very frequently. Although the organisms under these conditions may not get very large, the reproduction rate of species such as Asellidae and Oligochaeta probably is enough to keep the population stable albeit small. Low-frequency application of mechanical cleaning methods is less effective because important parts of distribution systems cannot be reached by mechanical methods, that is, dead ends, mains couplings, and, in case of corroding cast iron mains, encrustations. Chemical methods are more effective in killing these invertebrates, when used in combination with systematic flushing, although diffusion rates into dead spaces do limit the efficacy of these methods as well. Asellidae, Gammaridae, and Insecta are very sensitive to pyrethrins or permethrin, pesticides that are used in the United Kingdom for controlling these organisms under strict conditions (27). Oligochaeta can be controlled by maintaining 0.5 to 1.0 mg/L free chlorine residuals for weeks in combination with systematic cleaning of the mains (27).

Prevention of Multiplication of Invertebrates and Microorganisms

Multiplication of invertebrates in distribution systems probably depends on the presence of food sources, namely, biofilms and (biofilm-covered) sediments. Biofilm formation may be limited by maintaining a disinfectant residual in the distribution system and it may have a limiting effect on some invertebrates as well. An infestation with Oligochaeta can be prevented with residual concentrations of 0.2 mg/L free chlorine (27). Normal residuals of chlorine and chloramine in water leaving treatment works however are not very effective against most of the invertebrates found in distribution systems (27). Secondly, it is difficult to maintain a disinfectant residual throughout the distribution system. In the Netherlands, 65% of drinking water is produced from groundwater supplies, treated and distributed without a disinfectant, while surface water is treated in multiple barrier plants including chemical disinfection. Only 22% of all drinking water is chlorinated after treatment and in only 10% of all drinking water, a disinfectant residual is detectable, most of it barely (0.05 mg/L). Multiplication of microorganisms and invertebrates in distribution systems is limited by a far-reaching removal of biodegradable compounds in water treatment, aiming at achieving water with a high degree of biological stability. Biological stability of water can be assessed by measuring concentrations of easily assimilable organic carbon (AOC) (129,130), concentrations of biodegradable dissolved organic carbon (BDOC) (131,132), or by assessing the biofilm formation rate (BFR) (133).

Furthermore, maintaining biological stability in distribution systems also implies the use of biostable materials, for example, plastic materials that do not release biodegradable compounds into the water (134).

Benchmark Values

Guideline values for invertebrate numbers in distribution systems should be the result of balancing costs for invertebrate control and consumer perception of water quality. A quantitative relation between invertebrate numbers in distribution systems and consumer perception however is not yet available, partly because of the low number of consumer complaints. Benchmark values, based on percentiles of numbers found in distribution systems, can be used by water utilities for comparing their chances of consumer complaints.

If monitoring shows high numbers compared with benchmark values, chances of consumer complaints are higher as well, increasing the incentive for control. In the Netherlands, national benchmark values have been collectively set in 1993 for surface water supplies (136), which recently have been extended and amended to apply for all water supplies (Table 1). Finished water from surface water treatment plants is sampled monthly, independent of abundance. Other sampling frequencies depend on invertebrate numbers in previous samples. Samples are collected every five years if numbers previously found were low to normal, every three years when numbers were elevated, every year when numbers were high, and every six months when numbers were very high. One sample per series is collected from finished water of a (groundwater) treatment plant, and for each 5.10^9 L/yr drinking water distributed, one flushing-water sample is collected in the distribution system (but at least three flushing-water samples are collected for each treatment plant) (45,135). Benchmark values proposed in East-Flanders (a province of Belgium) for individual samples are less discriminating: 0.4/1000 L (elevated), >= 2/1000 L (high), and >= 20/1000 L (very high) for all large invertebrates (generally the same taxa as in the Netherlands, benchmarks valid per taxon, values per square meter converted to values per 1000 L, assuming an inner mains diameter of 100 mm). Values for small invertebrates are 1,000 times higher, values for very small organisms are approximately 2,000 times higher than values for large invertebrates (137). In the city of Antwerp, Belgium, guideline values for Asellidae and Gastropoda (snails) are below 10 per 100 m^2, that is, 4/1000 L (assuming 100 mm inner diameter) based on a relationship between complaints and abundance (22). The benchmark value for median numbers of Asellidae in the Netherlands is strikingly higher ($\geq 30/m^3$ is considered high), considering the limited numbers of complaints reported by the water utilities in the Netherlands (estimated 10 per year) (45).

Table 1. Benchmark Values in the Netherlands for Protozoans and Invertebrate Taxa in Finished Water from Treatment Plants and Water Flushed from Mains (Numbers/1000 L) [From J. H. M. Van Lieverloo, *J. Am. Water Works Assoc.*, (submitted) (45).]

	Finished Water From Treatment Plants[a]							Water Flushed from Distribution Mains[b] (both surface water and groundwater)			
	Elevated numbers[c]		High numbers		Very high numbers						
	Surface water	Groundwater	Surface water	Groundwater	Surface water		Ground water	Elevated	High	Very high numbers	
Numbers/1000 L	Median ≥	Sample ≥	Median ≥	Sample ≥	Median ≥	Sample ≥	Sample ≥	Median ≥	Median ≥	Median ≥	Sample ≥
Very small (<0.5 mm)											
Protozoa	10	30	30	100	500	3,000	1,000	Not determined			
— Ciliophora	5	nd[d]	10	nd	500	1,000	nd	These very small organisms are not collected			
— Gymnamoeba	5	nd	5	nd	5	100	nd	quantitatively by filtering through 30-μm mesh			
— Testacea	5	nd	10	nd	30	500	nd	plankton filters. Furthermore, these organisms			
Rotifera	300	30	1,000	100	5,000	10,000	500	are difficult to identify and quantify between the			
Gastrotricha	5	5	5	10	300	1,000	50	sediment particles in samples.			
Tardigrada	5	5	5	5	10	30	30				
Small (0.5–2 mm)											
Nematoda	30	5	80	10	100	1,000	100	50	300	1,000	10,000
Hydrachnellae	5	5	5	5	5	5	5	10	50	100	1,000
Cladocera	5	5	5	5	5	30	10	50	300	1,000	10,000
Ostracoda	5	5	5	5	5	5	5	10	10	10	100
Copepoda	5	5	80	10	100	1,000	50	30	300	1,000	5,000
Copepoda larvae	10	5	300	30	1,000	5,000	100	10	100	300	5,000
Large (>2 mm)											
Turbellaria	5	5	5	5	5	10	10	10	30	100	1,000
Oligochaeta	5	5	5	5	5	30	10	30	100	300	10,000
Gastropoda	5	5	5	5	5	5	5	1	1	5	50
Asellidae	5	5	5	5	5	5	5	1	30	100	100
Chironomidae	5	5	5	5	5	5	5	1	1	3	50

[a] 200 L from a finished water tap is filtered through a 10-μm mesh filter.

[b] 1,000 L is flushed out of mains via a hydrant at a flow increase (in mains) of 1.0 m/s and filtered through 500-μm mesh plankton filters. Simultaneously, approximately 100 L of the volume is also filtered through a 30-μm mesh filter (119).

[c] Median numbers per system (treatment plant, distribution system) or numbers in individual samples are classified 'low to normal numbers' when lower than the benchmark value for 'elevated numbers'. Numbers are 'elevated' when higher than or equal to the benchmark value for 'elevated numbers' but lower than the benchmark value for 'high numbers' etc. Benchmark values can be equal for different classifications due to detection limits. Detection limits are 5/1000 L for finished water from treatment plants and 10/1000 L for water flushed from mains.

[d] nd: not distinguished. Protozoan taxa are not distinguished separately in groundwater supplies.

CONCLUSION

The presence of invertebrates in drinking water can be a nuisance to consumers and therefore to water utilities, causing complaints about visible organisms, their body parts, or fecal pellets emerging from taps. The blocking of water supply, especially in water meters, and effects on taste, odor, and microbiological quality are known to occur as well. According to the World Health Organization, the presence of invertebrates in finished water from surface water supplies however is no health risk in developed countries (2). This view is not generally endorsed because of risks attributed to surface water invertebrates protecting pathogenic microorganisms from disinfectants in treatment plants (13,138). Protozoans are known to protect (micro-)organisms from disinfectants, and in some species *Legionella* bacteria may multiply. Therefore, protozoans and invertebrates, especially nematoda, are still under scrutiny from a health point of view (5,139,140).

Invertebrates and protozoans present in source water may immigrate into distribution systems by passing treatment systems, others may intrude via openings in the structure of treatment plants, finished water reservoirs, and distribution systems. In these systems, the organisms may multiply, provided they can adapt to the conditions and they can find enough and suitable food. Adult insects, for instance, are not able to survive in distribution systems because of the absence of air. Planktonic species and filter feeders will perish in most systems owing to a lack of suspended food particles. Benthic grazers, scrapers, and sediment feeders are most likely to flourish, especially when biofilm formation rate on water-exposed surfaces, including sediments, is high. Normal disinfectant residuals will only partly limit biofilm formation, invertebrate survival, and invertebrate multiplication, whereas periodical removal with pesticides, high disinfectant concentrations, or mechanical methods will only temporarily control invertebrate numbers. Because invertebrate populations are able to reestablish quickly, using biofilm and newly accumulated sediments, water utilities will be forced either to frequently repeat reactive control measures or to proactively limit biofilm formation rates and sediment accumulation rates. Considerations about increasing disinfectant residuals to control invertebrates would have to include a balance between consumers opinion about rarely detecting visible invertebrates and a continuous presence of a taste of disinfectants. The limitation of consumer complaints and microbiological deterioration of drinking water during distribution is likely to be attained most efficiently by limiting the availability of food sources for bacteria, protozoans, and invertebrates in distribution systems. An integral cost-benefit evaluation, including the costs of flushing and the use of bottled water as well as the benefits on other quality aspects such as microbiology, toxicology, and discoloration, is however necessary to substantiate further investments in treatment plants.

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Diagnosis of fouling problems of NF and RO membrane installations by a quick scan

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Abstract

Controlling fouling of membrane filtration installations (nanofiltration and reverse osmosis) is a major challenge for the water industry. Fouling affects the plant performance and increases the costs of plant operation. Diagnosis of the type(s) and extent of fouling is the essential first step for controlling fouling in order to maintain plant performance. A quick scan of the installation for the diagnosis of fouling involves an on-site study of a membrane element (autopsy), including evaluation of data of pretreatment. Comparison of the results of the quick scan with the database with results of autopsies related to plant performance (NPD, MTC), chemicals dosed (e.g. scale inhibitor) would show whether fouling is present and elucidates the types and extent of fouling present. The database also enables the evaluation of the risk on biofouling. Diagnosis of the type of fouling enables directed and more effective actions for prevention and control of (bio)fouling. Studies at (pilot) plants showed that operational problems can be caused by other types of fouling than expected for and that dosed chemicals can pose a risk for (bio)fouling. These case studies emphasize the significance of diagnosis of the type of fouling. Also, tools for prevention and prediction and studying the effect of cleaning strategies on fouling are presented.

Keywords: Biofouling; Fouling; Scaling; Diagnosis; Membranes; Reverse osmosis; Nanofiltration; Drinking water; Autopsy

1. Introduction

The use of membrane filtration in water treatment enables the production of high quality drinking water by removing microorganisms, inorganic and organic compounds, respectively. However, fouling of membrane elements may cause operational problems of membrane installations (e.g. increase of pressure drop and/or decrease of flux). Fouling increases the costs of plant operation

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and can be a threat for the production of drinking water. Fouling can ultimately lead to an early replacement of membrane elements. The fouling mechanisms of spiral wound membranes include scaling (inorganic, organic and colloidal) and biofouling. Scaling by inorganic compounds is usually controlled by the use of a scale inhibitor (polymer, acid) and scaling by colloidal material can be controlled by pretreatment (e.g. ultrafiltration). Biological filtration processes removing biodegradable compounds as pretreatment can reduce the risk on biofouling.

Different types of fouling can occur simultaneously, influencing each other [1]. Biofouling is considered to be a major (and most difficult to control) problem in NF and RO membrane filtration [2]. Therefore, controlling biofouling is a major challenge in operating membrane filtration installations [3,4].

Biofouling — defined, as accumulation of biomass on a surface by growth and/or deposition to such a level that it is causing operational problems — is difficult to quantify. However, the diagnosis biofouling is only justified when a relation is found between the encountered operational problems and biomass accumulation as determined with adequate parameters. Biofouling, which has been described extensively [1,5] may cause flux reduction (MTC decrease) and/or increased pressure drops (normalized pressure drop: NPD). A suite of biomass parameters and analytical tools are available [6], but the question remains which parameter(s) at which level is conclusive for identifying biofouling as the cause of the operational problems. Chemicals, such as scale-inhibitors (polymers and acids) used for scale control in NF and RO membranes differ greatly in their ability to promote growth of microorganisms and certain commercially available scale-inhibitors can cause biofouling [7]. These chemicals and chemicals dosed in pretreatment (e.g. flocculent) may also cause scaling [not published].

This report describes our efforts aiming at achieving methods and strategies for diagnosis of (bio)fouling, which enable prediction and prevention and control of (bio)fouling. This study is part of the integral approach of biological activity in water supply as developed at Kiwa.

2. Diagnosis

Identifying the cause of fouling of a membrane filtration (pilot) plant requires a destructive study (autopsy) of one or several spiral wound membrane elements. For obtaining reliable quantitative results it is essential to have skilled personnel and select the appropriate elements for autopsy and have certain equipment for analysis. Precautions must be taken to preserve the biomass composition, activity, spatial distribution and density as present under operational conditions in the membrane element. Autopsy includes the following steps — visual inspection — lengthways opening of the element — visual inspection — sampling and analysis [8]. The analysis involves adenosine-triphosphate (ATP) concentrations (a measure for the amount of active biomass), total direct cell counts (microscopic cell count, TDC), and heterotropic plate counts (HPC) on R2A medium (expressed in colony forming units, CFU) and inorganic compounds by ICP-MS (inductively coupled plasma mass spectrometry) analysis. The autopsy and the analysis can be performed on site using portable equipment. Conservation of samples enables the determination of parameters like colony counts, total direct cell counts and the concentration of inorganic compounds.

Autopsies have been conducted in 45 NF and RO membrane elements (several brands) obtained from 16 (pilot) plants. The various plants were supplied with water, which varied in: (i), type of source water (aerobic, anaerobic, ground water, surface water, tap water and seawater; (ii), pretreatment; (iii), temperature (from 1 to 30°C, stable and fluctuating) and (iv), dosage of chemicals (e.g. scale inhibitor), respectively. Ranges of concentrations of biomass parameters observed in membrane elements are shown in Table 1. Operational problems with certain plants include an increase of the NPD up to 300% or a decrease of the MTC

Table 1
Range of average concentrations of biomass parameters, iron and manganese in 45 RO membrane elements from 6 (pilot) plants with and without biofouling

Parameter	Range
Adenosinetriphosphate (ATP), pg ATP/cm^2	20–45,000
Total direct cell count (TDC), cell/cm^2	1×10^7–2×10^9
Heterotropic plate count (HPC), CFU/cm^2	3×10^3–3×10^7
Iron, mg/m^2	<1–250
Manganese, mg/m^2	<0.01–4

value of about 25% at the time of autopsy. Autopsies of membrane elements from these plants in general demonstrated high biomass concentrations (>1000 pg ATP/cm^2).

The quantitative information obtained from the autopsies provides a useful database concerning biofouling and scaling. Low biomass values in the membrane element have been observed in absence of operational problems and high levels of biomass have been observed in plants with severe operational problems. For values in between these extremes, the relationship between the involved parameters and the extent of the operational problems must be determined. Two case studies showed a combination of relatively low levels of biomass parameters with relatively high levels of inorganic compounds (up to 25,000 mg/m^2). These concentrations of inorganic compounds probably affected plant performance. At another case study of a plant with decreasing MTC-values the presence of uniformly shaped and sized fibers was observed at the feed side of the membrane. Fouling was caused by the accumulation of the fibers (which passed pretreatment) on the membranes.

A quick scan (autopsy and analysis of data obtained from the plant) will result in the diagnosis whether biofouling is present and includes a risk evaluation and recommendations to find the cause(s) of fouling if present. For prediction and prevention of fouling tools are available.

3. Tools for prediction and prevention and evaluation of cleaning

In order to predict and prevent biofouling also appropriate parameters for the assessment of the concentration of microorganisms and nutrients in water prior membrane filtration are needed. Parameters applied for the determination of the concentration of microorganisms in water are also applied during the autopsy (ATP, TDC and HPC-values). Parameters for assessment of the concentration of growth promoting substances in water are: AOC and the biofilm formation rate (BFR). The AOC-test is a bioassay with two well-defined pure cultures. From the maximum growth level of the two individual strains the AOC concentration is calculated and expressed as μg of acetate-C equivalents/l [9]. The BFR-value is determined with an on-line operated biofilm monitor at a continuous flow rate of 0.2 m/s. The accumulation of active biomass (ATP) on the surface of glass rings in this monitor is determined as a function of time and the BFR value is expressed as pg ATP/cm^2.d [10].

Severe biofouling has been observed in cases where the feed water had BFR-values — temporarily — exceeding 120 pg ATP/cm^2.d and/or the AOC-value exceeded 80 μg Ac-C/l [8]. BFR-values lower than 1 pg ATP/cm^2.d enabled stable operation for periods up to 2 years without cleaning. Relatively low values for these parameters have been observed in feed water of (pilot) installations with extensive pretreatment. These installations operated in general without biofouling problems and autopsies showed relatively low concentrations of biomass. High values (BFR, AOC) have been observed in (pilot) plants either treating surface water with only ultrafiltration as pretreatment or treated wastewater. Autopsies performed at these locations generally demonstrated high concentrations of biomass. However, extensive pretreatment does not guarantee absence of biofouling. Also variations in the concentration of microorganisms and/or growth promoting compounds in the RO-feed water have been observed. The

feed water quality can be influenced by (seasonal) variations of water quality prior pretreatment, which are not completely eliminated in the pretreatment. Dosage of — even one batch of impure — chemicals (flocculent, scale-inhibitor) to the feed water can cause serious biofouling, leading to early replacement of membranes [11,12]. Testing (AOC, BFR) of 14 commercially available scale-inhibitors based on polymers and dosage to single element units demonstrated that chemicals used to prevent scaling differ greatly in their ability to promote growth of microorganisms and confirmed the experiences in practice [7].

Biofouling can be prevented by (i) reducing the concentration of microorganisms and/or reducing the concentration of nutrients by pretreatment and/or (ii) performing preventive/curative cleanings. Tests with cleanings of membranes with biofouling showed that it is difficult to remove the biomass from the membrane [8].

A combination of pretreatment and cleaning may be the way to prevent biofouling. Membrane filtration plants with slow sand filtration — resulting in low values for AOC and BFR — as pretreatment were characterized as plants with low risks of biofouling.

The described methods are currently applied in practice for screening of the biofouling potential of water types and for monitoring (pilot) installations. This research is aiming at establishing more quantitative relationships between the concentrations of biomass and nutrients in the feed water and biofouling.

4. Summary

- Diagnosis of the type/cause of fouling is an essential first step aiming at controlling fouling. A quick scan (autopsy) gives conclusive information about the types and extent of fouling of the membrane filtration plant. This opens ways for prevention and control of fouling.
- Tools (AOC, biofilm monitor and autopsy) are available for (i) prevention and (ii) prediction and (iii) studying the effect of cleaning (strategies) on fouling.
- Chemicals (flocculent, scale-inhibitor) dosed during pretreatment can cause fouling. The tools (AOC, Biofilm monitor) can be used to test chemicals dosed on the risk of (bio)fouling.
- Three case studies showed that fouling may be controlled by looking closely at the plant set-up, pretreatment and chemicals dosed.

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Biomass production potential of materials in contact with drinking water: method and practical importance

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Abstract Synthetic materials in contact with drinking water may affect microbial water quality by releasing growth-promoting substances. Various tests are being used for assessing the microbial growth-supporting potential of such materials. The biofilm formation potential (BFP) method is based on determining the concentration of active biomass (as adenosine triphosphate (ATP)) on the surface of a material incubated in slow sand filtrate (surface to volume ratio 0.15/cm) at 25°C during a period of 16 weeks. In addition to attached biomass (biofilm), suspended biomass is also produced. The amount of suspended biomass is a significant fraction (20–70%) of the total biomass production, depending of the type of material. Therefore, it is concluded that the production of suspended biomass should be included in evaluating the growth-promoting properties of materials in contact with drinking water. Consequently, the BFP test has been adapted to the biomass production potential (BPP) test, with BPP including BFP and suspended biomass production (SBP), as pg ATP/cm^2. The defining criteria for BPP values for materials require further investigation into the effects of water quality on biofilm formation and the relationship between BPP values and regrowth problems.

Keywords Adenosine triphosphate; biofilm; biomass production potential; drinking water; *Legionella*; synthetic materials

Introduction

A wide range of plastic materials are available for use in contact with potable water, including drinking water distribution systems and installations in houses and other buildings. Typical materials applied in such systems include cross-linked polyethylene (PEX), polypropylene, polybutylene, chlorinated PVC, etc. In addition, the percentage of synthetic pipe materials is increasing, with unplasticized PVC (PVCu) as the dominant pipe material in The Netherlands and polyethylene materials used in the UK and in the USA. These materials have certain attractive properties in comparison with metals, concrete or asbestos cement. Certain materials cause environmental concern. The use of copper materials in contact with drinking water is a major cause of the release of copper in the environment (CUWVO, 1993) and the use of plastic systems is advocated. The intensive contact between materials and drinking water may affect water quality. Problems such as taste or odour, colour, the release of undesirable compounds and also the stimulation of microbial growth have been reported for synthetic materials, but also for natural materials in contact with drinking water (Burman, 1979; Colbourne and Brown, 1979; Schoenen and Schöler, 1983). Materials were also found to stimulate the growth of opportunistic pathogenic bacteria, in particular *Mycobacterium* spp. (Schulze-Röbbecke and Fischeder, 1989) and *Legionella* (Rogers *et al.*, 1994).

In the Netherlands a system for testing of materials in contact with drinking water is used for evaluating the suitability of products in contact with drinking water based on information about product composition and data about the toxicity of the constituents (the Positive List System) (VROM, 1992). Distribution of drinking water without a disinfectant residual is common practice in The Netherlands. Prevention of regrowth requires extended water treatment for removing biodegradable compounds from the water and the use of materials which do not stimulate regrowth. In determining the effect of water treatment regarding

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the removal of biodegradable compounds, methods have been developed for assessing the microbial growth potential of water, namely the AOC method and the method for determining the biofilm formation rate (Van der Kooij, 1992; Van der Kooij *et al.* 1995). Assessment of the biofilm formation rate is based on determining the linear growth rate of biomass accumulating on glass surfaces exposed to flowing water (0.2 m/s). Adenosine triphosphate (ATP) is used as the biomass parameter. ATP analysis enables fast detection of all active biomass, including both heterotrophic and autotrophic microorganisms. The technique has also been applied in determining the concentration of biomass ("biofilm") on the surfaces of pipes used for drinking water distribution.

Since ATP has been found to be an attractive parameter for biomass analysis, a test method for determining the biofilm formation potential (BFP) of materials in contact with drinking water has been developed using this parameter (Van der Kooij and Veenendaal, 1994). The BFP method only includes the use of the biomass present on the surface of the material to be tested. A number of materials release a significant proportion of growth-promoting components into the water, resulting in the formation of suspended biomass in addition to attached biomass. This paper describes the production of suspended biomass by materials in the BFP test and, based on these observations, the BFP test is adapted to the biomass production potential (BPP) test.

Methods and materials

ATP analysis

ATP is an energy-rich compound which is present in all living organisms. ATP analysis is based on extraction of the compounds from biomass using a nucleotide-releasing agent, followed by the light-generating luciferine–luciferase reaction. The generated light signal is measured as relative light units (RLU) after a 2 s delay time and a 10 s integration time with a luminometer (Lumac). The concentration of ATP is calculated from the RLU values using a conversion factor determined in calibration measurements.

BFP test

The BFP test is based on determining the concentration of active biomass on the surface of sample pieces of the material (each approximately 8 cm^2 external surface) incubated in biologically stable drinking water (slow sand filtrate) at 25°C during a period of up to 16 weeks. This water (600 ml) is contained in thoroughly cleaned, glass-stoppered Erlenmeyer flasks with a volume of 1 l. The material pieces are present with a total external surface area of approximately 100 cm^2, giving a surface to volume (S:V) ratio of approximately 0.15/cm. A small volume (5 ml) of membrane-filtered (pore size 1.2 µm) river water is added to each Erlenmeyer flask in order to ensure the presence of a wide variety of microorganisms, thus including a large metabolic potential for using compounds introduced with the materials. Duplicate flasks are incubated at 25°C in the dark and samples are collected after 1, 2, 4, 8, 12 and 16 weeks of incubation, respectively. Material pieces are collected from flask A and water samples are collected from flask B. Biomass concentrations are determined as ATP. Biomass is released from the material pieces by a series of six repeated low energy sonications, each for a duration of 2 minutes in autoclaved tap water. The ATP concentration in the obtained suspensions is determined and from this value, the ATP concentration of the biofilm is calculated. The BFP value is defined as the average ATP concentration on the material after 8, 12 and 16 weeks of exposure. The concentration of suspended biomass is determined in the water samples collected from flask B. The principle of the BFP test is based on the fact that the ATP concentration in biomass is directly related to biomass activity. In the exponential growth phase cells contain a large ATP concentration, which rapidly declines when the cells enter the stationary phase of growth (cf.

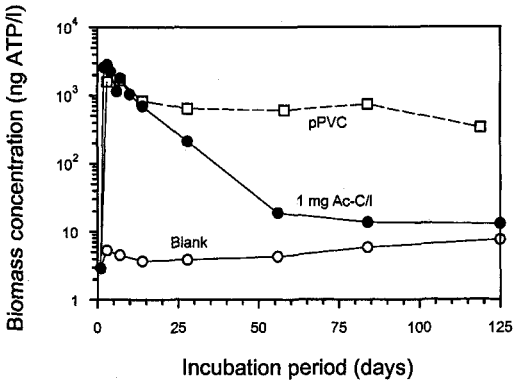

Figure 1 Effect of plasticized PVC (pPVC) and acetate (1 mg/l dosed at day 0) on the concentration of active biomass (ATP) in slow sand filtrate at 25°C

Figure 1). Hence, maintenance of a certain ATP concentration in the biofilm is a measure of the supply of biodegradable compounds to the biofilm. An incubation period of 16 weeks was found to be representative for this purpose.

Heterotrophic plate counts and *Legionella*

In addition, *Legionella* or other bacteria can be inoculated in the flasks and these and other bacteria can be enumerated in the biofilm and in the water using the appropriate techniques. *Legionella* is added to the samples as a mixed culture with aquatic bacteria grown in water containing pieces of a growth-promoting material. The heterotrophic plate counts (HPC) are determined using R2A medium incubated at 25°C over 10 days. Colony counts of *Legionella* are determined on buffered charcoal yeast extract agar (BCYEA) medium, with antibiotics. BCYEA without cysteine was used as a control to confirm the identity of typical colonies.

Results

Biofilm formation and suspended biomass production of PTFE, plasticized PVC (PVCp) and glass fibre-reinforced polyester

Samples of PTFE, PVCp and glass fibre-reinforced polyester (GRP) were incubated in the BFP test at 25°C and the ATP concentrations on the material (biofilm) and in the water were determined as a function of time over a period of up to 16 weeks. Figure 2 shows that the ATP concentration of water without material (blank) and with glass remained below 10 ng/l. This low level is indicative of a high degree of biostability and confirms that slow sand filtrate and well cleaned glass do not promote microbial growth. In the presence of materials, suspended biomass concentrations of up to 2000 ng/l were observed within 1–2 weeks with PVCp and GRP, respectively, but these values decreased upon further incubation and, after 50–75 days, plateau values were attained. Biofilm concentrations were also highest with PVCp and GRP and not detectable on glass (≤1 pg ATP/cm^2). These observations demonstrate that the various materials tested in this series show large differences in respect of biomass production, both attached and suspended. Suspended biomass production (SBP) can be calculated from the suspended biomass concentrations using the S:V ratio of the materials. Subsequently, these values can be added to the BFP values resulting in the biomass production potential (BPP) of the materials (after correction of the growth in the blank). The BFP, SBP and BPP values of the materials included in the test are presented in Table 1. This table reveals that the production of suspended biomass accounts for a significant part of the total BPP. The highest contribution was found for PVCp, which also had the highest BFP and BPP values.

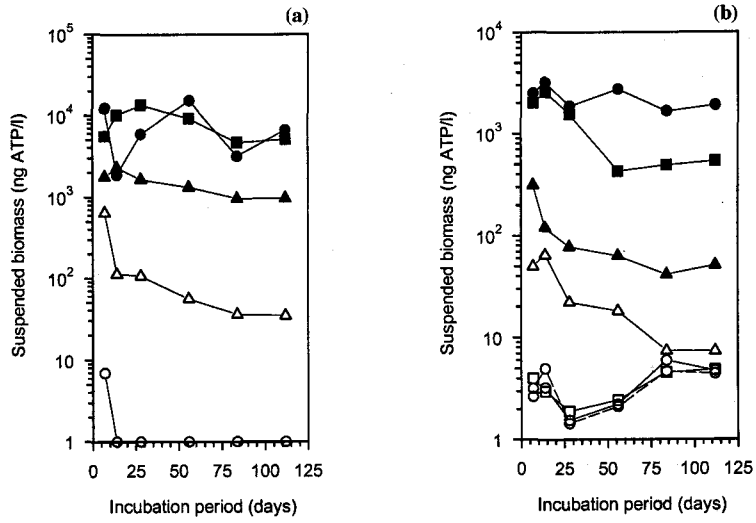

Figure 2 (a) Biofilm concentrations and (b) concentrations of biomass suspended in water (B) grown in the presence of various materials incubated at 25°C in slow sand filtrate (DOC = 2.0 mg/l). *Open circles, water* (blank); open squares, glass; open triangles, PTFE; l, PVCp; n, GRP 1; s, GRP 2

Table 1 BFP, SBP and BPP of materials in contact with drinking water

Material	BFP (pg ATP/cm²)	SBP (pg ATP/cm²)	BPP (pg ATP/cm²)	SBP:BPP (%)
Water	ND	25 (*)	25	100
Glass	1	<1	1	ND
PTFE	32	56	88	64
PVCp	6253	15111	21364	71
GRP 1	814	558	1372	41
GRP 2	4748	4330	9078	47

Relationship between ATP concentration and HPC

In the test series, the HPC values on the material and in the water were determined on day 112 in combination with ATP measurements in order to establish the relationship between these two parameters in water and in the biofilm. Figure 3 shows that the ATP values correlated well with the HPC values, in both suspended biomass and attached biomass, respectively. However, the HPC:ATP ratio differs one order of magnitude, i.e. approximately 10^3 CFU/pg ATP in water and 100 CFU/pg ATP in the biofilm. This difference may be explained by a higher ATP level in the cells in the biofilm than in suspended cells or a difference in population composition with less culturable but still active cells in the biofilm or a combination of these factors. Based on the CFU:ATP ratios and the data available on the ATP content of active cells (median value for bacteria in drinking water 2.5×10^{-5} pg ATP/cell) (D. Van der Kooij, unpublished results) it can be concluded that only a fraction of the cells were included in the HPC values.

Growth-promoting properties of PVCu and polyethylene

PVCu is the dominant pipe material in the Netherlands (46% of total pipe length). Polyethylene is used in a much smaller percentage (approximately 1.8%), with the main application of connecting houses or buildings with pipes in the street. Several of these materials were tested in order to determine BFP and BPP values. In addition, *Legionella* was added to the test flasks as an inoculum in order to determine the effect of these materi-

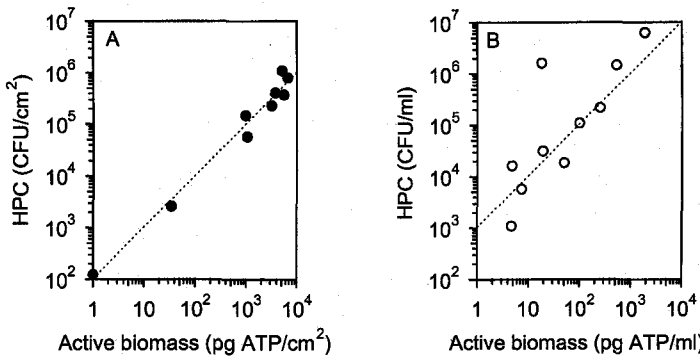

Figure 3 Relationship between ATP concentration and HPC values on the material and in water after 112 days of incubation. HPC was on R2A medium (10 days at 25°C). Dotted lines represent proportional relationships

als on *Legionella* at 25°C. This temperature can be attained in summer periods during the distribution of drinking water. The overall results of the test are shown in Table 2.

Table 2 shows that PVCu had BPP values close to 100 pg ATP/cm^2. The BPP values of the two polyethylene types were clearly higher. The lowest SBP:BPP ratios were observed for polyethylene. Under the test conditions, *Legionella* multiplied on the materials but growth was very limited on glass and on PVC. Relatively high numbers were observed on both polyethylene types, which also showed the highest BFP, SBP and BPP values. The growth of *Legionella* was slow and maximum values may not have been reached within the test period (112 days).

Discussion

Several test methods have been developed in the past 25 years for assessing the growth-promoting properties of materials in contact with drinking water during storage, transportation and distribution. In the UK the mean dissolved oxygen difference (MDOD) method is used, which determines the oxygen consumption in the presence of a material (Colbourne and Brown, 1979). The test applied in Germany, as described in Arbeitsblatt W270 (DVGW, 1990) is based on assessment of the volume of slime produced on the surface of plates of the material to be tested. These methods and the BFP method have been included in the prENV, as compiled in the framework of CEN (1999). A disadvantage of the MDOD and W270 methods is that the parameter used for determining growth cannot simply be used in determining biomass concentrations in water or on materials in practice. For this reason, a test method based on using ATP as the biomass parameter has been designed. ATP analysis has

Table 2

Material	BFP (pg ATP/cm²)	SBP (pg ATP/cm²)	BPP (pg ATP/cm²)	SBP:BPP (%)	Legionella (CFU/cm²)(2)
None (blank)	Nd	36(1)	36(1)	100	No growth
Glass	8	7	15	47	10 (day 84)
PVCu 1	51	35	86	41	25 (day 84)
PVCu 2	57	56	113	50	690 (day 112)
Polyethylene-40	1105	232	1337	17	4.2×10^3 (day 112)
Polyethylene-80	660	226	876	26	9.0×10^3 (day 112)

(1)Theoretical value based on the concentration of suspended biomass, the water volume and a surface area of 100 cm^2. This value is used for calculating the net BPP values of the materials.
(2)Maximum value observed in biofilm within 112 days; the day number at which a maximum was observed is indicated in parentheses.

many potential applications in the monitoring of bioactivity in water treatment and distribution systems. Moreover, the method is both rapid and sensitive, which are also important conditions for its application in water supply.

The BFP test provides information on the potential of a material to support a biofilm on its surface as the result of the release of biodegradable compounds from the material under static conditions. This condition may be regarded as a worst case situation; in practice the flow of water will remove biomass, but may also add biomass when compounds promoting biofilm formation are present. Such compounds may originate from the raw water, treatment effects (e.g. ozonation) or from the distribution system, for instance materials releasing biodegradable compounds. As a test period, 8–16 weeks was used. This period is slightly shorter than the period used in the W270 test (up to 6 months) but longer than the test period in the MDOD test (7 weeks). Still, it is possible that, after 16 weeks, certain materials do not reach a steady-state situation with regard to the release of biodegradable compounds. Another aspect is the relatively strong promotion of growth within the first 2 weeks of exposure. Hence, a material can be evaluated on the basis of growth promotion within a few weeks, within 16 weeks or an even longer period. Furthermore, inclusion of the production of suspended biomass gives a more complete measure of the potential of a material for promoting growth. Biodegradable compounds released into the water at a certain location in the distribution system can contribute to biofilm formation at other locations and, therefore, should be included in the test and the evaluation.

The results presented above show that various materials show large differences in growth-promoting properties. PVCu, which is the predominant material in distribution systems in The Netherlands, produces very little biomass production and is well suited for the distribution of biologically stable drinking water without disinfectant residual. Hence, PVCu can be used as a reference material. PVCp, with its very high BFP, SBP and BPP values (cf. Table 1), is unsuited to the distribution of drinking water. The other materials are between these two extremes, but a system for evaluating the suitability of a material to be used in contact with drinking water based on the results of the BFP/BPP test is needed. One approach is to compare BFP values with the biofilm concentrations observed in drinking water distribution systems. Biofilm concentrations in distribution systems in The Netherlands range from approximately 100 to 6000 pg ATP/cm^2 (on PVCu). Values greater than 1000 pg ATP/cm^2 are observed in distribution systems with regrowth of aeromonads (D. Van der Kooij, unpublished results). This would imply that BPP values should preferably be less than 1000 pg ATP/cm^2. Another approach is to determine the capability of a material for supporting multiplication of undesirable bacteria, in particular *Legionella.* Table 2 shows that polyethylene materials do support *Legionella* growth (under the static test conditions) much more strongly than PVCu. This observation is in agreement with the findings of Rogers *et al.* (1994) who also found that polyethylene materials support multiplication of *Legionella.* However, polyethylene is used as a major pipe material in a number of countries and evidence that this material promotes multiplication of *Legionella* in practice does not seem available. Relatively low water temperatures and sloughing of biofilm material may suppress the multiplication of *Legionella* in most situations.

Conclusions

The data presented above lead to the conclusion that the compounds released by a material and leading to the production of suspended biomass are a significant proportion of the BPP of a material. Therefore, it is concluded that both the BFP and SBP giving the BPP of a material should be determined. Further adaptations of the test may be needed (incubation period and temperature). Another approach is to adapt the test procedure by applying a regime of periodic replacement of the water. Such experiments and other investigations

may be included in the European Approval Scheme project that is expected to start at the end of the year 2000 with the financial support of the European Community.

At the present time insufficient information is available for clearly defining criteria for materials in terms of BFP and/or BPP values. It is possible that different criteria are needed for different situations. Further research is needed in order to determine the effects of these parameters on the microbial quality of drinking water.

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Diagnosis, prediction and prevention of biofouling of NF and RO membranes

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Abstract

Biofouling phenomena were studied in 30 membrane elements (autopsy) for nanofiltration (NF) or reverse osmosis (RO) collected from 13 plants. Biomass parameters included adenosinetriphosphate (ATP), Total Direct Cell counts (TDC) and Heterotropic Plate Counts (HPC), respectively. The concentrations of biomass were related to the extent of variation of operational parameters as flux (normalized flux: MTC) and/or pressure drops (normalized pressure drop: NPD), viz. highest biomass parameters were observed in plants with the highest changes of MTC and/or NPD. Monitoring of feed water from NF and RO plants was performed with biomass parameters (ATP and TDC), the easily assimilable organic carbon (AOC) test and the Biofilm Formation Rate (BFR) in a biofilmmonitor. Monitoring of the feed water showed that severe biofouling occurred in cases where the feed water had BFR-values — temporarily — exceeding 120 pg ATP/cm².d and/or the AOC-value exceeded 80 µg Ac-C/l. BFR-values lower than 1 pg ATP/cm².d enabled stable operating for periods up to two year without cleaning. For BFR-values between 1 and 120 pg ATP/cm².d as well as AOC-levels below 80 µg is still unknown whether biofouling occurs. Monitoring also showed that low concentrations of biodegradable compounds (µg/l) in the feed water could lead to biofouling. The results of monitoring of feed water agreed with the data from autopsies and extent of operational problems. Biofouling was observed in 12 of the 13 plants. Two plants suffered from biofouling caused by dosage of chemicals. Elucidation of the relationships between test parameters and extent of operational problems (NPD increase and MTC decrease) under different conditions requires more research. Based on such data more quantitative criteria for feed water quality can be defined enabling further prevention of biofouling. Also, further investigation is needed for the selection and preparation of effective cleaning chemicals and cleaning strategies.

Keywords: Antiscalant; Biofouling; Fouling; Biofilm formation rate; Membranes; Drinking water; Reverse osmosis; Nanofiltration; Autopsy

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1. Introduction

Application of membrane filtration in water treatment enables the production of microbiologically safe and biologically stable drinking water by removing microorganisms and inorganic and organic compounds, respectively. However, fouling may cause serious operational problems. Fouling mechanisms of nanofiltration (NF) and reverse osmosis (RO) membranes include: scaling (inorganic, organic and colloidal) and biofouling. Different types of fouling can occur simultaneously, influencing each other [1]. Controlling biological fouling is considered to be a major challenge in operating membrane filtration installations [2,3].

Biofouling — defined as accumulation of biomass on a surface by growth and/or deposition to such a level that it is causing operational problems — is difficult to quantify. However, the diagnosis 'biofouling' is only justified when a relation is found between the encountered operational problems and biomass accumulation as determined with adequate parameters.

Biofouling, which has been described extensively [1,4] may cause flux reduction (normalized flux: MTC) and/or increased pressure drops (normalized pressure drop: NPD). A suite of biomass parameters and analytical tools are available [5], but the question remains which parameter(s) at which level is conclusive for identifying biofouling as the cause of the operational problems. Chemicals such as antiscalants and acids used for scale control in NF and RO membranes differ greatly in their ability to promote growth of microorganisms and certain commercially available antiscalants can cause biofouling [6]. Criteria and a monitoring strategy are needed to detect biofouling in an early stage or for evaluation of the risks of biofouling (water type, pretreatment, chemicals dosed). Curative or preventive measures against biofouling are not always effective. Methods are needed for evaluation of the effects of cleaning methods and chemicals.

This report describes our efforts aiming at achieving methods and strategies for diagnosis of biofouling as well as prediction and prevention of biofouling.

2. Diagnosis

Identifying the cause of fouling of a membrane filtration (pilot) plant requires a destructive study (autopsy) of one or several spiral wound membrane elements. Skilled and trained personnel and certain equipment for analysis are essential for obtaining reliable quantitative results. A first step in autopsy is the selection of the appropriate elements. Precautions must be taken to preserve the original biomass composition, activity, spatial distribution and density as present under operational conditions in the membrane element. These precautions include (i) coverage of the end caps of the elements after removal from the installation, (ii) storage of the element on ice until analysis and (iii) analysis within restricted time — preferably within 24 h — after removal of the element from the installation. Autopsy includes the following steps: visual inspection; lengthwise opening of the element; visual inspection; sampling and analysis.

Visual inspection concerns both the intact membrane element and the membranes after opening. On several occasions deposition of (metal-like) particles have been observed on the influent side of membrane elements and telescoping at the effluent side of membrane elements. Points of interest during visual examination of the unfolded membrane surface are colour, odour, mucous nature of material on the membrane and spacers, presence of particles and special phenomena such as the presence of bladders, folds. Also photographs can be taken. Fig. 1 shows an RO membrane element from an

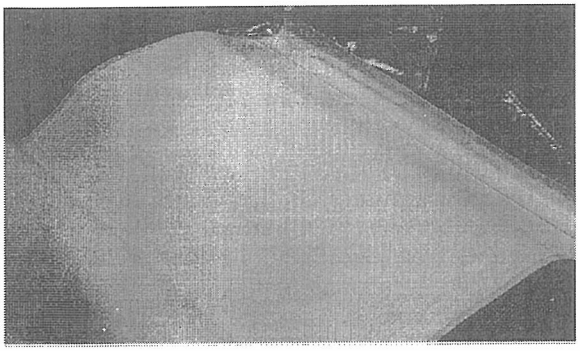

Fig. 1. Unfolded RO membrane element from a plant with severe biofouling.

installation suffering from severe and uncontrollable fouling. The membrane, feed spacer and product spacer all seemed relatively clean during visual examination. Analysis of the membrane element clearly demonstrated high concentrations of active biomass on the feed side of the membrane. Hence, it is difficult if not impossible to determine biofouling visually. Analyses of RO feed water of the involved plant revealed high concentrations of biodegradable nutrients. Therefore, also appropriate analysis of the water quality is needed to identify biofouling as the cause of the observed operational problems.

The concentration of biomass is determined over the length of the unfolded membrane element. For this purpose small segments with a surface area of about 7 cm^2 are sampled. Also the spatial distribution of biomass on the membrane surface, the feed spacer and the product spacer, respectively is determined by sampling and quantitative analysis of the individual parts. Finally, material from the feed side of the membrane is collected by scraping the entire external surface of several membrane envelopes. After determining the sampled surface area, the concentration of biomass and the weight of the sampled material samples of the collected material are stored in the –70°C freezer to enable further chemical analysis when needed. Membrane segments are also collected from the membrane element for quantitative analysis of inorganic elements to enable diagnosis of scaling.

Biomass suspensions are obtained by low energy ultrasonic treatment of membrane parts in sterile water. Adenosinetriphosphate (ATP) concentrations (a measure for the amount of active biomass), Total Direct Cell counts (microscopic cell count, TDC), and Heterotropic Plate Counts (HPC) on R_2A medium (expressed in colony forming units, CFU) are measured in the obtained suspensions. ATP gives an indication of the total amount of active biomass [7]. ATP concentrations are obtained by an enzymatic reaction using the luciferine–luciferase assay. The amount of light produced is determined and the ATP concentration is derived from the linear relationship between light production and reference ATP concentrations. TDC-values (cells/cm^2) are determined with epifluorescence microscopy using acridine orange as fluorochrome — applying a slightly adapted method to eliminate fading [8,9]. All fluorescing cells are counted. TDC does not discriminate between active and not active cells. The microscopic observations also give information about the variety and appearance of microorganisms present. The TDC-values usually have a large standard deviation. This is caused by the presence of large clusters of thousands of cells (Fig. 2). Ultrasonic

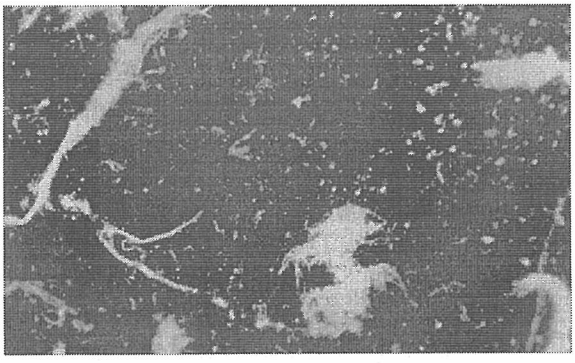

Fig. 2. Acridine orange stained (clusters) of microorganisms in suspension obtained from the feed side of an RO membrane with biofouling.

treatment used as pre-treatment of the cell count is obviously not capable of disrupting the cell clusters. Microscopic observations of the TDC samples also give information about the possible presence of extracellular polysaccharides (EPS).

HPC-values (CFU/cm²) were determined by spreading 0.05 ml volumes of water on nutrient poor R_2A medium [10]. Colonies were counted after 10 d of incubation at 25°C. HPC-values on R_2A medium have a relatively high yield of colony forming units when compared to other media. This high yield is obtained by the combination of a relatively low nutrient concentration in the medium and a long incubation time at a moderate incubation temperature. However, the HPC-values on R_2A medium usually are a minor part of the total number of cells present. The HPC-value generally is ≤1% of the TDC-value.

The use of chemicals for cleaning purposes affect the various biomass parameters in a different way; e.g. ATP and HPC-values can be reduced by bactericidal activity without reduction of the TDC-values. For this reason it is essential to perform all analysis mentioned.

Autopsies have been conducted in 30 NF and RO membrane elements (several brands) obtained from 13 (pilot) plants. The various plants were supplied with water, which varied in: (i), type of source water (aerobic, anaerobic, ground water, surface water, tap water and seawater; (ii), pre-treatment; (iii), temperature (from 1 to 25°C, stable and fluctuating) and (iv), dosage of chemicals (antiscalant and acid), respectively. Ranges of concentrations of biomass parameters in membrane elements are shown in Table 1. Operational problems with certain plants include an increase of the NPD up to 300% or a decrease of the MTC value of about 25% at the time of autopsy. Autopsies of these plants in general demonstrated high biomass values as shown in Table 1.

Concentrations of biofilm parameters can vary greatly over the length of a single membrane element and also between elements depending on the location in the plant, operation of the plant and type of fouling. Hence, appropriate selection of the membrane elements for autopsy and the samples on these elements is critical for diagnosis.

Table 1
Range of average concentrations of biomass parameters, iron and manganese in 30 RO membrane elements from 13 (pilot) plants with and without biofouling

Parameter	Range
Adenosinetriphophate (ATP),pg ATP/cm²	20–45,000
Total direct cell count (TDC), cells/cm²	1×10^7–2×10^9
Heterotropic plate count (HPC), CFU/cm²	3×10^3–3×10^7
Iron, mg/m²	<1–250
Manganese, mg/m²	<0.01–4

The range of distribution of active biomass (ATP) over the individual membrane layers (Table 2) shows that in most cases over 99% of the biomass is present at the feed side of the membrane, including the feed spacer. The feed spacer has a relatively low surface area when compared to the membrane surface, indicating that biomass concentrations on the feed spacer are relatively high. High concentrations of active biomass on the feed side of the membranes indicate that sufficient degradable nutrients are available for these microorganisms.

Table 2
Range of distribution of active biomass (ATP) over the individual layers of a membrane envelope

	Range (% of total)
Feed spacer	5–60
Membrane	40–95
Product spacer	<1–3

The quantitative information obtained from the autopsies provides a useful database concerning biofouling and scaling. Low biomass

values in the membrane element have been found in absence of operational problems and high levels of biomass have been found in plants with severe operational problems. For values in between these extremes, the relationship between the involved parameters and the extent of the operational problems must be determined.

3. Prediction and prevention

In order to predict and prevent biofouling also appropriate parameters for the assessment of the concentration of microorganisms and nutrients in water prior membrane filtration are needed. Parameters applied for the determination of the concentration of microorganisms in water are ATP, TDC and HPC-values. These parameters have been described above. Parameters for assessment of the concentration of growth promoting substances in water are AOC and the Biofilm Formation Rate (BFR). The AOC-test is a bioassay with two well-defined pure cultures. From the maximum growth level of the two individual strains the AOC concentration is calculated and expressed as μg of acetate-C equivalents/l [11]. The BFR-value is determined with a biofilm monitor. In the on-line operated biofilm monitor a continuous flow rate of 0.2 m/s is maintained and the accumulation of active biomass (ATP) on the surface of glass rings in this monitor is determined as a function of time [12]. The BFR is expressed as pg ATP/cm².d.

The range of the concentration of micro-organisms and growth promoting compounds in the feed water — including dosage of antiscalants and acid — of a selection of NF and RO membrane filtration installations is shown in Table 3. Relatively low values for these parameters have been observed in feed water of (pilot) installations with extensive pre-treatment. These installations operated in general without biofouling problems and autopsies showed relatively low concentrations of biomass (Table 1).

Table 3
Range of average concentrations of biomass parameters (ATP and TDC) and growth potential parameters (AOC and BFR) as observed in the feed water of pilot plants with NF or RO membrane elements

Parameter	Range
Adenosinetriphophate (ATP), ng ATP/l	<1–200
Total direct cell count (TDC), cells/ml	$<4\times10^2$–5×10^7
Easily assimilable organic carbon (AOC), μg Ac-C/l	3–1500
Biofilm formation rate (BFR), pg ATP/cm².d	<0.1–310

High values have been observed in (pilot) plants either treating surface water with only ultra-filtration as pre-treatment or treated waste water. Autopsies performed at these locations generally demonstrated high concentrations of biomass. However, extensive pretreatment does not guarantee absence of biofouling. Also variations in the concentration of microorganisms and/or growth promoting compounds in the RO-feed water have been observed. The feed water quality can be influenced by (seasonal) variations of water quality prior pre-treatment, which are not completely eliminated in the pre-treatment.

Dosage of — even one batch of impure — chemicals (acids, antiscalant) to the feed water can cause serious biofouling, leading to early replacement of membranes [13,14]. Testing of 14 commercially available antiscalants (AOC, BFR) and dosage to single element units demonstrated that chemicals used to prevent scaling differ greatly in their ability to promote growth of microorganisms and supported the experiences in practice [6].

Severe biofouling has been observed in cases where the feed water had BFR-values — temporarily — exceeding 120 pg ATP/cm².d and/or the AOC-value exceeded 80 μg Ac-C/l. BFR-values lower than 1 pg ATP/cm².d enabled stable operating for periods up to two year without

cleaning. A significant linear correlation between the concentration of acetate and BFR value has been reported for low concentrations of acetate added to water in a biofilm monitor, with a BFR-value of 35 pg ATP/cm².d for 1 μg of acetate-C/l [15]. Thus, a BFR-value of 120 pg ATP/cm².d — which is causing biofouling — is similar to an AOC-concentration of about 3 μg acetate-C/l. Low concentrations of — very easily biodegradable — nutrients (μg/l) in the feed water therefore can lead to biofouling of NF and RO membrane plants. A concentration of 10 μg of acetate-C/l also caused biological clogging of sand filter beds [16].

Prevention of biofouling can be obtained by (i) reducing the concentration of microorganisms and/or reducing the concentration of nutrients by pretreatment and/or (ii) performing preventive/curative cleanings. A combination of pretreatment and cleaning may be the way to prevent biofouling. Membrane filtration plants with slow sand filtration — resulting in low values for AOC and BFR — as pretreatment were characterised as plants with low risks of biofouling.

The described methods are currently applied in practice for screening of the biofouling potential of water types and for monitoring (pilot) installations. This research is aiming at establishing more quantitative relationships between the concentrations of biomass and nutrients in the feed water and biofouling.

4. Cleaning

Preventive and curative cleanings of a membrane installation may be needed to control biofouling. The effect of a selection of industrial cleaning chemicals [13] and other chemicals (not published) on pieces of membrane material and other materials with a biofilm has been tested. These tests showed that certain chemicals can effectively reduce biomass activity (ATP), but the biomass removal as measured with TDC values was much more limited.

A cleaning agent, which was effective in the laboratory test, was applied on RO membrane elements. The ATP, TDC and HPC-values observed on membrane parts collected from a membrane element without and after cleaning with this industrial agent are shown in Fig. 3. The two elements were taken from one particular installation. Cleaning of the element significantly reduced the concentration of active biomass (ATP with 94% and HPC with 99.8%) but did not significantly reduce the TDC-values. This means that most of the cells were inactivated (killed) but not removed from the membrane element. After the cleaning the remaining HPC count was about 10^3 CFU/cm² membrane surface area. It is possible that the surviving bacteria multiply rapidly in the presence of inactivated biomass. In order to avoid accumulation of biomass resistant to certain cleanings it is recommended to apply a variety of cleaning agents. Further improvement of cleaning methods and chemicals is needed to control biofouling.

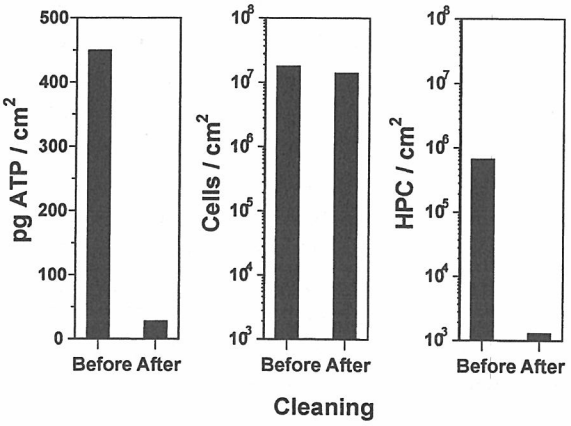

Fig. 3. Average biomass concentration on an RO membrane before and after treatment with an industrial cleaning agent.

5. Summary

- Visual inspection is not conclusive for diagnosis of fouling.

- Autopsies give conclusive information about the cause of fouling.
- Methods for determining the growth potential (AOC, BPP and BFR in the biofilm monitor) of feed water as described above show that low concentrations of biodegradable compounds (μg/l) in the feed water can lead to biofouling.
- Biofouling was observed at 12 of the 13 (pilot) plants, indicating that biofouling is a serious operational problem in NF- and RO-membrane filtration installations.
- Biofouling of 2 plants was caused by dosage of — one batch of impure — chemicals to the feed water.
- A combination of pre-treatment and cleaning may be the way to prevent biofouling.
- Autopsies can be used for studying the efficiency of a cleaning (strategy) on biofouling.
- Further research is needed to determine relationships between test parameters and extent of operational problems (NPD increase and MTC decrease) under different conditions. Based on such data more quantitative criteria for feed water quality can be defined enabling further prevention of biofouling.

6. Acknowledgments

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BIOLOGICAL STABILITY: A MULTIDIMENSIONAL QUALITY ASPECT OF TREATED WATER

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Abstract. Regrowth processes in drinking water distribution systems may lead to hygienic, aesthetic and technical problems. These complex processes depend on interactions between micro-organisms and (i), compounds serving as energy sources; (ii), environmental conditions (temperature, hydraulics) and (iii), physico-chemical processes (sedimentation, corrosion, disinfection), respectively. The concentration of growth-promoting compounds is considered as the main driving force for regrowth and a large variety of tests has been developed to assess the growth-promoting properties of treated water. These methods range from determining the decrease of the concentration of dissolved organic carbon in a batch test to the assessment of the Biofilm Formation Rate (BFR) in a flow-through test. Biostability assessment of treated water in the Netherlands includes the AOC test in combination with the BFR test. The growth-promoting properties of synthetic materials in contact with treated water are determined with the Biofilm Formation Potential (BFP) test. A complete understanding of regrowth processes enabling to define appropriate control measures requires further research including: (i), the effect of reactive surfaces on the availability of slowly degradable compounds, and (ii), improvement of mathematical models describing regrowth processes.

Keywords: biofilm, biological stability, drinking water, easily assimilable organic carbon (AOC), materials, regrowth

1.Introduction

Drinking water supply companies make many efforts to achieve a good quality of drinking water from water sources containing a variety of chemical and biological contaminants. Multiple treatment barriers, including (combinations of) physical, chemical and biological processes, are applied to reduce the concentrations of these contaminants below the Maximum Acceptable Concentration (MAC) values as defined in national legislation. Maintaining water quality during distribution is another challenge, because many factors may impair the quality of treated water in the distribution system. Factors of major importance are: (re)contamination from outside the distribution system, effects of materials in contact with treated water, and biological and/or physico-chemical processes. Good engineering practices, e.g., selection of proper materials and appropriate working procedures, are essential to protect drinking water from contamination. Water quality monitoring is used to detect the efficacy of the preventive measures and to intervene when needed to maintain water quality. Biological processes in the distribution system may cause hygienic (growth of opportunistic pathogens), aesthetic (taste and odour)

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or technical problems (corrosion). The main approaches in controlling these processes are: (i), maintaining a disinfectant residual and (ii), distributing biologically-stable drinking water in a system consisting of biostable materials in contact with water. Biostability is defined as the inability of water or a material in contact with water to support microbial growth in the absence of a disinfectant (Rittmann and Snoeyink, 1984). Maintaining a disinfectant residual can affect taste and odour (Bryan *et al.,* 1973) and/or results in the formation of disinfection by products with toxic properties (Bull and Kopfler, 1991). On the other hand, achieving biological stability, which implies a far reaching removal of biodegradable compounds, makes high demands on water treatment and on the quality of distribution system materials coming into contact with treated water, respectively. Methods and criteria to define biostability are required to either design or optimise water treatment and distribution systems for this purpose. Furthermore, a fundamental understanding of regrowth processes is needed for the development and interpretation of tests to assess the growth-promoting properties of water and materials in contact with water. The need to collect data about effects of measures in treatment and distribution has resulted in the development of a multitude of methods for measuring the growth-potential of drinking water. To a lesser extent also methods for determining the biostability of materials in contact with water have become available. This paper summarizes the complexity of regrowth processes and lists a number of tests developed to assess biostability of treated water. Also a brief description is given of the methods used in the Netherlands for biostability assessment and further research needs are identified.

2. Regrowth Processes and Methods for Biostability Assessment

2.1. REGROWTH PROCESSES

Problems caused by regrowth of micro-organisms range from colour caused by iron-precipitating bacteria (Clark *et al.,* 1967) to disease caused by opportunistic pathogens e.g., *Legionella* (Wadowsky *et al.,* 1982). Key processes in regrowth are: formation of biofilms, detachment of (micro)organisms from the pipe wall and accumulation of sediments, respectively. The presence of biodegradable compounds is a major driving force in these regrowth processes but also other environmental conditions (e.g., temperature, system hydraulics) and physico-chemical processes (adsorption, oxidation/reduction, sedimentation) have a significant effect. Elucidation of microbial growth processes in drinking water distribution systems is hampered by a number of reasons. These reasons include: (i), the large variety of suspended and attached micro-organisms involved; (ii), undefined compounds serving as energy sources at unknown concentrations; (iii), effects of different materials in contact with water; (iv), the hydraulic

complexity of distribution systems; (v), inaccessibility of sampling locations. Properties of both the predominant and the problem organisms are insufficiently known to predict their behaviour (growth, survival) in the described environment. Examples of problem organisms include *Legionella* spp. growing in hot water systems and coliforms multiplying in distribution systems (LeChevallier, 1990). Effects of only a few factors (mainly water temperature and disinfectants) have been studied for these organisms. For certain types of bacteria, e.g., *Pseudomonas* spp., *Spirillum* sp., *Flavobacterium* spp., *Aeromonas* spp. and also coliforms, some information has been collected about their ability to multiply at low substrate concentrations (Van der Kooij *et al.*, 1982; Van der Kooij and Hijnen, 1984, 1985, 1988; Camper *et al.*, 1991). However, substrate utilisation kinetics and conditions favouring growth or survival remain largely unknown for most bacteria multiplying in distribution systems. Based on the assumption that the concentration of biodegradable organic compounds is the main factor limiting regrowth, microbiologists have developed methods to assess the growth potential of treated water.

2.2. METHODS AND PARAMETERS TO ASSESS THE GROWTH-PROMOTING PROPERTIES OF TREATED WATER

Studies of bacterial multiplication in treated water aiming at determining the growth potential began at the end of the 19th century (Frankland and Frankland, 1894). It was observed that the number of culturable bacteria increased much more in stored samples of high quality deep well water with a very low concentration of organic compounds than in river water samples. The assumption was made that well water contained a relatively high concentration of biodegradable compounds, because of the absence of bacteria. However, already at that time it was known that well-closed bottles should be used for growth measurements instead of bottles with cotton plugs. (Frankland and Frankland, 1894). In the Netherlands, Beijerinck (1891) suggested to study the growth-promoting properties of water by inoculating selected pure cultures in sterilised samples of the water to be tested. Heymann (1928) described a method based on determining the effect of a series of passages through sand columns on the concentration of organic compounds in the water, measured as permanganate value. However, this method was not widely applied. Interest in regrowth phenomena strongly increased after 1970 when ozonation and granular activated carbon filtration were introduced in water treatment for the removal of persistent organic pollutants. In 1982, a method for determining the heterotrophic growth potential of treated water was described, which was based on determining the maximum level of growth of a selected pure culture in a water sample collected and contained in thoroughly cleaned, glass-stoppered Erlenmeyer flasks (Van der Kooij *et al.*, 1982). Within one decade many alternative methods for assessing the growth potential of drinking water were developed in Europe

and in the USA (Huck, 1990). Additional methods have been developed since 1990.

The methods can be classified on the basis of the test parameters and test conditions including:

- chemical (e.g., Dissolved Organic Carbon) or biological (biomass) parameter;
- suspended or attached (biofilm) growth;
- batch test (static) or plug-flow (dynamic) system;
- short /medium/long test period;
- on location (in situ) or in the laboratory;
- nature of biomass (pure cultures or mixed population);
- nature of biomass parameter (heterotrophic plate count, total direct count, adenosinetriphosphate, turbidity).

Table I lists the characteristics of the most commonly used tests.

TABLE I
Characteristics of methods for assessing the growth potential of treated water

Method	Characteristics	Reference
AOC	B, PC/HPC; SG/ST; L/MD/LD	Van der Kooij *et al.*, 1982,1984
	B, MP/ATP; SG/ST; L/MD	Stanfield and Jago, 1986
	B, PC/ATP; SG/ST; L/MD	LeChevallier *et al.*, 1993
	B, MP/TU; SG/ST; L/MD	Werner, P. 1985
BGP	B, MP/TDC; SG/ST, L/LD	Servais, *et al.*, 1987
BDOC	C (DOC), MP; AG/ST; L/MD	Joret and Levy, 1986
	C (DOC), MP; AG/DT; IS/SD	Lucena *et al.*, 1990
BFR	B, MP/ATP; AG/DT; IS/LD	Van der Kooij *et al.*, 1995

AG, attached growth (biofilm); AOC, assimilable organic carbon; ATP, adenosinetriphosphate; B, biomass-based parameter; BDOC, biodegradable dissolved organic carbon; BFR, biofilm formation rate; BGP, bacterial growth potential; C, chemical parameter (organic carbon); DT, dynamic (flow-through) test; DOC, dissolved organic carbon; HPC, heterotrophic plate count; IS, in situ; L, laboratory; MP, mixed population (indigenous flora); PC, pure culture(s); SG, suspended growth; SD, MD, LD, short (hours), medium (days), long (weeks) duration of test; ST, static (batch); SU, suspended growth; TDC, total direct count; TU, turbidity.

All these tests have their advantages and limitations, which will not be evaluated in this paper. Only a few comments will be made here:

- Most tests are batch tests conducted in the laboratory under defined conditions. These tests give information about the growth potential;
- The growth potential of treated water may also be affected by the presence of inorganic compounds (e.g., ammonia and sulfides) and methane. These compounds are not included in AOC and BDOC tests;

- Biofilm formation as occurring in distribution systems can be simulated with a variety of techniques and devices under conditions enabling quantitative measurements of biofilm parameters, e.g., Rototorque system (Van der Wende *et al.,* 1989), Robbins device, coupon test and a biofilm monitor (Van der Kooij *et al.,* 1995);
- A combination of growth tests, determining the (effects of) concentrations of rapidly and more slowly available compounds, as well as chemical analysis (e.g., for ammonia and methane), may be needed to assess the biostability of treated water. In the Netherlands biostability assessment of water is conducted using the AOC test and the BFR test;
- Assessment of biological stability of treated water may also require the inclusion of other parameters e.g., the concentration of biomass in the water entering the distribution system;
- The growth-promoting properties of materials in contact with drinking water should also be assessed.

3. Biostability Assessment in the Netherlands

3.1. AOC TEST

Assessment of the concentration of easily assimilable organic ca0rbon (AOC) is based on growth measurements of two selected pure cultures in a sample of pasteurised water contained in a thoroughly cleaned glass stoppered Erlenmeyer flask. The strains used in the test are: *Pseudomonas fluorescens* strain P17, which is capable of utilising a wide range of low molecular weight compounds at very low concentrations (Van der Kooij *et al.,* 1982) and a *Spirillum* sp. strain NOX, which only utilises carboxylic acids (Van der Kooij and Hijnen, 1984). These compounds appear to dominate the easily biodegradable low molecular weight compounds; carbohydrates were observed in much lower concentrations (Van der Kooij and Hijnen, 1985). In most types of drinking water the AOC test requires an incubation period of 1 to 2 weeks before the test strains reach their maximum level of growth. This period exceeds the residence time of drinking water in the distribution system. However, observations on water sampled from a number of distribution systems have shown that AOC concentrations decline rapidly in the distribution system, indicating that biofilm processes play an important role in AOC uptake. AOC reduction was limited at concentrations below 10 µg of C/l and at these low levels the heterotrophic plate counts (HPC) remained low. Based on these observations it was concluded that AOC values below 10 µg of C/l are indicative for drinking water with a limited regrowth potential for bacteria contributing to HPC values (van der Kooij, 1992).

The availability of AOC is also demonstrated in an AOC-reduction test. In such a test additional water samples are collected and incubated at 15°C without

pasteurisation. After a defined incubation period (e.g., 7 days) these samples are also pasteurised and the AOC concentration is determined. Typical results are presented in Figure 1, which shows the decrease in AOC-concentration in ozonated water to a level of about 10 µg of C/l. Even in treated water with AOC concentrations below 10 µg of C/l some further reduction was observed.

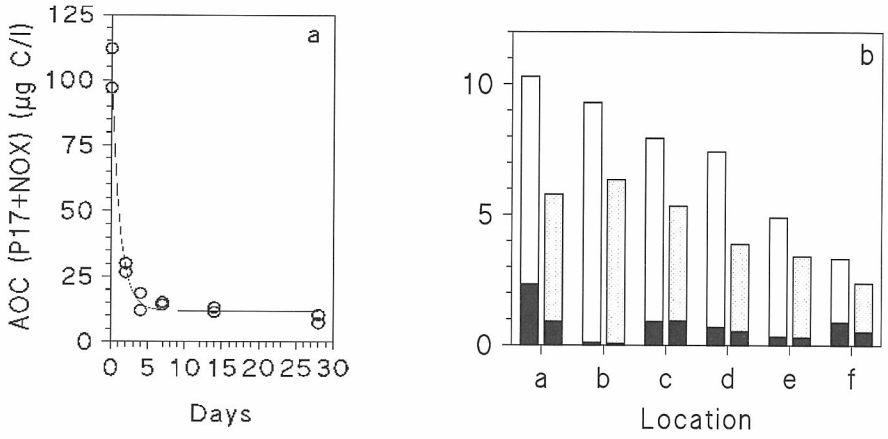

Fig. 1. a: AOC decrease in unpasteurised samples of ozonated water stored at 15 °C; b: AOC decrease in samples of 6 types of treated water stored at 15°C for 7 days. Black proportion of the bars is the fraction of AOC utilised by strain P17; the dotted bars are AOC values in the stored samples.

3.2. BIOFILM FORMATION

The Biofilm Formation Rate (BFR) value of (treated) water is assessed with a biofilm monitor, consisting of a vertically-placed glass column (L = 60 cm; Ø 2.5 cm) containing glass cylinders on top of each other. Water flows downward through this column with a flow rate of 0.2 m/s. Cylinders are collected from the column at regular intervals, sonicated in sterile water and the suspended biomass concentration is assessed with ATP-analysis (Van der Kooij *et al.,* 1995). Subsequently the BFR value is calculated from the linear increase of the biomass concentration on the surface of glass cylinders as a function of time. BFR values in treated water usually range from below 1 pg ATP/cm^2.d (slow sand filtrate) to about 75 pg ATP/cm^2.d. The highest BFR values in treated water have been observed in ground water supplies using anaerobic ground water as the raw water source. This water is treated by intensive aeration(s) and filtration(s), respectively. The AOC concentration in treated water in most cases is clearly below 10 µg C/l. Dosage experiments revealed that a concentration of 10 µg acetate-C/l causes a BFR value of 365 pg ATP/cm^2.d (Van der Kooij *et al.*, 1995b). Hence, concentrations of a few µg of easily available compounds can cause a significant biofilm formation. Such compounds may also include

methane, ammonia and sulfides. Therefore, combining the AOC test with an assessment of the BFR value gives information about the growth potential for heterotrophic bacteria and the rate at which the water can promote biofilm formation. In unchlorinated ground water supplies a clear relationship has been found between BFR values and the regrowth of aeromonads in the distribution system. From this relationship it was calculated that the risk of exceeding the guideline value for aeromonads in the distribution system (200 CFU/100 ml as 90-percentile over a one-year period) was less than 20% when the BFR value was less than 10 pg ATP/cm^2.d (Van der Kooij *et al.,* 1999).

3.3. EFFECTS OF SYNTHETIC MATERIALS

Biofilm formation on the wall of the pipes is caused by compounds present in the water and by compounds originating from the pipe material. In the Netherlands PVC is the main pipe material. The Biofilm Formation Potential (BFP) test has been developed to assess the growth promoting properties of synthetic materials in contact with water (Van der Kooij *et al.,* 1994). In this test the ATP concentration on pieces of material incubated in slow sand filtrate at 25°C is assessed as a function of time. Typical results are presented in Figure 2.

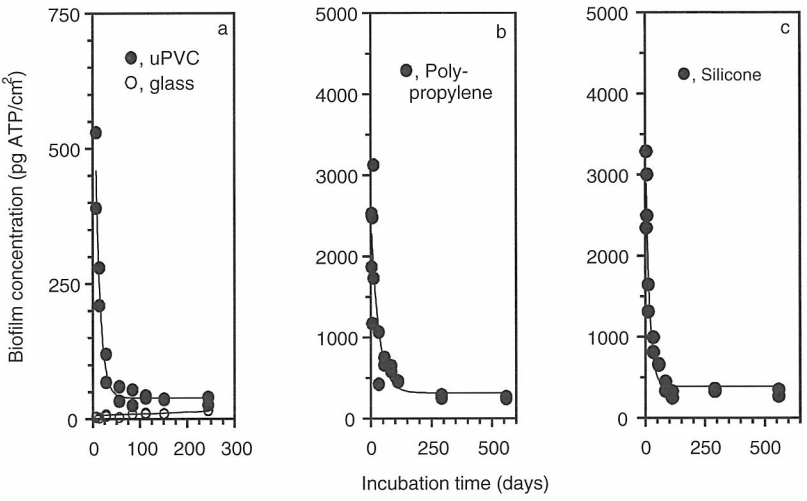

Fig. 2. The biofilm concentration (pg ATP/cm^2) on glass, unplasticized PVC (a), polypropylene (b), silicone tubing (c), as a function of time in the BFP test conducted at 25 °C.

The ATP-concentration on the material declines as a function of time. The plateau level, which for many materials is reached after about 8 to 12 weeks is defined as the BFP-value. Unplasticized PVC has a very low BVP value (< 100 pg ATP/cm^2). BFP values of PE materials range from about 350 to 2700 pg ATP/cm^2.

3.4. UNIFIED BIOFILM APPROACH

Assessment of the concentration of biofilm on the pipe wall gives direct information about the biological stability of the involved distribution system. Biofilm concentrations ranging from about 40 pg ATP/cm^2 to 5800 pg ATP/cm^2 (median value: 650 pg ATP/cm^2) have been observed on segments of PVC pipes of about 20 distribution systems, mainly ground water supplies. Biofilm concentrations on pipe walls, BFR values of the water and the BFP-value of materials provide data based on one and the same parameter (biomass activity). This suit of cohering parameters has been defined as the Unified Biofilm Approach for assessing the biostability of water and materials in contact with water (Van der Kooij *et al.,* 1999).

4. Evaluation

In the Netherlands water supply companies aim at limiting regrowth by distributing biologically stable water in distribution systems using biostable materials in contact with water. Biostability assessment of water includes the AOC test and the BFR test. The biostability of materials is tested with the BFP test. From the data obtained in the investigations, values for the test parameters have been derived which either are indicative for a high degree of biostability or for situations where biofilm formation causes problems. The use of one simple biomass parameter (ATP) in the biofilm-formation based tests and in observations in practice makes it easier to draw conclusions from the obtained data, especially in situations with inert pipe material, e.g., PVC. The situation is more complex when a reactive material, e.g., cast iron comes in contact with water. It has been shown that such material considerably enhances biofilm formation (Camper *et al.*, 1996). The most likely explanation is that organic compounds absorbed from the water by the corroding metal surface become available to attached micro-organisms. In this way, the contact time between organics and micro-organisms is greatly enlarged and certain slowly biodegradable compounds can also contribute to biofilm formation and regrowth. Another process affecting regrowth is the accumulation of biomass in certain parts of the distribution system. Sloughing of biofilms plays a role in sediment accumulation, but the concentration of biomass present in treated water may also contribute to this process. Sediment accumulation is also affected by hydraulic conditions (e.g., fluctuations in flow rate).

The described regrowth processes and test procedures clearly show that biostability is a multidimensional quality characteristic of treated water. One single test is not sufficient to define biostability. Further investigations needed to elucidate regrowth processes and biostability assessment include:

- the effect of surface reactivity on biofilm formation and biostability;

- the relationship between AOC uptake and biofilm formation in distribution systems.

Mathematical modelling of biofilm formation and regrowth processes in distribution system is another approach in elucidating regrowth phenomena (Dukan *et al.*, 1996; Servais *et al.*, 1995). However, a number of processes as described above are not included in these models, despite their complexity. Furthermore, in these models only BDOC is used as the critical parameter for regrowth potential. Improvement of mathematical modelling of regrowth processes is needed to enable improvement of distribution system design and/or the definition of effective preventive and curative measures for regrowth limitation.

Acknowledgements

The methods for determining the biostability of drinking water and materials in the Netherlands have been developed in the framework of the Joint Research Program of the Water Supply Companies in the Netherlands. Many people, including Harm Veenendaal, Hans Vrouwenvelder and Wim Hijnen contributed to the investigations.

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Biofouling potential of chemicals used for scale control in RO and NF membranes

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Abstract

The potential of 14 different antiscalants (AS) to promote biofouling was determined with the easily assimilable organic carbon (AOC) test and the Biomass Production Potential (BPP) test, respectively. The AOC concentrations of slow sand filtrate supplemented with 50 mg/l of AS ranged from 4 to 112 μg of C/l, and maximum concentrations of adenosinetriphosphate (ATP) from 7 to 380 ng/l, respectively. Concentrations of AOC and BPP were not related to the organic carbon content of the AS's tested. Biofilm Formation Rate (BFR) values of three selected AS's, as determined with the biofilm monitor at a concentration of 2.5 mg/l, were 245, 18.1 and <1.0 pg ATP/cm².d, respectively and confirmed the ranking as based on the AOC and BPP tests. Dosage (2.5 mg/l) of one selected AS with a relatively high growth potential caused severe fouling in a 4" RO membrane element supplied with tap water. ATP concentrations on the membrane, including spacers, were 4 times higher in the element supplied with tap water containing AS than in the control. These experimental results agreed with practical experiences, viz. severe biofouling was observed with an AS with high AOC and BPP values and a high BFR value, but no operational problems were observed with an AS with low values for these parameters. Practical experiences also showed that impure mineral acids can cause biofouling. Therefore, selection of chemicals used for scale control on the basis of their growth-promoting properties is needed to limit operational problems. Quality classes based on growth potential tests are proposed for AS's, but further comparison between test results and practice is needed to improve this classification.

Keywords: Antiscalant; Scaling; AOC; Biomass production potential; Biofouling; Biofilm formation rate; Membranes; Drinking water; Reverse osmosis; Nanofiltration; Autopsy

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1. Introduction

Membrane filtration techniques are very promising for the preparation of microbiologically safe and biologically stable drinking water because of their removing capacities for micro-organisms, and for inorganic and organic compounds, respectively. However, fouling of membranes may cause serious operational problems. Biofouling — growth of biomass, i.e. biofilms — may cause flux reduction and/or increased pressure drop during nanofiltration (NF) and reverse osmosis (RO) [1,2]. Organic antiscalants (AS) and mineral acids are added to water prior to NF or RO membrane units to avoid scaling of the membranes with barium sulfate or carbonates, respectively. The AS concentration in the feed water of a membrane installation depends on the type of water, recovery, salt retention and water temperature and generally is between 1 and 6 mg/l, but also concentrations up to 35 mg/l are applied [3]. Dosage of a mineral acid, viz. sulfuric acid or hydrochloric acid, can prevent calcium carbonate scaling, but does not control barium sulfate scaling. In practice membrane plants often use a mineral acid and an AS for scale prevention [4]. Operational problems such as an increase of the normalized pressure drop at NF- and RO-pilot plants have been ascribed to biofouling, which was mainly caused by chemicals such as AS's and impure acids [5,6]. However, quantitative data about the microbial growth potential of these chemicals and their effects on biofouling of membrane installations are scarce. Therefore a study was conducted to assess the biofouling potential of a variety of commercially available AS's.

Materials and methods

2.1. Antiscalants

A total of 14 AS's (A to O: I not included) was supplied by different manufacturers. The active compound of AS A is based on polyacrylic acid and AS O is based on (organo) phosphonates, while the AS's B to N contain mixtures of active compounds. Details about the composition of these products were not provided for commercial reasons.

2.2. Growth tests

Two types of batch growth tests were used to determine the concentration of growth promoting properties of AS's dissolved in water. These tests included the assessment of the concentration of easily assimilable organic carbon (AOC) and the Biomass Production Potential (BPP) test, respectively. The AOC-test is based on determining the maximum colony counts of two selected bacterial cultures growing in a sample of pasteurized water, contained in thoroughly cleaned glass-stoppered Erlenmeyer flasks incubated at 1°C. The selected bacterial cultures, which have been isolated from drinking water, are strain (P17) of *Pseudomonas fluorescens,* which can utilize a wide range of organic compounds and *Spirillum* sp. strain NOX, which only utilizes organic acids. The AOC concentration is calculated as μg of acetate-C equivalents/l from the maximum colony counts of the individual strains and their yield factor for acetate [7,8].

The BPP test is performed by determining the maximum concentration of adenosinetriphosphate (ATP) of the indigenous bacterial population in a water sample collected and incubated in an Erlenmeyer flask at 25°C [9]. The BPP value of a product (or water) is defined as the maximum ATP concentration per mg of product or per liter of water, respectively. This test is useful in situations where biodegradable chemicals are present which cannot be utilized by the strains used in the AOC test. The AOC and BPP test are determined in duplicate flasks.

Slow sand filtrate with low values for AOC and BPP was used to determine the effect of dosage of AS's on microbial growth. Two con-

centrations (5 and 50 mg /l of AS) were tested because the AS concentration in feed water of a membrane installations can vary significantly depending on the type of water, membranes, and installation. Also, the AS concentration on the feed side of the membrane increases due to the effect of the membrane filtration process. A high concentration of an easily degradable compound (acetate) was added to a blank sample to verify that carbon was the growth-limiting factor.

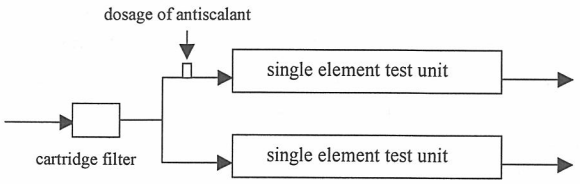

Fig. 1. Scheme of test unit with two 4" RO-membrane elements. Each single element test unit was monitored and controlled individually.

2.3. Biofilm formation

The Biofilm Formation Rate (BFR) of tap water without (blank) and supplemented with an AS to a concentration of 2.5 mg/l was determined with a biofilm monitor. In the biofilm monitor a flow rate of 0.2 m/s is maintained and the accumulation of active biomass (ATP) on the surface of glass is determined as a function of time at ambient temperature [10,11,12]. The tap water available at the laboratory (temperature ranging from 12 to 14°C) is prepared from ground water (aeration, rapid sand filtration) without the use of a chemical disinfectant during treatment and distribution.

2.4. Pilot plant with two single element units

Two single element test units containing a spiral wound 4" RO-membrane element (Hydranautics, type ESPA3-4040), were operated simultaneously and independently with a constant feed flow per test unit of 1.2 m^3/h and concentrate and permeate flows of 1.0 and 0.2 m^3/h, respectively (20% recovery). Tap water was used as feed water. Polypropylene cartridge filters with a pore size of 40 μm were installed prior to the test units to remove suspended particles from the feed water and were replaced weekly. The AS to be tested was added to the feed water of one test unit at a concentration of 2.5 mg/l. The setup of the experiment is shown in Fig. 1.

The RO membrane elements were removed simultaneously to determine the concentration of biomass in the membrane elements when the normalized pressure drop (NPD, pressure drop normalized for water temperature and flow) had increased >40% in one of the RO elements.

2.5. Analytical parameters

Biomass parameters used to determine the concentration of biomass on the membranes (biofilm) included adenosinetriphosphate (ATP), total direct cell counts (TDC) and heterotrophic plate counts (HPC). ATP (pg ATP/cm^2) is a measure for the total amount of active biomass [13]. ATP concentrations were obtained by an enzymatic reaction using luciferin and firefly luciferase. Light production was determined and the ATP concentration was derived from the linear relationship between light production and reference ATP concentrations.

TDC values (cells/cm^2) were determined with epifluorescence microscopy using acridine orange as fluorochrome, applying a slightly adapted method to eliminate fading [14,15]. All fluorescing cells were counted. Microscopic observations also give information about the variety and appearance of microorganisms present.

HPC values (CFU/cm^2) are determined by spreading 0.05 ml volumes of water in triplicate on nutrient poor R_2A medium [16]. Colonies were counted after 10 days of incubation at

25°C. Biomass parameters have been determined in suspensions obtained after ultrasonic treatment of the membrane envelope components and in the material scraped from defined surface areas from the feed side of the membrane, respectively.

DOC values were determined with a Shimadzu DOC-5000A of a sample of ultra pure water (<0.1 μS/cm) supplied with AS. Pretreatment of the samples involved acidification with hydrochloric acid followed by stripping with high purity oxygen. The remaining organic carbon (also called non purgable or non volatile organic carbon) is determined as carbon dioxide with an infra-red gas analyzer.

3. Results

3.1. Growth potential tests

The AOC concentrations of slow sand filtrate supplemented with 50 m/l of each AS ranged from 4 to 112 μg C/l. The AOC concentration of the blank was about 2–3 μg C/l. Growth curves as observed with a few AS types are presented in Fig. 2, demonstrating that strain P17 multiplied more rapidly in the presence of AS than strain NOX. Standard deviations of the AOC tests were less than 3.5% in all cases. AOC concentrations per mg of added AS were calculated and ranked in decreasing order (Fig. 3). The AS's A to D had the highest values with about 2 μg AOC/l per mg of added AS. The AOC fraction utilized by strain P17 was more than 72% of the observed AOC value with 11 of the AS's tested, indicating that compounds other than carboxylic acids were responsible for the observed growth.

Maximum ATP concentrations at an AS concentration of 50 mg/l ranged from 7 up to 380 ng ATP/l with a value of 7 ng ATP/l in the blank. Standard deviations of these values ranged from 2 to 42% (median value 15%). BPP values (ng ATP/mg AS) were calculated from these maximum biomass concentrations (Fig. 3).

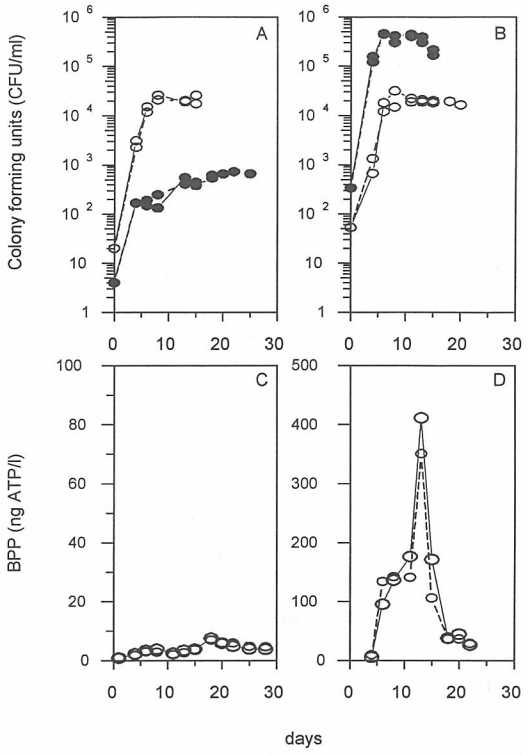

Fig. 2. Growth curves of *Pseudomonas fluorescens* strain P17 (●), and *Spirillum* sp. strain NOX (○) in slow sand filtrate (A) and (B), slow sand filtrate supplemented with 50 mg/l of antiscalant A. Concentrations of ATP in slow sand filtrate (C) and (D), in slow sand filtrate supplemented with antiscalant D, respectively.

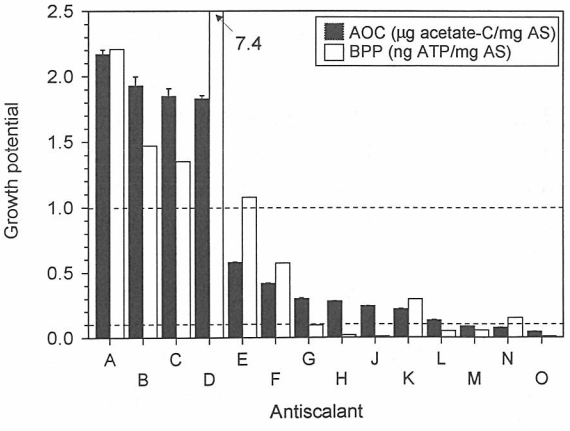

Fig. 3. Growth potential (AOC concentration per mg and ATP yield per mg) of AS's determined in biologically stable slow sand filtrate at a concentration of 50 mg AS/l).

Table 1
Values (with relative standard deviation) for DOC, AOC, BPP and BFR per unit of weight for three different antiscalants

Antiscalant	DOC ($\mu g\ C.mg^{-1}$ AS)	AOC ($\mu g\ C.mg^{-1}$ AS)	BPP ($ng\ ATP.mg^{-1}$ AS)	BFR ($pg\ ATP.cm^{-2}.d^{-1}.mg^{-1}$AS)
A	134	2.2 (1.6%)	2.2 (38%)	98 (14%)
K	45	0.22 (0.5%)	0.30 (3%)	7.2 (8%)
O	29	0.04 (0.1%)	<0.01 (27%)	<0.1 (8%)

These values confirmed the large variety in growth promoting properties of the tested AS products. For most AS's the numerical values for AOC and BPP were similar. BPP values of AS D and to a lesser extend also of AS E were clearly higher than the corresponding AOC values indicating that biodegradable compounds were present which were not utilized in the AOC test. AS dosages did not significantly influence the pH value of the blank water (pH 8.2–8.5) and in none of the AOC or BPP tests toxicity was observed.

AOC tests conducted with water supplemented with 5 mg/l of AS revealed stronger growth than in the blank only with the AS's A to D. In the BPP test AS's A to E had stronger growth than in the blank. The growth potential test values (AOC and BPP) per mg AS of the AS's A to D gave similar values as observed at 50 mg AS/l.

The concentration of dissolved organic carbon (DOC) of the 14 AS's ranged between 20 and 162 g C/kg. The AOC values were a minor fraction of the DOC concentrations (0.1 to 3.5%) and did not correlate with the DOC values. This is illustrated by comparing AS F (highest DOC value: 162 g C/kg AS) and AS G (27 g C/kg AS), which had a relatively limited difference in growth potential (cf. Fig. 3). Hence, DOC is not an appropriate parameter for determining the growth potential of an organic AS.

Biofilm formation in the biofilm monitor with and without the addition of one of three AS's (A, K and O) at a concentration of 2.5 mg/l revealed large differences between these AS's (Fig. 4). The BFR values calculated per mg of AS are listed in Table 1 together with the values for AOC and BPP. The obtained BFR values confirmed the ranking as obtained in the batch tests (cf. Fig. 3).

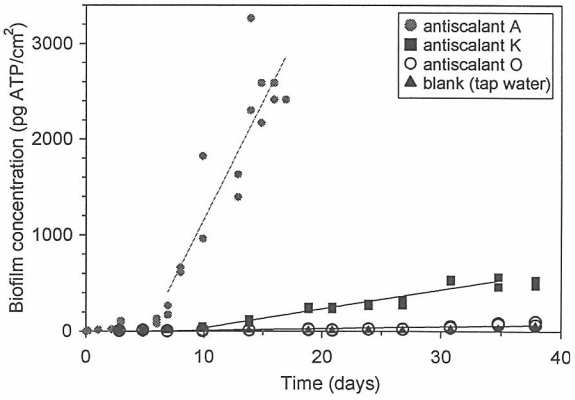

Fig 4. Effect of dosage (2.5 mg/l) of anti scalant on biofilm formation in the biofilm monitor.

3.2. Biomass accumulation in membrane element

Two 4" RO membrane elements installed in parallel in a test unit were supplied with tap water during one week to obtain stable and reproducible values for the mass transfer coefficient (MTC or normalised flux) and the NPD of both

Table 2
Composition of fouling layer present on the membrane envelope*

Membrane element	ATP, pg/cm^2	TDC, cells/cm^2	HPC, CFU/cm^2	Fe, mg/m^2	Mn, mg/m^2
Blank	510±160	$3\times10^7\pm3\times10^6$	$1.3\times10^4\pm6.1\times10^3$	143±33	2.1±4.6
AS A (2.5 mg/l)	2200±420	$4\times10^7\pm7\times10^6$	$1.3\times10^5\pm9.5\times10^4$	282±57	17±1.0

* The membrane envelope includes the feed spacer, the feed side and the product side of the membrane and the product spacer, respectively.

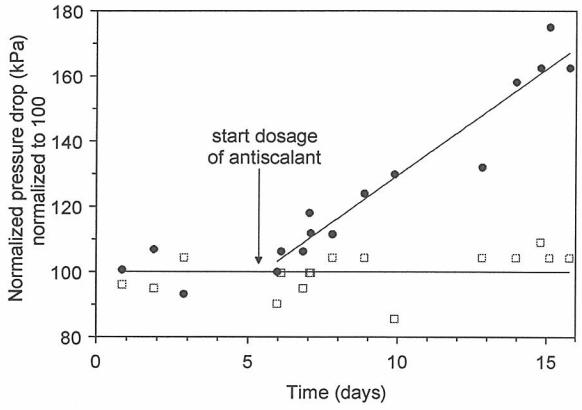

Fig. 5. Normalized pressure drop as observed over membrane element in time fed with tap water with (●) and without (❐) dosage of an AS with a relatively high growth potential and BFR-value.

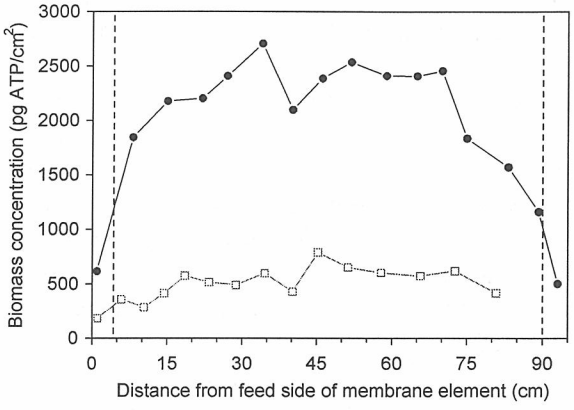

Fig. 6. Biomass concentration (ATP) over the length of a RO membrane element with (●) and without (❐) dosage of antiscalant A after an operation time of 16 days (see Fig. 3). The presented concentration is the sum of the biomass on three layers (feed spacer, membrane and product spacer).

installations. Subsequently, AS A was added to the feed water of one test unit at a concentration of 2.5 mg/l. This dosage caused an increase of the NPD, reaching about 60% within 11 days (Fig 5). The MTC value did not differ significantly between both test units and did not demonstrate a declining trend within the test period.

After an operation time of 16 days both membrane elements were taken from the test unit directly after measuring the NPD. Subsequently, autopsy was performed on these elements for visual inspection and the analysis of biomass and chemical components. A slightly brown colored mucous layer was present on the feed side of the membrane of both elements, but visual inspection did not reveal clear differences.

The average values of the parameters of the biofilm on the membrane envelope differed clearly between the two elements (Table 2). The average concentrations of active biomass (ATP) and the heterotrophic plate count (HPC) values were 4 and 10 times higher in the membrane element with AS A dosage than in the blank. Fig. 6 shows the spatial distribution of the

concentration of active biomass on the membranes (including feed spacer, membrane and product spacer, respectively). The distribution of biomass (ATP, TDC and HPC) over the length of the membrane all revealed relatively low biomass densities at the influent and effluent sides of the elements were glue lines of about 5 cm wide were present. Most (>99%) of the biomass (ATP, HPC and TDC) was present at the feed side of the membranes (membrane and feed spacer), and 10 times more material was present on the feed side of the membrane than on the product spacer. Higher concentrations of iron and manganese were observed on the AS supplied membrane than on the blank membrane (Table 2). The accumulation of these compounds probably was related to biofilm formation.

TDC values did not differ significantly. The large standard deviation of this parameter (20%), as caused by the presence of large clusters of thousands of cells, hampered comparison. Microscopic observations of the TDC samples revealed that the cells on the membrane with AS dosage were larger and were more strongly fluorescing than cells present on the control membrane. These observations confirmed that addition of AS A stimulated biofouling of the membrane element.

4. Discussion

4.1. Testing methods

The obtained results clearly show that chemicals used to prevent scaling differ greatly in their ability to promote growth of microorganisms. These observations confirm observations in practice and in pilot plants, where an AS, with high AOC, BPP and BFR values in this study, caused severe biofouling and an AS with low values for these parameters did not cause problems [6]. Therefore, assessment of the growth potential of antiscalants with the AOC, the BPP and the BFR tests is needed to quantify the biofouling potential of such chemicals and to enable selection of AS types which do not enhance biofouling. DOC analysis is not appropriate for determining the growth potential of organic AS's, but may be used for this purpose with mineral acids [17].

Assessment of the growth potential was performed with the AOC and BPP tests at AS concentrations of 5 and 50 mg/l, respectively. The AOC test has been designed for testing the biostability of drinking water [7], and the BPP test has been derived from a similar test [18]. Both tests enable the detection of growth promoting compounds to a level of a few micrograms per liter. The AOC test gives information about classes of the growth-promoting compounds, because strain NOX is only able to utilize carboxylic acids, whereas *P. fluorescens* strain P17 can growth on a wide range of both naturally occurring and synthetic compounds, with a great preference for biomass derived substances (amino acids, proteins) [7]. The ATP yield for growth is about 1 ng ATP/μg of C [9,18]. The BPP test with the indigenous bacterial population may utilize even more biodegradable compounds, but this study shows that the BPP value was higher than the AOC value with only a few AS types (cf. Fig. 3). A disadvantage of the BPP test is a rapid increase followed by a rapid decline of the ATP-concentration, which may lead to underestimation of the maximum value [9]. The AOC method gives a more gradual increase of biomass ensuring a more reproducible measurement, but not all easily biodegradable compounds may be included. Performing these tests at two different AS concentrations enables to obtain information about the presence of growth-inhibiting compounds and the dose-effect relation of the AS tested. In the experiments described above, no growth inhibition or toxicity was observed. Conserving compounds such as benzoate or sorbate sometimes are AS ingredients in a con-

centration of about 0.5% (personal communication with supplier). These compounds, which inhibit bacterial growth in the undiluted product, may contribute to the growth potential at relatively low concentrations as obtained after dilution. In practice the AS dosage may range from of few mg/l to much higher values. Furthermore, the concentrating effect in the membrane filtration process increases the AS concentration. The test at 50 mg/l is particularly important to obtain accurate information about low concentrations of growth-promoting compounds in the involved AS type.

Also the BFR test gives accurate information at low concentrations, but this test takes more effort and space than the batch tests. A significant linear correlation between the concentration of acetate and BFR value has been reported for low concentrations of acetate added to water in a biofilm monitor, with a BFR-value of 35 pg ATP/cm².day for 1 μg of acetate-C/l [11]. The data presented in Table 1 show that the AOC to BFR ratios for the AS's were similar to the BFR value for 1 μg of acetate-C/l. Hence, the growth promoting compounds present in these AS's obviously are easily biodegradable. It is not clear whether these compounds were either impurities of the AS composition, or an essential part of the formulation.

Many membrane plants use both a mineral acid and an AS for scale inhibition [4]. Impure hydrochloric acid was found to be the cause of acute biofouling in a NF membrane filtration pilot plant [6,17]. The acid dose increased the AOC concentration from 8 to 27 ug C/l and a maximum BFR-value of 310 pg ATP/cm².day of the feed water for the NF membranes. A strong increase of the NPD (100%) was observed. A series of cleanings with industrial agents were performed but were not sufficiently effective and membrane elements were replaced.

4.2. Classification

Judgement of the growth promoting properties of the AS's depends on the concentration at which the compound is used, the type of membrane installation, the degree of concentration and water quality factors such as composition and temperature. Furthermore, many interactions occur between processes of biofouling and fouling caused by dissolved compounds and particles, e.g. the accumulation of iron(hydr)oxides. Presently, insufficient knowledge is available to define the relationship between growth promoting properties of feed water and the rate of biofouling. On the other hand, a large data base exists with information about the growth promoting properties of treated water in the Netherlands, because the tests used to analyze the AS's were developed to determine the biostability of drinking water. Treated water in the Netherlands have AOC values ranging from a few μg/l to about 20 to 30 μg/l, with BFR values less than 1 pg ATP/cm².d (for slow sand filtrates) to 75 pg ATP/cm².d for drinking water prepared from anaerobic ground water. AOC utilization in the distribution systems is negligible below 10 μg C/l and regrowth of aeromonads is limited at BFR values below 10 pg ATP/cm².d [8,19]. Still, evaluation of AS's in terms of biofouling risk based on the tests described in this report is complicated. As mentioned above, rapid fouling has been observed at BFR values of 250 to 300 pg ATP/cm².d. Furthermore, an operational period of about 350 days without NPD increase was observed in a RO membrane filtration plant supplied with slow sand filtrate with a low AOC value (6 μg C/l) and BFR value <1 pg ATP/cm².d [6]. Consequently, under ideal conditions the contribution of AS dosage to the AOC concentration and the BFR value of the feed water should be negligible.

Table 3
Classes proposed for the growth potential of antiscalants added to water prior NF and RO membrane installations for minimising the risk on biofouling

Class	Risk of biofouling	Growth potential of antiscalants AOC[1] and BPP[2]
I	Low	≤0.1
II	Moderate / Unclear	>0.1–<1
III	High	≥1

1, AOC expressed as µg C/mg AS;
2, BPP expressed as ng ATP/mg AS

Table 3 proposes a classification for AS products with respect to the risk of causing bio-biofouling in three classes on the basis of the growth potential. AS types in class I do not cause operational problems such as increased NPD or reduced MTC values and include AS M, N and O, respectively. Class III includes AS types causing severe biofouling problems. The position of the AS types in class II is not yet clear. For prevention of biofouling AS types with very low values for AOC, BPP and BFR are preferred.

Feed water itself may play a major role in (bio)fouling and extended pretreated is needed to remove biodegradable compounds to a low concentration. Water treatment with ozone increases the growth potential of the water and biological filtration generally reduces the AOC values [7]. The optimum pretreatment for a specific situation also depends on treatment costs and the costs of operating the membrane filtration unit, including cleaning procedures. At this stage it is not possible to define the required pretreatment. The AOC and BFR test, which can be used for (semi) continuous monitoring, are useful tools to detect effects of water quality, seasonal effects and fluctuations in treatment and to define the relationship between water quality and rate of biofouling. Such studies may lead to quantitative criteria for the biofouling potential of feed water and chemicals used for scale inhibition in membrane filtration installations.

5. Conclusions

The results presented in this paper lead to the following conclusions:

(i) Chemicals used to prevent scaling differ greatly in their ability to promote growth of microorganisms.

(ii) The described AOC, BPP and BFR tests can be used to determine the biofouling potential of chemicals including organic antiscalants and mineral acids, but also the feed water. DOC analysis is not suited for determining the growth potential.

(iii) Low concentrations of nutrients (µg/l) in the feed water can lead to biofouling.

(iv) Classes of antiscalants with respect to risks on biofouling are proposed, but further investigations are needed to substantiate and eventually refine this classification.

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2 Publicaties in H_2O

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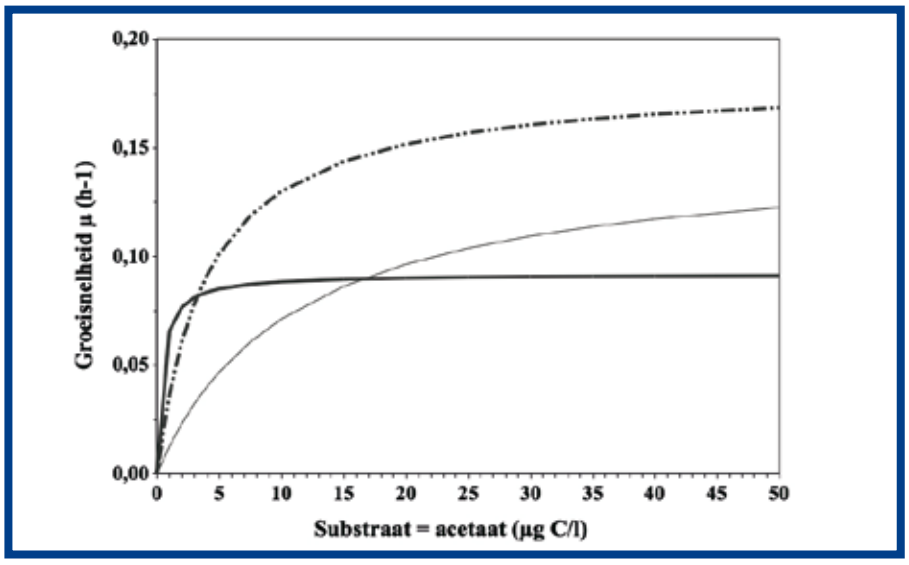
Biofouling spiraalgewonden membranen al bij lage concentraties afbreekbare stoffen in voedingswater

Biofouling veroorzaakt operationele problemen bij de toepassing van spiraalgewonden membranen bij de waterbehandeling en ontzouting van zeewater. Vervuilde membranen moeten periodiek worden gereinigd en soms ook worden vervangen. Zeer lage concentraties van afbreekbare stoffen in water kunnen bacteriegroei en biofilmvorming veroorzaken. Uit laboratoriumexperimenten blijkt dat de drempelconcentratie voor biofouling eveneens slechts enkele microgrammen gemakkelijk afbreekbare stoffen per liter voedingswater is. Beperking van biofouling vereist dus een vergaande AOC-verwijdering bij de voorzuivering en selectie van antiscalants op groeibevorderende eigenschappen.

Spiraalgewonden membranen worden steeds meer toegepast voor de ontzouting van zeewater en brakwater (omgekeerde osmose) en voor de behandeling van zoet water (nanofiltratie). De reden hiervoor is de effectieve verwijdering van (an)organische stoffen, deeltjes en pathogene micro-organismen. Verstopping van de voedingskanalen in de membraanelementen door biofilmvorming (biofouling) is echter een lastig probleem. In de afgelopen jaren is bij KWR in BTO-verband en in het kader van het Europese project MEDINA (Membrane-based Desalination: an Integrated Approach) onderzoek verricht naar het verband tussen de groeipotentie van het voedingswater en biofouling van de membranen.

Afb. 1: Invloed van het acetaatgehalte op de groeisnelheid van enkele reincultures in drinkwater bij 15 C.



De belangrijkste oorzaak van biofouling is de aanwezigheid van afbreekbare stoffen in het voedingswater. Bacteriën kunnen groeien bij concentraties van slechts enkele microgrammen van gemakkelijk afbreekbare stoffen in water. Dit is aangetoond met groeiproeven in drinkwater onder goed geconditioneerde condities [1),2),3)]. Met deze *batch*-proeven is het mogelijk om het verband te bepalen tussen de exponentiële groeisnelheid (µ) van bacteriën en de concentratie van een afbreekbare verbinding. Dit verband wordt beschreven met de Monod-vergelijking:

$$\mu = \mu_{max} * \frac{[S]}{k_s + [S]}$$

Hierin is μ_{max} (uur) de maximale groeisnelheid en k_s de substraatconcentratie (S) in µg C/l, waarbij de groeisnelheid µ de helft is van μ_{max}. Verschillende soorten bacteriën die kunnen groeien op bijvoorbeeld acetaat, hebben onderling verschillende waarden voor μ_{max} en k_s (zie afbeelding 1).

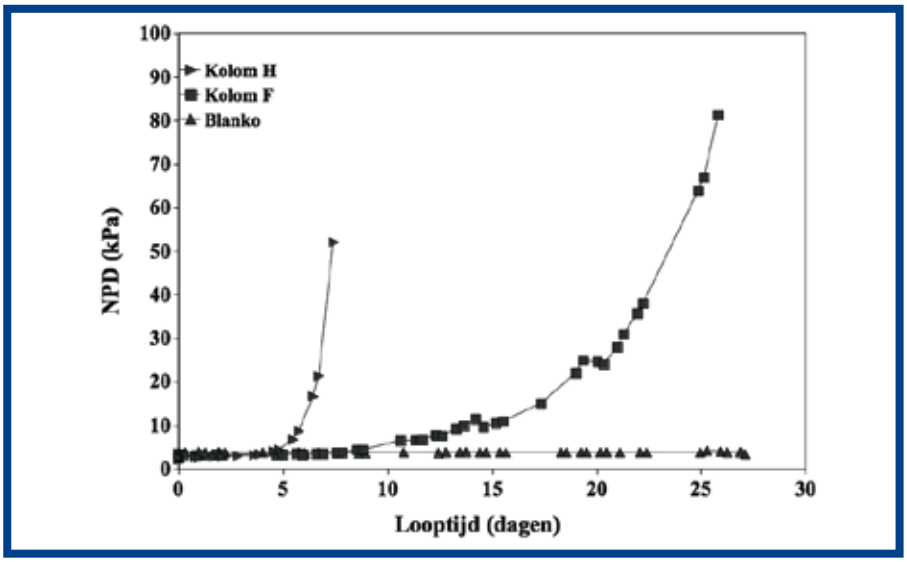
Polaromonas, een bacterie die is geïsoleerd

uit een actieve koolfilter[4], heeft een zeer lage k_s-waarde voor acetaat, namelijk 0,4 µg C/l en groeit onder deze condities sneller dan de andere twee bacteriën. De bacterie heeft echter ook een lage maximale groeisnelheid van 0.9 uur en verliest bij concentraties hoger dan 3 en 17 µg C/l de concurrentie van respectievelijk AOC-stam *Pseudomonas fluorescens* P17 en *Aeromonas* die dan sneller groeien.
In biofilms op membranen zijn *Spingomonas*-soorten aangetroffen[5]. De groeikinetiek van deze bacteriën is mogelijk vergelijkbaar met die van *Polaromonas*. Naast groeikinetiek op individuele verbindingen zijn ook mogelijkheden in het benutten van typen afbreekbare verbindingen (koolhydraten, aminozuren, carbonzuren en aromaten) van invloed op de biodiversiteit van bacteriën in water of biofilms. Hierbij is ook de groeiopbrengst belangrijk. Uit metingen in batchcultures bleek dat omzetting van 1 µg C kan leiden tot de vorming van 4 x 10^6 tot 2 x 10^7 bacteriecellen[1),2)].

De vraag is nu welke lage concentraties van gemakkelijk afbreekbare verbindingen nog biofouling van SW-membranen kunnen veroorzaken. Een volgende vraag is of de methoden voor de bepaling van de biologische stabiliteit van drinkwater (AOC en biofilmvormingsnelheid) bruikbaar zijn voor de beoordeling van de biofoulingspotentie van het voedingswater van SW-membranen. Voor de beantwoording van deze vragen is informatie nodig over de relatie tussen de concentratie van afbreekbare stoffen in het voedingswater en de vorming van biofilms door bacteriegroei in het membraanelement, die drukvaltoename (door verstopping van het voedingskanaal) en/of afname van de doorlaatbaarheid van het membraan (productieverlies) tot gevolg hebben. Er is onderzoek uitgevoerd in het laboratorium en er zijn metingen verricht bij installaties in de praktijk om deze vragen te beantwoorden.

Laboratoriumexperimenten

Laboratoriumexperimenten zijn uitgevoerd om te bepalen bij welke drempelconcentratie van gemakkelijk afbreekbare stoffen nog drukvaltoename optreedt en wat het verband is tussen de concentratie van acetaat en de snelheid van verstopping.
Bij dit onderzoek is de Membrane Fouling Simulator (MFS)[6),7)] gebruikt. Deze installatie, met een effectieve lengte van 20 centimeter, is een nabootsing van een deel van het voedingskanaal in een SW-membraanelement. De hydraulische condities in de MFS met daarin een deel van een nanofiltratiemembraan met voedingsspacer waren vergelijkbaar met die in een SW-membraanelement.
Bij een cross-flow van 0.1 m/s werd in een schoon voedingskanaal een genormaliseerde drukval (dP_o bij 12,5°C) van gemiddeld 2.8 kPa gemeten. Er was geen permeaatproductie tijdens de proeven. De MFS-installatie werd gevoed met drinkwater met een laag AOC-gehalte (3-5 µg/l) en een gemiddelde temperatuur van 15°C. Door een voorfiltratie over cartridges met poriegrootten van 10 en 1 µm was het drinkwater vrij van deeltjes en lag het ijzergehalte laag (0,008 mg/l). Aan dit water werden acetaatconcentraties gedoseerd variërend van 1 tot 1.000 µg C/l.



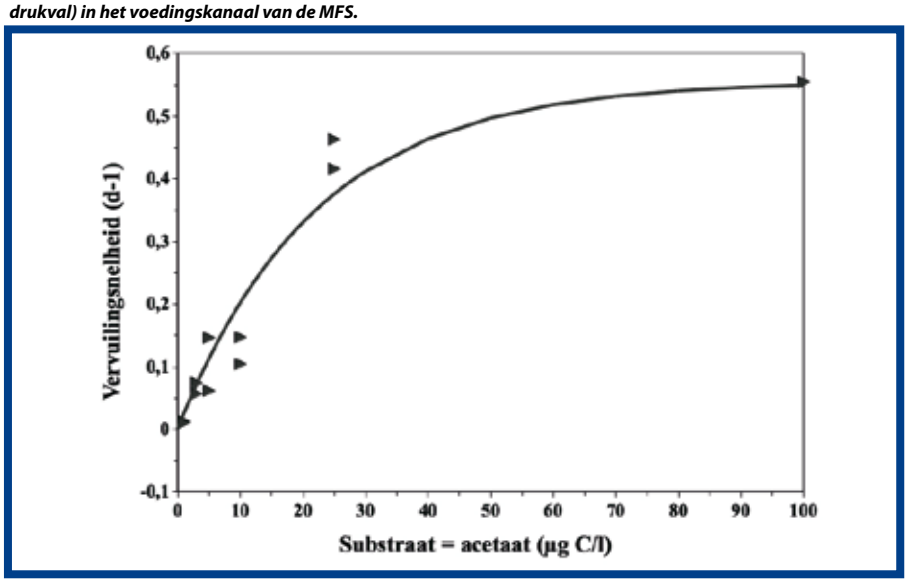
Afb. 2: De genormaliseerde drukval (kPa) over het voedingskanaal van de MFS gevoed met drinkwater zonder (blanco) en drinkwater met verschillende concentraties acetaat.

Daarnaast werden ook installaties gevoed met het drinkwater zonder acetaat en met drinkwater zonder voorfiltratie (blanco). Tijdens de looptijd werd de drukval over het voedingskanaal bepaald en genormaliseerd naar een temperatuur van 12,5°C.

Drempelconcentratie: 1 microgram acetaat-C per liter

De blanco MFS-installaties vertoonden in de eerste 100 dagen geen toename in drukval (zie afbeeldingen 2a en 2b), maar bij acetaatconcentraties van 10 en 1.000 µg C/l nam de drukval snel toe. Een trage toename in de drukval werd waargenomen bij een concentratie van 1 µg C/l (zie afbeelding 2c). In de twee installaties gevoed met ongefiltreerd drinkwater nam de druk na circa 90 dagen langzamer toe dan in de installaties die gevoed werden met 1 µg acetaat-C/l (afbeelding 2b). De invloed van het acetaatgehalte op de ontwikkeling van drukval werd ook duidelijk aangetoond door de dosering tijdelijk te onderbreken. Uit de gegevens blijkt dat verstopping van het voedingskanaal van SWM-elementen door bacteriegroei optreedt vanaf een (drempel) concentratie van 1 µg acetaat-C/l.

Afb. 3: Invloed van de acetaatconcentratie in het voedingswater op de vervuilingsnelheid (toename van de drukval) in het voedingskanaal van de MFS.



Kinetiek van het biofoulingsproces

Met behulp van de formules gepresenteerd door Shock en Miquel in 1987[8)] en de kinetiek van de biofilmgroei kan worden afgeleid dat de drukval in een voedingskanaal van een SW-membraan eerst lineair en daarna exponentieel toeneemt. Dit verloop werd ook gevonden bij de doseerexperimenten (zie afbeelding 1)[7)]. Voor alle acetaatconcentraties is de exponentiële verstoppingsnelheid R_f (d) berekend. Het verband tussen R_f en de acetaatconcentratie (zie afbeelding 3) bleek evenals de groeikinetiek van bacteriën (afbeelding 1) te kunnen worden beschreven met een verzadigingsfunctie.
De maximale waarden van R_f werden bereikt bij acetaatconcentraties vanaf 25 µg C/l. Beneden 25 µg C/l nam de verstoppingsnelheid snel af en was 50 procent van de maximale waarde bij een concentratie van 15 µg C/l (afbeelding 3). Deze concentratie is

locatie	verstoppings-snelheid Rf(d) (gemiddelde en marge)	groeipotentie van het voedingswater			
		AOC (.g C/l		BVS (pg ATP/cm2.d)	
		voor antiscalant	na antiscalant	voor antiscalant	na antiscalant
blanco*	0,0	3-5	na**	-d	na
6	0,0	-***	6,5	-	3
5	0,0	6,3	11,7	5	7
1	0,05 (0,04-0,06)	11,3	-	16	-
3	0,13 (0,07-0,28)	12,6	21,6	5-94	-
2	0,14 (0,07-0,22)	14,9	23,6	15-79	270
4	0,15 (0,14-0,15)	9,0	14,6	48	296

* = blanco van de laboratoriumdoseerproef (<90 dagen) ** = geen antiscalant *** = niet bepaald

Exponentiële verstoppingsnelheid R_f van SW-membranen in de testbank en de groeipotentie van het voedingswater (AOC en BVS), gemeten in dezelfde periode bij een installatie.

hoger dan de k_s-waarden van bacteriën voor groei met acetaat (afbeelding 1). Het verstoppingproces is een gevolg van groei van bacteriën in een biofilm bij hoge afschuifkrachten. Een mogelijke verklaring voor het verschil tussen de k_s-waarden voor groei en de waargenomen k-waarde voor biofouling is het optreden van transportlimitatie in de biofilm en van de vertraging van de biofilmvorming door uitspoeling van biomassa.

Veldstudie

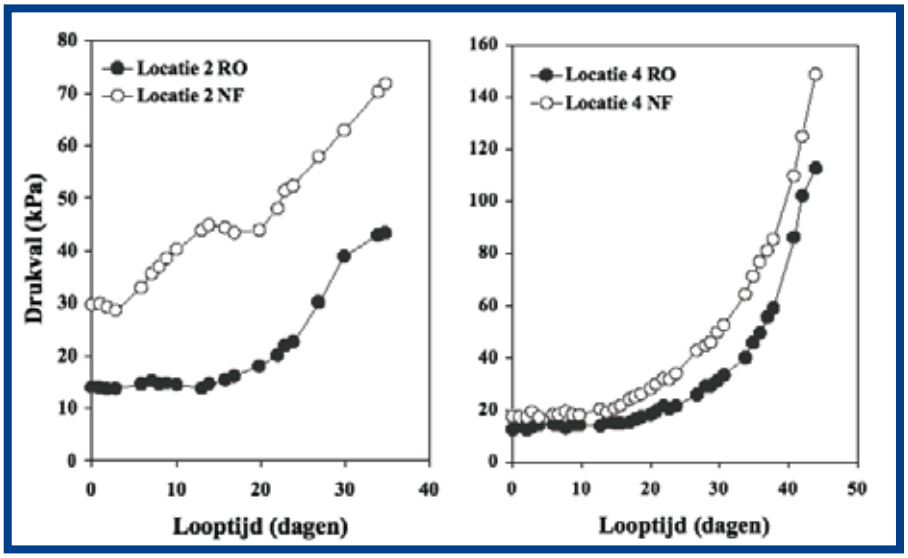
Een veldstudie is uitgevoerd om na te gaan bij welke concentraties van afbreekbare verbindingen in het voedingswater onder praktijkcondities biofouling van SW-membranen optreedt. Door de waargenomen lage drempelconcentratie voor biofouling zijn de parameters DOC en BOD ongeschikt voor de bepaling van de biofoulingspotentie van het voedingswater. Daarom zijn metingen van AOC- en biofilmvormingssnelheid uitgevoerd. Een zestal locaties met RO/NF-membraaninstallaties is geselecteerd voor dit onderzoek. Locatie 1 betrof een proefinstallatie[9]; de andere locaties waren full-scale installaties[5),10),11)]. De verstoppingpotentie van het voedingswater werd bepaald met een testbank met een RO- en een NF-membraanelement en op locaties 5 en 6 met een MFS-installatie.

AOC, BVS en membraanvervuiling

Bij de locaties 1 tot en met 4 nam de druk in de voedingskanalen van de membraanelementen toe. De verstopping bleek onafhankelijk van het type membraan (NF of RO). Het verloop van de drukval kwam overeen met het verloop in de laboratoriumproeven (zie afbeelding 4). Uit dit verloop is de exponentiële verstoppingsnelheid berekend (zie de tabel); op de locaties met de hoogste AOC- en BVS-waarden in het voedingswater bedroegen de waarden 0,13 tot 0,15. Bij lage AOC- en BVS-waarden, zoals op locatie 5 en 6, werd geen vervuiling waargenomen gedurende de looptijd van meer dan 60 dagen.

Deze waarnemingen bevestigen eerder gepubliceerde gegevens[11),12)]. Hieruit blijkt dat de AOC- en BVS-waarden een positieve correlatie hebben met het optreden van biofouling en de snelheid van het proces. Bij alle locaties werd een antiscalant aan het voedingswater toegevoegd. Op de locaties waarbij metingen van AOC en BVS zijn uitgevoerd voor en na deze dosering, bleek dat de groeipotentie was toegenomen na de dosering (zie de tabel).

Afb. 4: Het verloop van de genormaliseerde drukval in het RO- en NF-membraanelement van de testbank, gevoed met water na dosering van antiscalant op locaties 2 en 4.



Discussie: AOC en BVS als biofoulingparameters

De AOC-concentraties in het voedingswater bij de praktijklocaties varieerden van 6,5 tot circa 24 µg acetaat-C equivalenten/l (zie tabel). De hierbij waargenomen verstoppingsnelheden waren echter duidelijk lager dan bij dezelfde concentraties van acetaat in het laboratoriumonderzoek (afbeelding 3). Dat AOC in water niet overeenkomt met acetaat, werd ook duidelijk uit de doseerproef zelf, waarbij het voedingswater met een AOC-waarde van 3-5 µg C/l geen verstopping veroorzaakte (blanco in de tabel).

De verklaring voor dit verschijnsel is dat het AOC-gehalte wordt bepaald door groeimetingen van de teststammen gedurende meerdere dagen tot een week in monsters van het voedingswater. Daarbij worden eerst de gemakkelijk afbreekbare stoffen benut en daarna ook de moeilijker afbreekbare stoffen die waarschijnlijk een minder belangrijke rol spelen in het biofoulingproces. Het AOC-gehalte geeft relatief snel een indicatie van de biofoulingpotentie van het voedingswater, maar is om bovengenoemde reden als enige parameter niet voldoende.

De BVS-bepaling is gebaseerd op het meten van de ontwikkeling van de biofilmvorming op een oppervlak dat, evenals in een membraanelement, wordt blootgesteld aan voedingswater met een zeer korte contacttijd. Voor de acetaatconcentratie van 1 µg C/l, waarbij biofouling in het voedingskanaal kan optreden, is op basis van doseerproeven een BVS-waarde van 35 pg ATP/cm^2.d afgeleid[13)]. Deze waarde ligt tussen de BVS-waarden gemeten bij de locaties 5 en 6 zonder biofouling en de locaties 1 tot en met 4 met duidelijke biofouling (zie de tabel). Dit toont aan dat de BVS-waarde een directe relatie heeft met de verstoppingpotentie van het voedingswater. Het vaststellen van een richtwaarde voor de BVS waarbij biofouling wordt voorkomen, vergt meer onderzoek.

Ook is nog onduidelijk wat de invloed is van traag opneembare voedingsstoffen en van deeltjes (bijvoorbeeld ijzer en biomassa) op de biofouling van SW-membranen.

Waarnemingen[14] bevestigen dat dosering van chemicaliën voor het verhinderen van scaling kan leiden tot een toename van de groeipotentie van het voedingswater. Gezien de zeer lage drempelconcentratie voor biofouling is het van belang om chemicaliën te doseren die niet groeibevorderend zijn. AOC- en BPP-testen kunnen worden gebruikt voor de beoordeling van de groeibevorderende eigenschappen van de chemicaliën[15].

Conclusies

- Enkele microgrammen per liter van gemakkelijk afbreekbare verbindingen in het voedingswater kunnen biofouling van SW-membranen veroorzaken;
- De methoden die zijn ontwikkeld voor de bepaling van de biologische stabiliteit van drinkwater (AOC en BVS), zijn bruikbaar voor het meten van de biofoulingpotentie van (zoet) voedingswater;
- Beperking van biofouling vereist een vergaande verwijdering van groeibevorderende stoffen in de voorzuivering en het gebruik van chemicaliën die niet groeibevorderend zijn.

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Verwijdering deeltjes met ultrafiltratie verlaagt snelheid van biofilmvorming

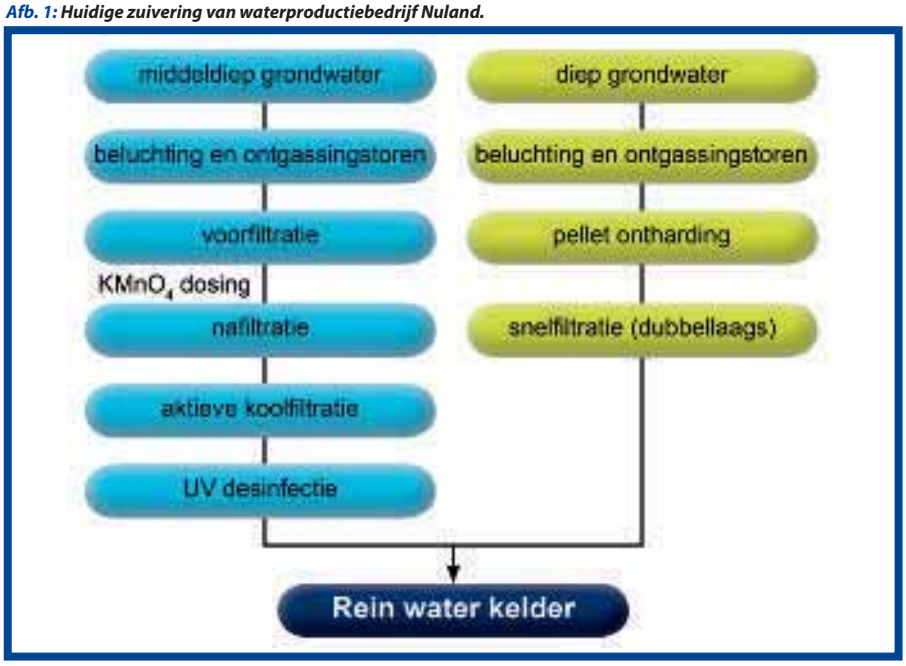
Om problemen met nagroei en vervuiling van het distributiesysteem te voorkomen streven de waterleidingbedrijven naar de productie van biologisch stabiel drinkwater. Waterproductiebedrijf Nuland (Brabant Water) maakt uit grondwater drinkwater dat voldoet aan alle wettelijke kwaliteitseisen, maar een hogere biofilmvormingssnelheid (BVS) heeft dan de streefwaarde van 10 pg ATP. $cm^{-2}.d^{-1}$, ook na aanpassingen in de zuivering. Uit bedrijfstakonderzoek bleek dat toepassing van ultrafiltratie op het reine water van Nuland tot een aanzienlijke verlaging van de BVS-waarde leidt. De NOM-concentratie wordt daarbij niet verlaagd. Dit betekent dat de in dit water aanwezige bacteriën en deeltjes en/of daaraan gebonden componenten die door ultrafiltratie worden tegengehouden, een belangrijke rol spelen bij de biofilmvorming. Het exacte mechanisme en de relatie met de biologische stabiliteit van het water zijn nog niet duidelijk. Deze aspecten worden binnen het bedrijfstakonderzoek nader onderzocht.

Uit onderzoek dat Vitens uitvoerde, bleek dat de biofilmvormingssnelheid van het water bij waterproductiebedrijf Oldeholtpade na toepassing van ionenwisseling daalde van 4 naar 0,6 pg ATP.$cm^{-2}.d^{-1}$[1]. Deze verlaging werd vooral toegeschreven aan de gedeeltelijke verwijdering van NOM. Ionenwisseling lijkt dan ook een geschikte technologie om de biologische stabiliteit te verbeteren. Uit het bedrijfstakonderzoek van de drinkwaterbedrijven dat bij Vitens is uitgevoerd op waterproductiebedrijf Spannenburg, kwam naar voren dat toepassing van ultrafiltratie een afname van de biolfilmvormingssnelheid met 60 procent veroorzaakte[2].
Ook optimalisatie van de nitrificatie in de bestaande zuivering bij Spannenburg leidt tot een verbetering van de biologische stabiliteit[3]. Op basis van de hypothese van de expertgroep Klassieke Zuivering, dat deeltjes en natuurlijk organisch materiaal in water van invloed kunnen zijn op de biologische stabiliteit, is een onderzoek opgezet waarbij deeltjes en NOM geheel of gedeeltelijk uit het water worden verwijderd. Bij dit onderzoek zijn de effecten van ultrafiltratie en ionenwisseling onderzocht.

Verwijdering van NOM door ionenwisseling

NOM bestaat uit veel verschillende typen organische verbindingen waarvan circa 60 procent negatief geladen is, met name humus- en fulvinezuren[4]. Dit maakt ionenwisseling geschikt voor een gedeeltelijke verwijdering van NOM. Humuszuren zijn verantwoordelijk voor de kleur van het water. Daarom wordt op waterproductiebedrijf Oldeholtpade ionenwisseling toegepast voor kleurverwijdering. Aangenomen wordt dat humuszuren moeilijk afbreekbaar zijn.

***Afb. 1:** Huidige zuivering van waterproductiebedrijf Nuland.*



Een sterke afname van de biolfilmvormingssnelheid door toepassing van ionenwisseling werd daarom niet verwacht.

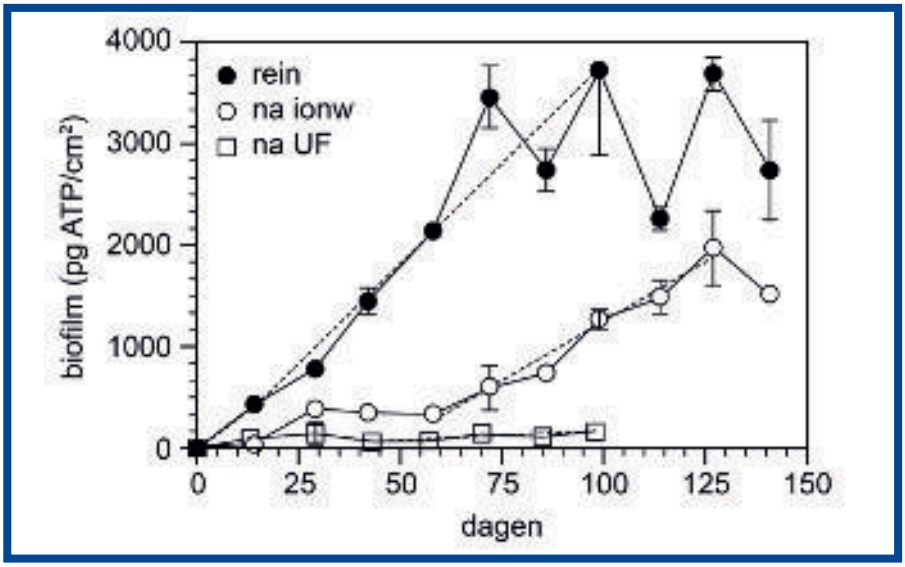
Vorming en verwijdering van deeltjes

Deeltjes ontstaan bij de zuivering van grondwater door beluchting en hebben meestal een basis van ijzer- en/of mangaan(hydr)oxiden. Deze deeltjes worden door snelfiltratie niet volledig verwijderd. Daarnaast komen deeltjes in het drinkwater door filterspoelingen. Ze worden vervolgens geïntroduceerd in het distributiesysteem. Ook bij vastbed-ionenwisseling wordt een deel van de deeltjes verwijderd door filtratie. Met ultrafiltratie worden alle 'deeltjes' verwijderd die groter zijn dan circa 20 nanometer, afhankelijk van het gekozen membraan. Dit betekent dat ook polymeren met een molecuulgewicht groter dan circa 150 kDa worden verwijderd.

Onderzoek Nuland

Bij waterproductiebedrijf Nuland wordt diep en middeldiep grondwater (gewonnen op 108,9 tot 135,9 meter, respectievelijk 34,5 tot 76 meter beneden maaiveld) gezuiverd tot drinkwater (zie afbeelding 1).

Beide grondwatertypen worden apart gezuiverd, waarna het water wordt gemengd en gedistribueerd. Het drinkwater van Nuland voldoet aan alle wettelijke kwaliteitseisen en aan de bedrijfsnormen van Brabant Water (zie tabel 1). Het AOC-gehalte (8 µg/l) is iets lager dan de streefwaarde van 10 µg/l acetaat-C voor biologisch stabiel drinkwater[5] en voldoet daarmee aan de bedrijfsnorm. In 2005 is de zuivering van Nuland gemoderniseerd, omdat het drinkwater niet aan alle bedrijfsnormen voldeed en de zuivering op leeftijd was. Hierbij is onder meer een centrale ontharding toegevoegd. Ondanks deze aanpassingen bleef de BVS-waarde (18 pg ATP.cm^{-2}.d^{-1}) van het geproduceerde drinkwater (van beide straten na menging) hoger dan de streefwaarde van 10 pg ATP. cm^{-2}.d^{-1} [6]. Deze relatief hoge waarde leidt mogelijk tot nagroei in het distributiesysteem (*Aeromonas* en/of dierlijke organismen) en in binneninstallaties (*Legionella*). Daarom is in BTO-verband bij waterproductiebedrijf Nuland onderzoek uitgevoerd naar mogelijkheden om deze relatief hoge BVS-waarde te verlagen.



***Afb. 2:** Biofilmvorming in de biofilmmonitors die werden gevoed met het reine water, het reine water na ionenwisseling en het reine water na ultrafiltratie.*

	rein water	na ionenwisseling	na ultrafiltratie
BVS (pg ATP cm^{-2}·d^{-1})	38,7 ± 4,2	23,2 ± 2,1	1,0 ± 0,5
FeAS (mg Fe m^{-2}·d^{-1})*	1,00 ± 0,19	0,095 ± 0,01	0,031 ± 0,084 ??
MnAS (mg Mn m^{-2}·d^{-1})**	0,42 ± 0,08	0,04 ± 0,01	0,008 ± 0,02 ??
ATP (ng/l)	8,9	4,9	4,2
totaal aantal bacteriën (N/ml)	2,1 x 10^5	2,1 x 10^5	1,1 x 10^4
AOB (N/ml)	4,4 x 10^2	1,8 x 10^2	<1,5 x 10^1
NPOC (mg C/l)	2,9	1,1	2,8
UV-absorptie (m^{-1})	9 ± 2	1 ± 1	9 ± 2
PO_4 (mg/l)	12	10	11
deeltjes (N/ml)	157	60	20

* = ijzerafzettingssnelheid; ** = mangaanafzettingssnelheid.

***Tabel 2:** Invloed van ionenwiseling en ultrafiltratie op de watersamenstelling.*

Effect op watersamenstelling

Met drinkwater van waterproductiebedrijf Nuland is onderzocht welke invloed natuurlijk organisch materiaal en deeltjes hebben op de biofilmvormende eigenschappen van het water. Het water is behandeld met ultrafiltratie (geen verwijdering van NOM, wel volledige verwijdering van deeltjes) en ionenwisseling (gedeeltelijke verwijdering van NOM en deeltjes). De effecten van deze behandelingsprocessen zijn bepaald met behulp van biofilmmonitors, deeltjestelling en NOM-karakterisering. De metingen zijn uitgevoerd tussen april en september 2008. In afbeelding 2 zijn de resultaten van de biofilmmonitors weergegeven. De resultaten met betrekking tot de BVS, NOM- en deeltjesverwijdering staan in tabel 2.

***Tabel 1:** Samenstelling van ruw en rein water bij waterproductiebedrijf Nuland (gegevens 2006).*

parameter	ruw middeldiep	ruw diep	rein	bedrijfsnorm
CH_4 (mg/l)	1,5	2,5	<0,01	<0,1 na ontgassing
NH_4 (mg/l)	1,0	1,1	0,02	<0,05
NO_3 (mg/l)	<0,5	<0,5	3,4	<25
NO_2 (mg/l)	<0,01	<0,01	<0,01	<0,05
Fe (mg/l)	8,0	2,0	0,01	<0,05
Mn (mg/l)	0,4	0,08	<0,01	<0,02
HCO_3 (mg/l)	236	262	190	>120
EGV (mS/m)	45	91	52	<80
TOC (mg C/l)	9,8	2,7	3,2	<5
pH	7,1	7,4	7,8	7,8<pH<8,3
AOC (µg/l ac-C)	onbekend	onbekend	8	<10

Biofilmvorming

De BVS van het reine water (38,7 pg ATP. cm^{-2}.d^{-1}) was in de onderzoeksperiode veel hoger dan de streefwaarde en tweemaal hoger dan de eerdere meting in 2007. Ook de ijzerafzettingssnelheid (FeAS) is hoger dan de eerdere meting in 2007 (0,5 mg Fe.m^{-2}.d^{-1}). De FeAS- en MnAS-waarden zijn relatief hoog in vergelijking met water van andere productiebedrijven in Nederland[7]. Een hoge BVS-waarde kan leiden tot nagroei van

Aeromonas. In het distributiesysteem van Nuland worden verhoogde aantallen *Aeromonas* waargenomen, maar de wettelijke kwaliteitseis (< 1000 kve per 100 ml) wordt niet overschreden.
De BVS-waarde van het water na ionenwisseling was gedurende de eerste 60 dagen laag, maar nam daarna toe tot 23,3 pg ATP. $cm^{-2}.d^{-1}$ (zie afbeelding 2). Deze toename is mogelijk het gevolg van het veranderen van de wijze van regenereren, maar dat is niet nader onderzocht. De BVS-waarde van het water na ultrafiltratie (1,0 pg ATP.$cm^{-2}.d^{-1}$) was veel lager dan de streefwaarde voor biologisch stabiel water. Ultrafiltratie had vrijwel geen effect op het gehalte natuurlijk organisch materiaal in het water. Dit betekent dat deeltjes en/of colloïden die met ultrafiltratie uit het gemengde water werden verwijderd, een grote invloed hebben op de biofilmvormende eigenschappen van het reine water. Aan deeltjes gebonden nutriënten en sporenelementen zouden hierbij een rol kunnen spelen, maar ook biomassa (van bacteriën) kan bijdragen aan de biofilmvorming.

Deeltjes

Uit tabel 2 blijkt dat 90 procent van de deeltjes die groter zijn dan 1 µm, door ultrafiltratie is verwijderd. Gezien de poriegrootte van ultrafiltratiemembranen was volledige verwijdering verwacht. Een mogelijke verklaring is dat de deeltjesmeting plaatsvond in de buffertank na de ultrafiltratie en niet direct in het permeaat.
Opvallend is de significante verwijdering (60 procent) van deeltjes door ionenwisseling. Uit deeltjestelling (> 1 µm) blijkt dat de grootste fractie (90 procent) van deeltjes in de range van 1-3 µm valt.
Dit kan betekenen dat ook een aanzienlijk aantal deeltjes aanwezig was met afmetingen kleiner dan 1 µm, die wel door ultrafiltratie worden verwijderd maar niet met deeltjestelling worden waargenomen. Tot deze deeltjes behoren tevens de bacteriën. Uit tabel 2 blijkt dat het totale aantal bacteriën in het reine water circa 2 x 10^5 per ml bedraagt. De lage BVS-waarde na ultrafiltratie is mogelijk een gevolg van de vergaande verwijdering van bacteriën uit het water.

NOM

Ultrafiltratie verwijderde vrijwel geen natuurlijk organisch materiaal; ionenwisseling verwijderde het voor 62 procent (zie tabel 2). Uit nadere analyse met LC-OCD blijkt dat vooral de humuszuren werden verwijderd door ionenwisseling (93 procent), gevolgd door de fractie laagmoleculaire neutrale verbindingen (32 procent). Gezien de significante verwijdering van deeltjes (die niet meegenomen worden in de NOM-karakterisering) is niet duidelijk wat de oorzaak is van de beperkte verlaging van de BVS-waarde door ionenwisseling.
Deze verlaging is waarschijnlijk slechts in beperkte mate het gevolg van de verwijdering van NOM, vanwege de geringe bijdrage van de moeilijk afbreekbare humuszuren aan de biofilmvorming. Nadere analyse met gefluïdiseerde ionenwisseling - waarbij deeltjesverwijdering een geringere rol speelt - kan uitwijzen of NOM-verwijdering invloed heeft. Een analyse van de NOM-fracties, aangevuld met AOC-metingen, zou kunnen uitwijzen of ionenwisseling ook biologisch afbreekbare fracties verwijdert.

Samenvatting

Ultrafiltratie van het reine water van waterproductiebedrijf Nuland leidde tot een aanzienlijke afname van de snelheid van de biofilmvorming, terwijl vrijwel geen natuurlijk organisch materiaal werd verwijderd. Dit betekent dat de in dit water aanwezige bacteriën, deeltjes, colloïden en/of de daaraan gebonden nutriënten een grote invloed hebben op de biofilmvorming. Eerder onderzoek naar de biofilmvormende eigenschappen van het reine water, uitgevoerd in opdracht van Brabant Water, leidde tot de hypothese dat deeltjes belangrijke nutriënten voor bacteriën bevatten waaronder fosfaat[6]. In BTO-verband wordt onder andere op Nuland verder onderzoek uitgevoerd naar de invloed van bacteriën, deeltjes en NOM op de biofilmvorming.

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Waterproductiebedrijf Nuland (foto: Brabant Water).

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ATP-metingen geven informatie over kans op nagroeiproblemen bij drinkwaterdistributie

Het gehalte actieve biomassa in het gedistribueerde drinkwater, gemeten als adenosinetrifosfaat (ATP), verschilt per distributiesysteem en is afhankelijk van de watersamenstelling (DOC, AOC en biofilmvormingssnelheid). Dit blijkt uit ATP-metingen in de distributiesystemen van zes drinkwaterpompstations. Uit de metingen bleek ook dat de afstand tot het pompstation, het seizoen en aanpassingen in de waterzuivering de ATP-concentratie in het leidingwater beïnvloeden.

Resultaten van spuiacties in het distributienet tonen aan dat het effect van schoonmaken met ATP-metingen betrouwbaar kan worden gevolgd. Met metingen van troebelheid of ijzergehalte was dit minder goed mogelijk. Tussen het ATP-gehalte en het koloniegetal van *Aeromonas* of het koloniegetal van heterotrofe bacteriën (KG 22) in gedistribueerd drinkwater is geen sterk direct verband aangetoond. ATP-metingen zijn dus geen surrogaat voor de wettelijke parameters KG22 of het koloniegetal van *Aeromonas*. Wel is een sterk direct lineair verband gevonden tussen ATP en het totaal aantal bacteriecellen in het water. ATP-metingen geven snel, eenvoudig en goedkoop informatie over de kans op nagroeiproblemen in het leidingnet.

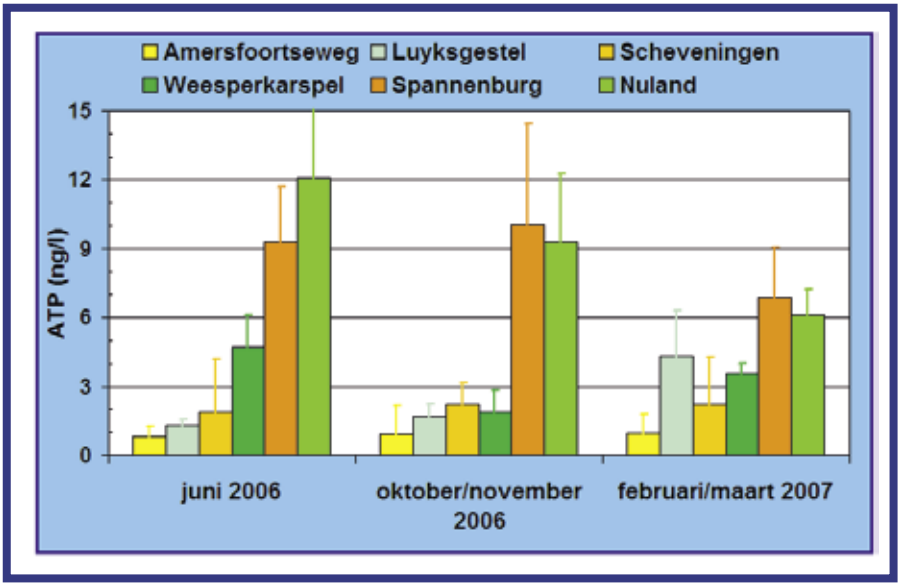
Nagroei van micro-organismen in het distributiesysteem en de binneninstallatie is ongewenst, omdat daarbij vermeerdering van ziekteverwekkende micro-organismen kan optreden. Daarnaast kan nagroei leiden tot klachten van consumenten over troebelheid, afwijkende geur en smaak en groei van dierlijke organismen. In Nederland wordt nagroei in het leidingnet beperkt door drinkwater te distribueren met een lage concentratie aan groeibevorderende stoffen. Waterleidingbedrijven meten periodiek het koloniegetal op glucosegistextractagar na drie dagen incuberen bij 22°C (KG22) en het koloniegetal van *Aeromonas* bepaald na 20 tot 24 uur incuberen bij 30°C.

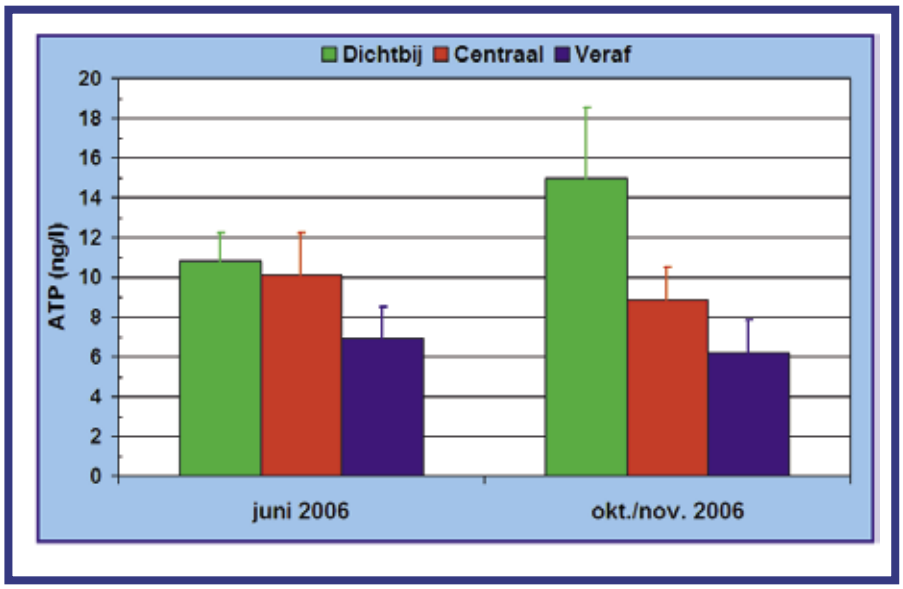
Deze metingen zijn als bedrijfstechnische parameters opgenomen in het Waterleidingbesluit. In de afgelopen jaren is de wettelijke norm voor KG22 (geometrisch jaargemiddelde van 100 kve/ml) in Nederland niet overschreden, maar in een aantal distributiegebieden ligt het koloniegetal van *Aeromonas* herhaaldelijk hoger dan de wettelijke norm (1000 kve/100 ml[1]).

Deze wettelijke parameters hebben echter enkele nadelen. Ten eerste vertegenwoordigen de koloniegetallen slechts een klein deel van het totale aantal levende micro-organismen en geven daardoor geen informatie over de totale concentratie actieve biomassa in drinkwater. Daarnaast is de analysetijd relatief lang (KG22: 72 uur, *Aeromonas*: 24 uur).

Een beter bruikbare parameter voor de concentratie van actieve biomassa in het drinkwater is adenosinetrifosfaat (ATP), een energierijke verbinding die wordt aangemaakt in actieve cellen van alle levende organismen, dus ook in micro-organismen[2]. Het ATP-gehalte is daardoor een goede maat voor de microbiële activiteit in het drinkwater. De ATP-concentratie in drinkwater wordt bepaald met behulp van een eenvoudig in te zetten enzymatische

***Afb. 1:** ATP-concentraties van het drinkwater in het leidingnet van zes pompstations in de periode juni 2006, oktober/november 2006 en februari/maart 2007.*





Afb. 2: ***ATP-concentraties in water bemonsterd op locaties in het leidingnet dichtbij, centraal en veraf van pompstation Spannenburg.***

toetsing. Voordelen van ATP als parameter voor nagroei ten opzichte van KG22 en *Aeromonas* zijn dat de analyse binnen een paar minuten is uitgevoerd, relatief goedkoop is en een lage detectiegrens heeft (0,5 ng/l). In Nederland is geruime tijd ervaring opgedaan met het bepalen van ATP in drinkwater. Over het algemeen ligt de ATP-concentratie in het Nederlandse drinkwater lager dan 10 ng/l[3),4)].

In het kader van het bedrijfstakonderzoek voor de waterbedrijven is, samen met Dunea, Waternet, Brabant Water en Vitens, onderzoek verricht naar de ATP-concentraties van het drinkwater in het leidingnet en van het spuiwater. Hierbij zijn de effecten onderzocht van watersamenstelling, seizoen en afstand tot het pompstation op het ATP-gehalte van het drinkwater. Daarnaast is getoetst of een relatie bestaat tussen de ATP-concentratie en andere microbiologische parameters in het water. Ten slotte is onderzocht of ATP als parameter gebruikt kan worden om de effectiviteit te bepalen van spuien als middel om het distributiesysteem schoon te maken.

In drie seizoenen (voorjaar, herfst en winter) zijn in de distributiesystemen van zes pompstations 30 monsters genomen van het drinkwater, verdeeld over locaties dichtbij, centraal en veraf van het pompstation. Deze monsters zijn geanalyseerd op ATP-gehalte, KG22, *Aeromonas* en totaal aantal cellen van micro-organismen. In de distributiesystemen van twee pompstations (Amersfoortseweg en Spannenburg) is spuiwater geanalyseerd op microbiologische, chemische en fysische parameters voor het beoordelen van de effectiviteit van het spuien op het schoonmaken van het leidingnet en om te achterhalen of een relatie bestaat tussen ATP en fysisch/chemische parameters (troebelheid, ijzer, mangaan).

Invloeden op ATP-gehalte

De gemiddelde ATP-concentraties van het drinkwater in het leidingnet van de zes pompstations varieerden tussen de 0,8 en 12,0 ng/l (zie afbeelding 1). In de distributiesystemen van pompstations Nuland en Spannenburg bedroeg de ATP-concentratie 6-12 ng/l. Dit niveau is significant hoger dan de concentraties in het water van de pompstations Amersfoortseweg, Luyksgestel, Scheveningen en Weesperkarspel. In dezelfde periode zijn ook het gehalte opgelost organisch koolstof (DOC), afbreekbaar organisch koolstof (AOC) en de biofilmvormingssnelheid (BVS) van het reinwater van de pompstations bepaald (behalve voor Luyksgestel). De resultaten laten zien dat het reinwater van Nuland en Spannenburg ook hogere AOC- en DOC-gehaltes heeft en een hogere BVS-waarde dan het water van de drie andere pompstations. De samenstelling van het reinwater beïnvloedt dus de ATP-concentratie in het distributiesysteem.

In februari/maart 2007 was de ATP-concentratie van het drinkwater in het leidingnet van Nuland en Spannenburg significant lager dan in juni 2006 en in oktober/november 2006 (zie afbeelding 1). De gemiddelde temperatuur van het drinkwater in het distributienet van Nuland en Spannenburg bedroeg respectievelijk in de periode februari/maart 2007 8,1 en 10,6°C. Dat is significant lager dan in de twee andere perioden (12,5 tot 19,5 °C). De lagere microbiële activiteit in de winterperiode is een gevolg van de lagere temperatuur in het distributiesysteem.
Opvallend is dat in de winterperiode het gemiddelde ATP-gehalte van het drinkwater in het leidingnet van pompstation Luyksgestel significant hoger was dan in juni of oktober 2006 (zie afbeelding 1). Ook de ATP-concentratie in het reinwater van pompstation Luyksgestel was in maart 2007 twee tot drie keer hoger dan in de twee andere perioden. Onduidelijk is welke factoren deze hogere ATP-concentratie in de winterperiode veroorzaakten. Het ATP-gehalte in het gedistribueerde water en reinwater van pompstation Weesperkarspel varieerde in de drie perioden tussen 1,9 en 4,7 ng/l (zie afbeelding 1) en was significant verschillend in elk van de onderzochte perioden.
Eén van de waterbehandelingsprocessen in de zuivering van Weesperkarspel is ozonisatie. Tijdens de onderzochte periode was de gedoseerde ozonconcentratie niet constant, met als gevolg een veranderende hoeveelheid AOC in het water. Deze variabele hoeveelheid AOC beïnvloedt de mate van biologische activiteit en dus de hoeveelheid ATP in het reinwater. Deze resultaten laten zien dat bij sommige pompstations het seizoen (watertemperatuur) een invloed heeft op het ATP-gehalte in het distributiesysteem, terwijl resultaten bij andere pompstations laten zien dat de watersamenstelling een grotere invloed heeft op het ATP-gehalte in het water dan het jaarseizoen (watertemperatuur).

Bij de meeste pompstations had de afstand van het monsterpunt in het leidingnet tot het pompstation geen effect op de hoeveelheid ATP in het gedistribueerde water. Alleen de ATP-concentraties in water in het verafgelegen deel van het uitgestrekte distributienet van Spannenburg (6,2-6,9 ng/l) waren significant lager dan in het deel nabij het pompstation (10,8-15,0 ng/l) (zie afbeelding 2). De lagere ATP-concentratie in het verafgelegen deel van het leidingnet wordt waarschijnlijk veroorzaakt doordat afbreekbare stoffen in het voorste deel van het leidingnet door micro-organismen in de biofilm aan de leidingwand worden opgenomen. Hierdoor bevat het water in het achterste deel van het leidingnet minder afbreekbare stoffen, waardoor de microbiologische activiteit (en daarmee het ATP-gehalte) lager wordt.

Parameter voor nagroei in drinkwater

Uit de correlatieanalyse bleek dat slechts een zwakke directe correlatie bestaat tussen ATP en KG22 of *Aeromonas* (zie de tabel). Hierdoor kan ATP niet als vervangende parameter voor deze wettelijke parameters worden gebruikt. ATP en het totaal aantal bacteriecellen zijn wel sterk lineair met elkaar gecorreleerd (zie de tabel). ATP en celtellingen zijn dus geschikte parameters om de biomassahoeveelheid en de mate van nagroei te beschrijven in het drinkwater. Celtellingen worden momenteel uitgevoerd met fluorescentiemicroscopie. Deze methode is arbeidsintensiever dan de gebruikte enzymatische methode om ATP te bepalen, waardoor ATP-metingen in de praktijk aantrekkelijker zijn.

Tussen het ATP-gehalte en de aantallen *Aeromonas* in het leidingwater is geen directe relatie gevonden, maar bij de pompstations met een relatief hoge ATP-concentratie in het distributiesysteem (pompstations Spannenburg en Nuland) zijn wel de hoogste aantallen *Aeromonas* in het drinkwater van het leidingnet waargenomen. Met behulp van ATP-metingen kunnen dus relatief snel en eenvoudig watertypen of locaties in distributiesystemen worden opgespoord waar mogelijk problemen zijn met nagroei in het leidingnet. In het verleden is een databank opgezet van de hoeveelheid ATP in het reinwater van 241 pompstations

in Nederland[3),4)]. Het ATP-gehalte van het drinkwater wordt beschouwd als hoog wanneer de 95-percentielwaarde van de ATP-concentratie wordt overschreden (9,4 ng/l ATP). Uit de resultaten in het distributiegebied van de zes pompstations bleek dat overschrijding van deze waarde werd waargenomen in het distributiegebied van Nuland en Spannenburg.

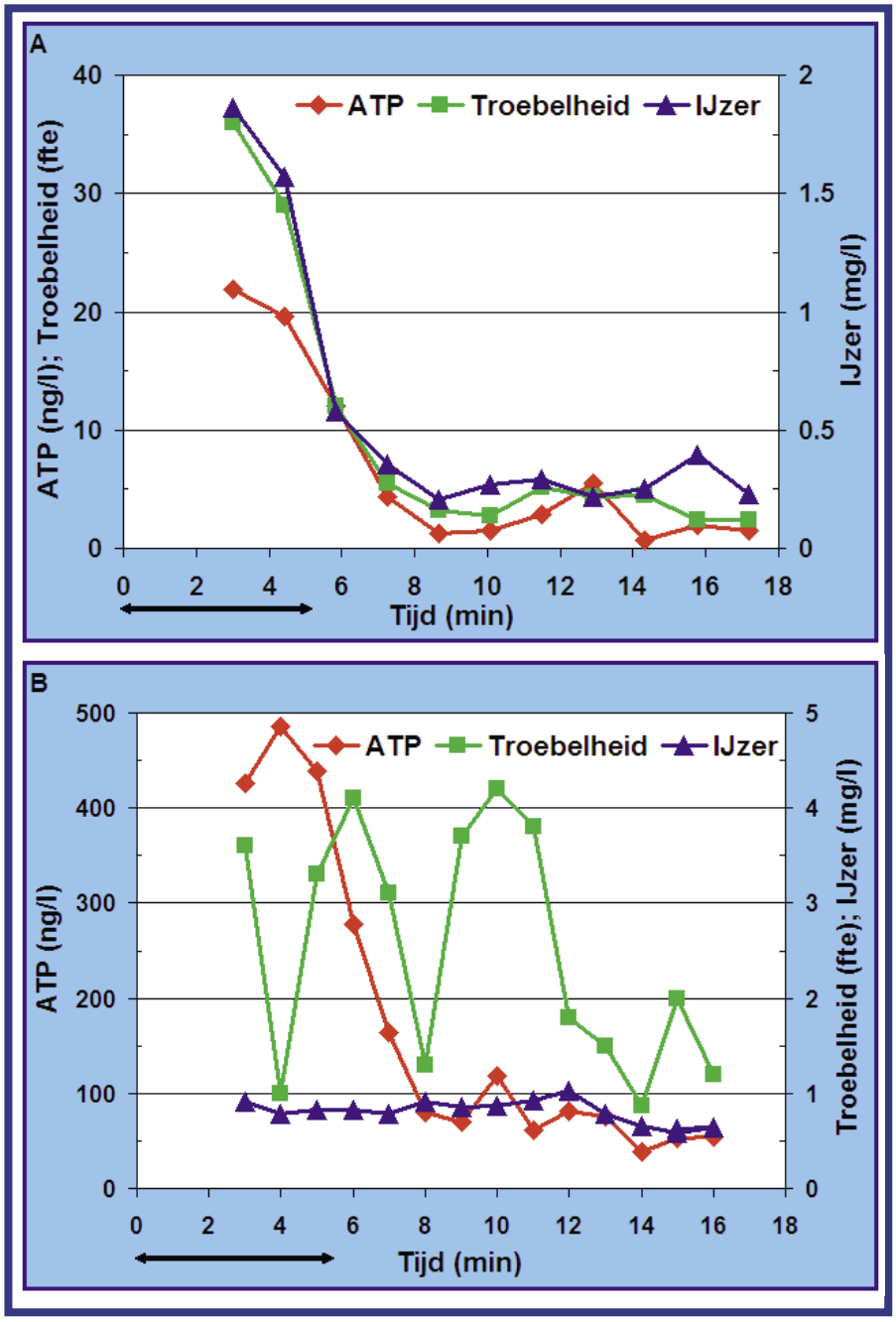
correlatie	periode	N	p	R^2
ATP - totaal aantal cellen	oktober/november 2006	48	<0.01	0,82
ATP - totaal aantal cellen	februari/maart 2007	42	<0.01	0,55
ATP - KG22	juni 2004	499	<0.01	0,20
ATP - *Aeromonas*	oktober/november 2006	112	<0.01	0,25

Uitkomsten van de lineaire correlatieanalyse.
N = aantal waarnemingen, p = significantieniveau, R^2 = Pearson's correlatiecoëfficiënt.

Monitoren van schoonmaakacties leidingnet

Tijdens de spuiactie in het distributiesysteem van pompstation Amersfoortseweg nam de ATP-concentratie toe tot 21,9 ng/l tijdens de eerste verversing van de leiding (zie afbeelding 3). Het verloop van de ATP-concentratie was tijdens deze spuiactie hetzelfde als het verloop van de troebelheid en ijzer- en mangaanconcentratie. In het distributiesysteem van pompstation Spannenburg nam de ATP-concentratie in het spuiwater toe tot 485 ng/l tijdens de eerste verversing van de leiding.
In tegenstelling tot het spuiwater van pompstation Amersfoortseweg correleerde de ATP-concentratie in het spuiwater van pompstation Spannenburg niet significant met troebelheid, ijzer en mangaan. De hoge ATP-concentratie in het spuiwater van Spannenburg en de afwezigheid van relaties tussen ATP en fysisch/chemische parameters duiden erop dat het sediment in het leidingnet van Spannenburg wordt gedomineerd door een biologische component. De hoge ATP-concentratie in het spuiwater van Spannenburg komt overeen met de relatief hoge ATP-concentratie van het drinkwater in het leidingnet van Spannenburg (zie afbeelding 1).
Opvallend is dat de troebelheid van het spuiwater in het distributiesysteem van pompstation Amersfoortseweg ongeveer tien keer hoger is dan de troebelheid van het spuiwater in het distributiesysteem van pompstation Spannenburg, terwijl de actieve biomassa in het spuiwater van Amersfoortseweg ongeveer 20 keer lager is dan in het spuiwater van Spannenburg. Dit betekent dat wanneer veel biomassa in het leidingnet aanwezig is, de troebelheid van het drinkwater in het leidingnet geen goede indicatie geeft van de mate van vervuiling van het distributiesysteem. Daarom kan de troebelheid niet altijd worden gebruikt om te bepalen of het distributiesysteem moet worden schoongemaakt. Uit de resultaten blijkt ook dat het raadzaam is om tijdens het schoonmaken van leidingen zowel microbiologische (ATP) als chemische (ijzer en mangaan) en fysische (troebelheid) parameters te bepalen in het spuiwater.

***Afb. 3:** Troebelheid, ATP-gehalte en ijzerconcentratie in het spuiwater van de pompstations Amersfoortseweg (A) en Spannenburg (B). De lengte van de pijl geeft de tijd voor de eerste verversing van de leiding aan.*



Conclusies

Waterleidingbedrijven kunnen ATP-metingen inzetten om snel, eenvoudig en goedkoop een beeld te krijgen van de hoeveelheid actieve biomassa in het drinkwater en het spuiwater. Daarmee kunnen mogelijke nagroeiproblemen in het leidingnet worden voorspeld of kan het effect van schoonmaakacties worden bepaald. Tevens kunnen ATP-bepalingen in reinwater dienen om de effecten te bepalen van aanpassingen in de zuivering op de biologische activiteit in het reinwater en in het distributiesysteem.

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Moleculair-biologische analyse van processen in actieve koolfilters

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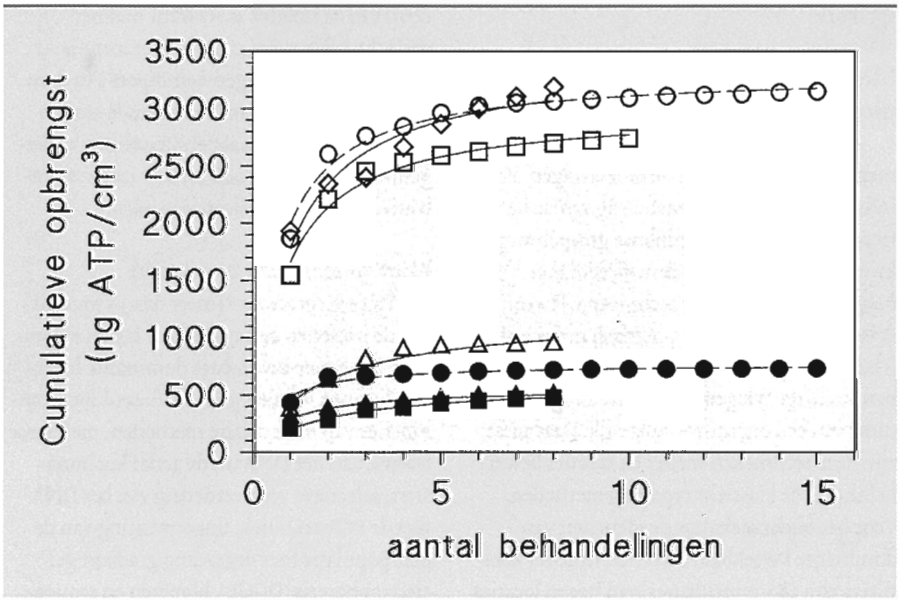
Filtratie met behulp van actieve kool wordt in Nederland al 25 jaar toegepast voor de verwijdering van organische verbindingen die een ongewenste invloed hebben op de kleur, geur of smaak van drinkwater. Bovendien vormt filtratie met actieve kool met of zonder vooroxidatie een effectieve barrière tegen bestrijdingsmiddelen en andere organische microverontreinigingen. Biologische processen in de actief-koolfilters kunnen een bijdrage leveren aan de verwijdering van genoemde typen verbindingen, maar zijn met name van belang voor de verwijdering van gemakkelijk afbreekbare stoffen. Ozonisatie of een ander oxidatieproces voor actieve koolfiltratie gaat gepaard met de vorming van afbreekbare organische koolstof en leidt tot hogere biologische activiteit in de koolfilters[3]. Het oxidatieproces en de biologische omzettingen verlagen de belasting van de adsorptiecapaciteit van actieve kool en leiden tot een verlenging van de looptijd. Een lagere regeneratiefrequentie is aantrekkelijk uit oogpunt van kosten en gunstig voor het milieu[2].

In 2000 begon een onderzoek naar de invloed van vooroxidatie op de (interacties tussen) biologische processen en adsorptie in actieve koolfilters. Dit zogeheten biologisch actieve koolfiltratie-onderzoek wordt uitgevoerd door Waterleidingbedrijf Amsterdam, Hydron, Norit, Kiwa, de Technische Universiteit Delft en Wageningen Universiteit met subsidie van het Ministerie van Economische zaken (Senter).

Het onderzoek beoogt het effect van oxidatie op de biologische omzetting en adsorptie zodanig te optimaliseren dat een minimale regeneratiefrequentie wordt bereikt. Voor het realiseren van dit doel worden de biologische omzettingen en de adsorptie in één integraal model opgenomen[1].

De ontwikkeling van dit model wordt ondersteund met een analyse van de microbiologische processen in de koolfilters. Hierbij wordt onder meer gebruik gemaakt van moleculair-biologische methoden.

Afb. 1: Cumulatieve opbrengst van actieve biomassa bij ultrasone behandeling van monsters uit actieve koolfilters uit verschillende zuiveringssystemen.



Bepalen actieve biomassa met ATP

De hoeveelheid (actieve) biomassa en de aard van de aanwezige micro-organismen bepalen de intensiteit en de aard van de omzettingsprocessen in actief koolfilters. Diverse methoden zijn beschikbaar voor de bepaling van de concentratie van de biomassa in deze filters, waaronder zuurstofverbruik, koloniegetal, totale directe microscopische celtelling en fosfolipiden. Deze methoden zijn echter bewerkelijk en/of leveren geen eenduidige informatie. Zo zijn koloniegetallen niet goed bruikbaar, omdat lang niet alle bacteriën kweekbaar zijn en maakt microscopische telling geen onderscheid tussen dode en levende cellen of grote en kleine cellen. Voor de kwantificering van actieve biomassa in de koolfilters is daarom de keuze gevallen op de analyse van adenosinetrifosfaat (ATP). Deze verbinding, die alleen aanwezig is in levende (micro-)organismen, kan worden geanalyseerd met behulp van een snelle, gevoelige en specifieke methode. Deze methode is gebaseerd op een enzymreactie waarbij in aanwezigheid van ATP licht vrijkomt (luminescentie). De gemeten ATP-concentraties kunnen worden vergeleken met een databestand van ATP-concentraties in biofilms en drinkwater, wat de interpretatie vereenvoudigt.

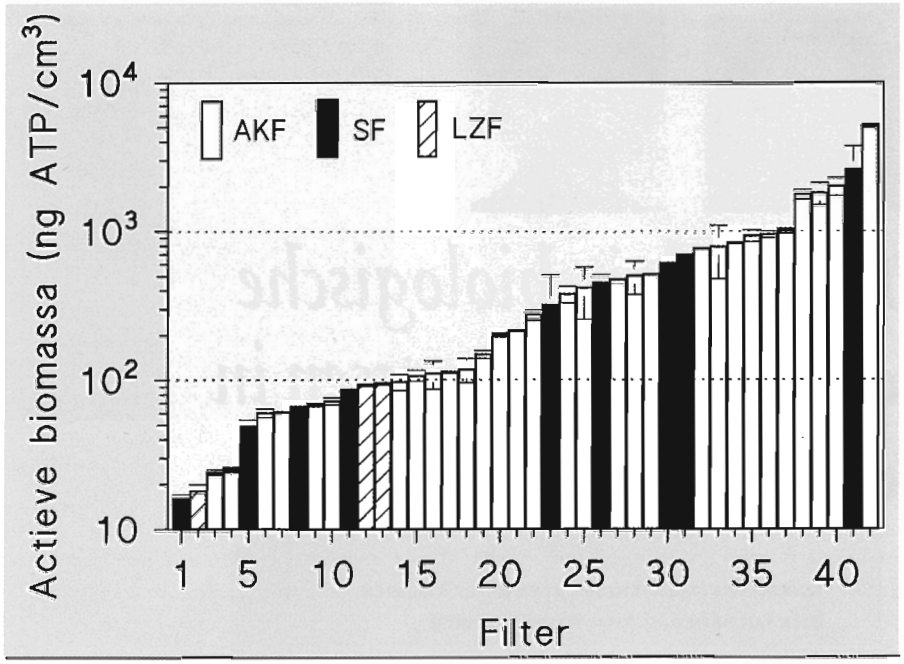
De kwantitatieve bepaling van de microorganismen op actieve kool verloopt in twee stappen: desorptie van de biomassa en meten van de concentratie van deze biomassa. Voor de eerste stap wordt behandeling met ultrasoon geluid (sonificatie) toegepast. Deze techniek is effectief, maar heeft als nadeel dat de betrokken micro-organismen worden beschadigd, waardoor de kweekbaarheid afneemt. Voor het toepassen van de analyse met behulp van ATP is dit echter geen probleem.

Ultrasone behandeling met hoge energie bleek het meest effectief voor de verwijdering van de biomassa van het oppervlak aan actieve kool. Wel is een serie behandelingen nodig, omdat de bacteriën zich sterk hechten op actieve kool (afbeelding 1). Op basis van de bevindingen is een procedure opgesteld waarmee meer dan 90 procent van de op actieve kool aanwezige biomassa kan worden gemeten met behulp van ATP. Bacteriën tussen de koolkorrels vormen slechts een kleine fractie (minder dan tien procent) van de biomassa in actief koolfilters.

Biomassa in actieve koolfilters

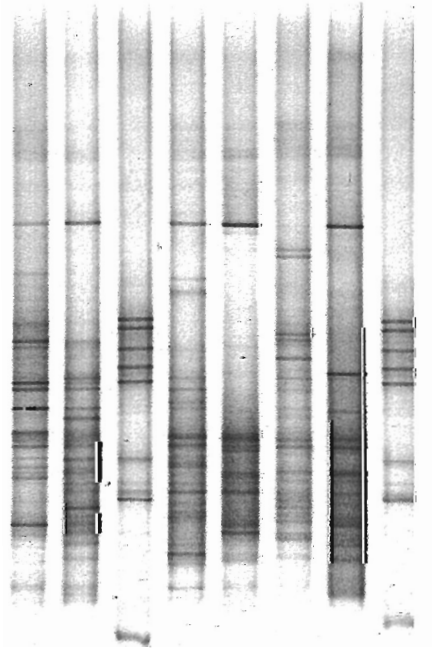
Afbeelding 2 toont de concentraties van biomassa, waargenomen in een aantal actief koolfilters en (langzame) zandfilters. De locaties en filters zijn geselecteerd om een indruk te krijgen van de effecten van verschillende operationele parameters (watersamenstelling, voorbehandeling, looptijd) op de concentratie van biomassa. Naast de ATP-concentratie is het totale aantal cellen (TDC) bepaald met behulp van microscopie en is het koloniegetal (HPC) bepaald. Het ATP-gehalte was significant gecorreleerd met de TDC-waarden. Opvallend was dat de mediaanwaarde van het ATP-gehalte per cel in de actieve koolfilters (circa 2 x 10^{-8} pg ATP/cel) meer dan tien maal lager was dan in de snelfilters. Dit betekent dat de bacteriën op actieve kool veel minder actief zijn dan in de snelfilters. Het ATP-gehalte vertoonde geen statistisch significante correlatie met de koloniegetallen, die een kleine fractie (minder dan vijf procent) vormden van de TDC-waarden.

De ATP-concentraties bij langzame zandfiltratie zijn lager dan in actieve kool en zandfiltratie die binnen eenzelfde range liggen (afbeelding 2). Uit de in verhouding lage ATP-gehaltes in langzame zandfilters kan worden afgeleid dat het aanbod aan afbreekbare verbindingen (watersamenstelling x filtratiesnelheid) van grote invloed is op de concentratie actieve biomassa. De onderzochte actioeve koolfilters vertonen grote onderlinge verschillen wat de concentratie actieve biomassa betreft. Deze verschillen zijn het gevolg van de looptijd van de filters en de aard van de voorbehandeling (watersamenstelling). De hoogste concentraties van biomassa zijn gemeten in de



Afb. 2: Concentraties actieve biomassa in actieve koolfilters (AKF), snelfilters (SF) en langzame zandfilters (LZF) van verschillende waterbehandelingsinstallaties. De monsters zijn enkele centimeters onder de bovenkant van het filterbed genomen.

Afb. 3: Fingerprnts van bacteriepopulaties in verschillende koolfilters, bepaald met behulp van de DGGE-methode.



actief koolfilters met een lange looptijd die gevoed worden met geozoniseerd water. Onduidelijk is nog of de eigenschappen van actieve kool ook van invloed zijn op de ontwikkeling van biomassa. De concentraties van biofilm op actieve kool, berekend uit de ATP-gehaltes en de aanname dat het uitwendige oppervlak van de actieve kooldeeltjes ongeveer 100 cm^2 per gram bedraagt, liggen tussen 500 en 10^5 pg ATP/cm^2. Deze concentraties zijn veel hoger dan de biofilmconcentraties die zijn waargenomen in leidingnetten en in membraansystemen[5),6)]. Wordt echter ook het oppervlak van de poriën met een diameter groter dan 1 µm bij de berekening betrokken, dan ligt de gemiddelde biofilmconcentratie zo'n tien maal lager en kan worden afgeleid dat van een echte biofilm op de actief koolkorrels geen sprake is.

Identificatie van bacteriën met moleculaire technieken

De aard van de aanwezige micro-organismen bepaalt de aard van de omzettingen. Voor informatie over de samenstelling van de bacteriepopulatie zijn de dominante groepen van kweekbare bacteriën geïdentificeerd met behulp van moleculaire technieken. Hierbij staat de analyse van het genetisch materiaal (DNA) centraal. Met deze technieken is een nauwkeurige fylogenetische plaatsing (classificatie) van een organisme mogelijk. Daarnaast zijn deze technieken sneller en minder bewerkelijk dan de klassieke typeringsmethoden. Voor het onderzoek naar de identiteit van dominante kweekbare bacteriën in actief koolfilters zijn 260 'reincultures' van negen locaties in Nederland verzameld. Deze 'cultures' zijn onderling vergeleken met behulp van DNA-fingerprinting. Deze vergelijking resulteerde in een clustering in tien groepen met vijf tot 24 isolaten per groep. Vervolgens is van een aantal isolaten uit elke groep na toepassen van de polymerase-kettingreactie (PCR) de volgorde van de bouwstenen (sequentie) van het 16s RNA-gen bepaald. Uit een vergelijking van verkregen sequenties met de sequenties van bekende bacteriesoorten bleek dat 66 procent van de isolaten behoort tot de familie *Comamonadacea*. Dit betreft de hernoemde en nieuwe geslachten *Acidovorax, Aquaspirillum, Comamonas, Hydropgenophaga, Polaromonas* en *Variovorax*, waarover nog relatief weinig bekend is. De meeste isolaten zijn verwant met *Polaromonas* en *Hydropgenophaga*. Bijna een kwart (24 procent) van de isolaten is verwant met het geslacht *Sphingomonas*. In de literatuur is beschreven dat vertegenwoordigers van deze geslachten bepaalde milieuvreemde stoffen kunnen omzetten, maar onduidelijk is of dergelijke omzettingen ook bij zeer lage concentraties in actief koolfilters optreden.

Niet-kweekbare bacteriën

Het overgrote deel (meer dan 95 procent) van de bacteriën op actieve kool is niet kweekbaar. Deze niet-kweekbare dominante bacteriën kunnen worden geïdentificeerd met combinaties van moleculaire methoden, met name isolatie van het DNA uit de actief koolmonsters, selectieve vermeerdering van het DNA met de PCR-techniek, fingerprinting van de hele populatie met degrading gradient gel electrophoresis (DGGE), kloneren en sequen-

tie-analyse. DGGE is een techniek voor het vaststellen van verschillen in de populatiesamenstelling in verschillende monsters of voor het volgen van de veranderingen van de populatiesamenstelling. In dit onderzoek wordt DGGE toegepast om de effecten van verschillende procescondities op de samenstelling van de bacteriële populaties te bestuderen (afbeelding 3). Van de populaties die op basis van DGGE-fingerprints duidelijk verschillend zijn, worden vervolgens dominante soorten geïdentificeerd door middel van sequentie-analyse van 16s DNA-klonen.

De tot nu toe verkregen resultaten bevestigen dat ook deze bacteriën behoren tot nog vrijwel niet beschreven typen. Om vast te stellen welke omzettingen de kweekbare en de niet-kweekbare bacteriën bewerkstelligen in de actieve koolfilters, worden experimenten met geselecteerde verbindingen uitgevoerd. De informatie over de voedingsbehoeftes en de groeikinetiek van deze bacteriën zal worden gebruikt in het model voor de optimalisatie van de biologische processen in de biologisch actieve koolfiltratie. ¶

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