PAPER

Check for updates

Flow cytometric fingerprinting to assess the microbial community response to changing water quality and additives†

Benjamin Buysschaert, [iD] [ab] Jorien Favere,[a] Lotte Vermijs,[a] Valerie Baetens,[a] Agathi Naka,[a] Nico Boon[a] and Bart De Gusseme*[ab]

Water is used for a very broad range of industrial applications with different water quality requirements. In all cases, the microbial water quality remains of importance as the microbial community can cause biofouling, microbial induced corrosion, odor problems, or health hazards. A close and accurate monitoring of the microbial water quality is therefore relevant for all water types used in industrial applications. Flow cytometry and additionally flow cytometric fingerprinting have been proposed before as methods to monitor the aquatic microbial communities but it remains unclear on how sensitive the fingerprinting method is for detecting quality changes in practice for different types of water. In this paper, we compared the microbial dynamics of coarsely filtered surface water, tap water, and demineralized water by challenging these waters with different concentrations and types of nutrients (C, N, and P) and additives such as corrosion inhibitors and biocides. We demonstrated that the cytometric fingerprints of the aquatic microbial communities differed in function of the type and concentration of product added, but that these differences are dependent on the type of water. Flow cytometry proved to be sensitive enough to detect subtle changes in microbial communities and to measure bacterial regrowth in different types of water. As a result, we conclude that cytometric fingerprints can be considered as indirect indicators of the physical–chemical composition of the water and a tool to monitor water biostability, as a tell-tale for minor environmental changes.

Water impact

Monitoring small changes in water quality are very important but represent a challenge today. Aquatic bacteria communities react quickly to changing water quality and can be measured through single cell analysis. In this paper, flow cytometry is used as a tell-tale for minor water quality changes to optimize the water quality and biostability during and after treatment.

1 Introduction

Water is fundamental for industrial applications where it is used in a wide variety of ways such as for rinsing and cleaning, cooling, as base material, as solvent, or for transportation. As a consequence, the water quality requirements are different for each application. The used water type varies from coarsely filtered surface water, which may serve as cooling water, to purified and demineralized water that can be used for food processing or steam production. Due to the rising water demand, consciousness of our environmental impact, and climate change, water reuse systems are increasingly implemented.[1] This evolution gives rise to additional challenges concerning water quality as deviations in the water quality can be an important source of problems, regardless of the water type. Monitoring the microbial water quality is of major concern, since aquatic bacteria can cause biofouling of membranes, induce corrosion (MIC), produce undesired odors, and potentially affect public health (*e.g. Legionella*).[1–5]

Aquatic bacteria became of interest in the 19th century when attempts were made to resolve waterborne diseases by scientific research. Later, research showed that every water source contains bacteria, regardless of its quality.[6] These aquatic bacteria typically live in complex and dynamic communities that are shaped by environmental factors.[7] An important factor is the amount of available nutrients. For example, lake waters are divided into eutrophic, mesotrophic, and

[a] Center for Microbial Ecology and Technology (CMET), Department of Biochemical and Microbial Technology, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, B-9000 Gent, Belgium.
E-mail: Bart.DeGusseme@UGent.be; Web: www.cmet.UGent.be;
Fax: +32 (0)9 264 62 48; Tel: +32 (0)9 264 59 76
[b] FARYS|TMVW, Stropstraat 1, B-9000 Gent, Belgium
† Electronic supplementary information (ESI) available. See DOI: 10.1039/c9ew00283a

oligotrophic water, containing a decreasing concentration of nutrients respectively.[8] Besides the quantity, also the balance between different nutrients impacts the microbial community.[9] Insights in the dynamics and composition of the microbial community can thus indirectly provide information about the environmental factors shaping aquatic microbial communities. Ideally, process conditions have a limited impact on the microbiological stability of the water as biostability is believed to be a sustainable solution for water quality management while issues can be detected easily. However, analyzing aquatic microbial communities in industrial settings is difficult. The plate count method, which is still considered as the golden standard, is typically used.[10] Disadvantages of the method are the necessity for skilled technicians for the sterile preparation of the plates, and the time delay between sampling and results as the plates need to incubate for at least 24 hours.[11] Furthermore, only a very small fraction (<0.01%) of the aquatic bacteria can be cultivated, making this method less accurate.[12] Because of all these impracticalities, the microbial quality of process water is not considered in current monitoring activities. Instead, microbiologically induced issues are commonly mitigated by the addition of biocides to the water which is done by a periodic addition of high concentrations of chemicals.[13,14] The addition of chemicals may be an effective solution, though their presence can be harmful for the environment or impede water reuse. Thus, a close and online monitoring of the microbial water quality is a useful tool to improve water quality management, to reduce downstream costs, and to mitigate health and environmental hazards.[15]

Several methods for online monitoring of aquatic bacteria have been developed in the last few years. A first approach consists of detecting biomolecules that correlate with bacterial concentrations. Common examples are measurement of ATP concentration, enzymatic activity, or tryptophan fluorescence.[16–18] However, these methods can be inaccurate because they rely on indirect measurements of the bacteria. Consequently, as aquatic bacteria communities differ in every water, no universal conversion to cell concentration can be done.[18] An alternative method measures the refractive index of the water to determine changes in the water quality such as microbial regrowth.[19] The disadvantage of this method is that it cannot discriminate among different sources inducing the change in refractive index. Approaches based on particle counting are therefore more reliable as they measure the microbial concentration directly.[20] Flow cytometry in particular has been proposed multiple times as tool to monitor the microbial water quality.[21–23] As this method relies on fluorescent dyes for detection, physiological traits can be assessed on the single-cell level. This can be particularly useful to assess decontamination efficiency.[12] To extract more information from the complex cytometric data, flow cytometric fingerprinting has been proposed and showed to provide useful information regarding the aquatic microbial communities.[11,23–25] Both Props, Monsieurs[25] and Prest, Hammes[23] showed with different fingerprinting approaches that changes in taxonomic

diversity correlate well with changes in cytometric diversity. Moreover, Besmer, Sigrist[26] showed that these approaches are complementary to cell counts. In this paper, we assessed the possibilities and limits of flow cytometric fingerprinting for the monitoring of the microbiological water quality and biostability with industrially relevant examples. We choose to work with coarsely filtered surface water, tap water, and demineralized water because of their different properties and because of their frequent use in practice. Different types of substrate and additives were added in different concentrations to the waters to evaluate the effect on the flow cytometric fingerprints.

2 Materials and methods

Experimental design

Surface water from a river in Belgium (Coupure, Gent) containing 8.19 ± 0.005 mg TOC per L was collected for the experiments. For a first experiment, a dilution series up to 1:1000 of the surface water was prepared in 0.22 μm-filtered autoclaved Milli-Q water (Merck, Germany). Immediately thereafter, samples were taken for flow cytometry. Subsequently, nutrients were added as a complex substrate or a defined substrate in concentrations resulting in a carbon concentration approximately ten times higher than the measured carbon concentration in the water to ensure growth. Yeast extract (YE, Carl Roth, Belgium) was used as complex substrate and was added at a concentration of 159 mg L^{-1} which is equivalent to 81 mg L^{-1} TOC according to our measurements. The defined substrate was prepared as a mixture of acetate (C), NH_4Cl (N), and K_2HPO_4 (P) with a controlled molar C:N:P ratio of 20:5:1 of which was also added at a concentration of 81 mg L^{-1} TOC according to our measurements. Blank samples and samples with the different types of substrate were incubated for 24 hours at 28 °C at 100 rpm. All samples were prepared as biological triplicates. After 24 hours, all samples were analyzed with flow cytometry.

For a second experiment, undiluted surface water (8.19 ± 0.005 mg TOC per L), tap water (1.56 ± 0.010 mg TOC per L) and demineralized water (148 ± 4.51 μg TOC per L) were aliquoted and both the complex and the defined substrate were added to different samples. For surface water and tap water, acetate, NH_4Cl, and K_2HPO_4 were also added individually to different samples to determine the contribution of each nutrient. For each condition several concentrations were tested, relative to the measured TOC concentration of the water type. Samples to which no substrate was added were considered as blanks. All samples were prepared as triplicates. An overview of the relative increase of each nutrient for the different types of water is given in Table 1. Tap water samples and demineralized water samples were prepared in AOC-free borosilicate glass vials of 40 mL (35 mL total sample volume) with screw caps containing PTFE-faced liner[27] to avoid TOC cross contamination (TraceClean TOC

This journal is © The Royal Society of Chemistry 2019

Environ. Sci.: Water Res. Technol., 2019, 5, 1672–1682 | 1673

Table 1 The concentrations of all nutrients added to the batch experiment expressed in mg L^{-1}. The concentrations were based on the initial TOC concentration of the different water types. Solutions with a relative concentration of 1/10, 1/2, 1, 2, and 10 of the initial TOC concentration were prepared individually. The individual nutrients were added in the same concentration as in the defined substrate for surface water and tap water

Surface water

Complex substrate	Defined substrate	Individual nutrients			TOC concentration relative to the water sample
		Carbon	Nitrogen	Phosphorus	
mg L^{-1} TOC	mg L^{-1} TOC	mg L^{-1} TOC	mg L^{-1} N	mg L^{-1} P	
0.81	0.80	0.80	0.07	0.03	1/10
4.04	4.06	4.04	0.34	0.15	1/2
8.09	8.15	8.11	0.68	0.30	1
16.18	16.29	16.22	1.37	0.60	2
74.01	81.46	81.08	6.83	2.98	10

Tap water

Complex substrate	Defined substrate	Individual nutrients			TOC concentration relative to the water sample
		Carbon	Nitrogen	Phosphorus	
mg L^{-1} TOC	mg L^{-1} TOC	mg L^{-1} TOC	mg L^{-1} N^{-1}	mg L^{-1} P	
0.16	0.16	0.16	0.01	0.01	1/10
0.78	0.78	0.78	0.07	0.03	1/2
1.55	1.56	1.55	0.13	0.06	1
3.11	3.12	3.10	0.26	0.11	2
15.53	15.58	15.51	1.31	0.57	10

Demineralized water

Complex substrate	Defined substrate	TOC concentration relative to the water sample
µg L^{-1} TOC	µg L^{-1} TOC	
14.8	14.8	1/10
74	74	1/2
148	148	1
296	296	2
1480	1480	10

free vial, VWR, Belgium). Surface water samples were prepared in sterile 6-well plates with a total sample volume of 5 mL. Samples were incubated at 28 °C at approximately 100 rpm. Samples were taken every 12 to 24 hours.

In a third experimental setup, a biocide and a corrosion inhibitor were added to demineralized water in different concentrations, based on the used concentration in industry. A biocide containing quaternary ammonium salts (F-7050, Kurita, Germany) and a corrosion inhibitor containing phosphonates (S-1035, Kurita, Germany) were used. Dilution factors of 1 : 1000, 1 : 500, 1 : 100, 1 : 50, and 1 : 10 were used for both products. The recommended dilution factor is 1 : 100 for both. Samples were prepared and incubated at 28 °C at 125 rpm for 144 hours and bacterial regrowth was followed up through sampling every 24 hours.

TOC analysis

TOC analysis was performed using a Sievers 900 Portable TOC Analyzer (GE Analytical Instruments, Belgium). Preceding the TOC analysis, inorganic carbon (IC) was removed on a Sievers 900 Inorganic Carbon Remover (ICR) (GE Analytical Instruments, Belgium). Borosilicate glass vials of 40 mL with screw caps containing PTFE-faced liner were used for analysis (TraceClean TOC free vial, VWR, Belgium). Glassware was

prepared according to Hammes and Egli[27] in order to be free of any assimilable organic carbon (AOC) that may interfere. Samples were diluted to a range from 10 to 5000 ppb TOC and analyzed on the TOC analyzer. Samples were analyzed in quadruplicate.

Staining protocol and flow cytometry

An optimized staining protocol was used from Van Nevel et al. (2013).[28] Bacteria were stained with 10 µL mL^{-1} of SYBR Green I (SG, Invitrogen, Belgium, 100× diluted in DMSO from stock) for total cell counting. The samples were then incubated for 13 minutes at 37 °C. Samples of the first experiment were analyzed on a FACSVerse cytometer (BD Biosciences, Belgium). The blue laser (488 nm, 20 mW) was used for the excitation of the stains. Two optical filters were used for green fluorescence emission (527/32 nm) and for the red fluorescence emission (700/54 nm). Samples of the other experiments were analyzed on an Accuri C6+ (BD Biosciences, Belgium) with a blue (488 nm, 20 mW) and red (640 nm, 14.7 mW) laser. Standard optical filters were used and included FL-1 (530/30 nm), FL-2 (585/40 nm) and FL-3 (670 LP) for the blue laser and FL-4 (675/25 nm) for the red laser. The performance of both instruments was monitored daily with their corresponding CS&T beads (BD Biosciences, Belgium).

Data analysis

All data was extracted from the proprietary Accuri C6+ CSampler software or FACSuite software in the flow cytometry standard (FCS 3.0) format and subsequently imported into R v3.4.0 (ref. 29) through the functionality offered by the flowCore package v1.42.2.[30] Data was first log transformed and then normalized by dividing all values by the maximal green fluorescence intensity. No compensation was applied. Sample quality was assessed visually by looking at the signal stability in function of time. Samples with unstable signals were removed prior further analysis. Gating to reduce the background was performed in R studio using the flowCore package. A 0.22 μm-filtered control was used to determine the position of the background and a universal gate was constructed to remove as much background as possible. Next, a single-step discretization ('binning') and Gaussian bivariate density estimation was performed on the selected parameters (green and red fluorescence, FSC, and SSC) using the KernSmooth package v2.23.15.[31] An equally spaced grid (binning grid) of 128 × 128 was fixed for each bivariate density estimation using the flowFDA package v1.0. All bivariate density estimations were concatenated to a one-dimensional feature vector which we refer to as the fingerprint. Subsequently, cytometric fingerprinting indices were calculated according to the publication of Props, Monsieurs[25] where Hill number diversity indices are applied to describe the cytometric diversity within and between samples. Three indices are used; D_0, D_1, and D_2. The difference between the indices is the weight given to the most abundant conceptual phenotypes. D_0 does not account for the relative abundance of these conceptual phenotypes while D_2 gives the most weight to the most abundant conceptual phenotypes. Alternatively, the dissimilarity between the flow cytometric fingerprints can be visualized with principal coordinate analysis (PCoA) based on the quantitative Jaccard distance measure (Ružička index) as implemented in the function vegdist from the vegan package v2.5.2.[32] When relevant, ANOSIM analysis was used to determine the dissimilarity between the groups with the anosim function from the vegan package v2.5.2.

3 Results

3.1 Surface water

Results of the first experiment with surface water incubated with two different types of substrate are presented in Fig. 1. Regrowth was the highest for samples incubated with a complex substrate and was higher for less diluted samples. Samples incubated with the defined substrate also showed regrowth but to lesser extent, with a comparable final bacterial concentration for all dilutions after 24 hours. Furthermore, the control samples where no substrate was added, showed comparable or even more regrowth than samples incubated with defined substrate. A more in-depth analysis of the cytometric data by fingerprinting shows that the combination of cell density and flow cytometric diversity index D_2 exhibits a clear distinction between the different types of samples (Fig. 2). The results confirm that the cytometric diversity of all samples changed after incubation, and that the change was the strongest for samples incubated with a complex substrate. Samples incubated with a defined substrate could also be distinguished from the other conditions. The level of dilution affected the cytometric diversity D_2 and how D_2 changed after 24 hours of incubation, although discrimination among dilutions was less clear than before incubation.

Differences between cytometric fingerprints of all samples were visualized by principal coordinates analysis (PCoA) and show that the cytometric fingerprint of all samples changed after 24 hours of incubation (Fig. 3). The separation between
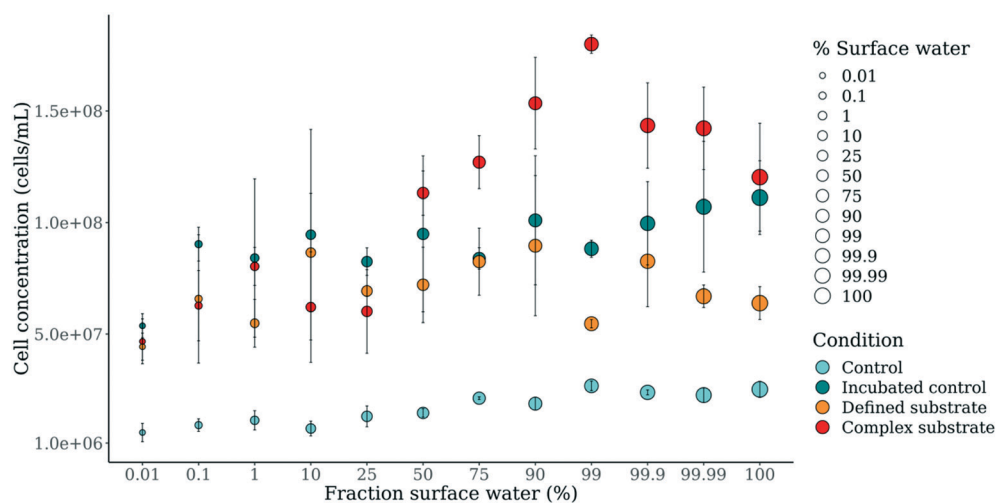

Fig. 1 Average bacterial concentration and the standard deviation in function of the degree of dilution (% surface water, n = 3). Diluted surface water was incubated with a complex substrate or a defined substrate with a C : N : P molar ratio of 20 : 5 : 1. Controls to which no substrate was added are shown before and after incubation. Microbial regrowth can be noticed in all samples but is strongest for samples incubated with the complex substrate.
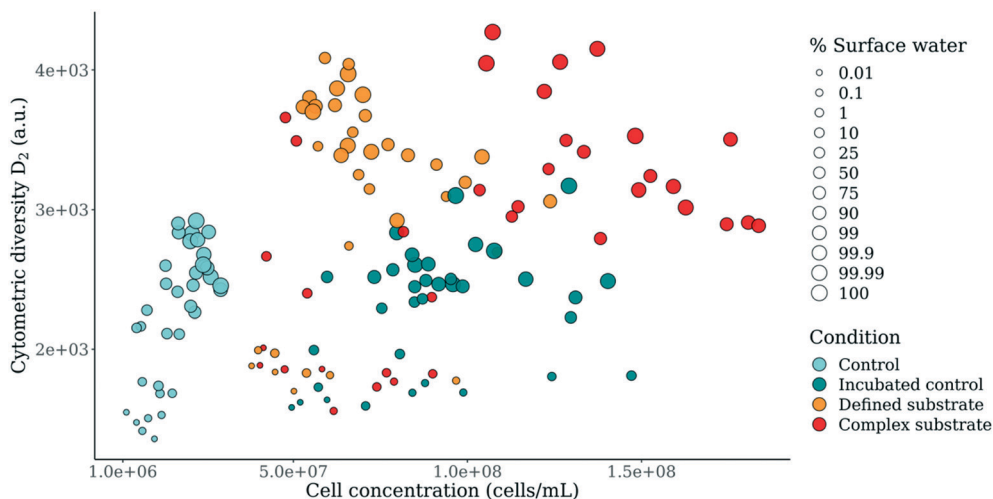
Fig. 2 Flow cytometric diversity index D_2 in function of the cell concentration of all samples (in cells per mL). All samples are were prepared as biological triplicates and are shown individually. The marker size indicates the degree of dilution (% surface water). A distinction is visible between samples incubated with a complex substrate and a defined substrate. The control samples changed after incubation though to a lesser extent than when substrate was added. The effect of dilution is only clear for the control samples prior incubation.

the fingerprints according to the four conditions was confirmed by ANOSIM (R = 0.529, p = 0.001). The cytometric fingerprints of blank samples changed to a lesser extent than when substrate was added, although no distinction between substrate type was observed. On the other hand, the level of dilution clearly affected the cytometric fingerprints before dilution and their change after 24 hours of incubation. Cytometric fingerprints of more diluted samples showed less change after incubation. For undiluted samples, temporal data was also acquired and all samples, including the blanks, showed regrowth but samples where carbon was supplied as a complex substrate (yeast extract) or as defined substrate (acetate) showed the clearest regrowth. Again the complex substrate sustained more and faster growth than the defined

substrate. Samples spiked with high concentrations of defined substrate showed a growth delay when compared to the lower concentrations. Samples spiked with only phosphorous showed a reverse effect of concentration, as lower concentrations sustained more regrowth. Flow cytometric diversity indices changed for all samples relative to the baseline changes of the blank water, although it is lacking a general or substrate-specific trend (Fig. S1†).

3.2 Drinking water

As tap water is frequently used in diverse industrial applications, experiments were also performed on this water type. After one day of incubation, bacterial regrowth was visible for
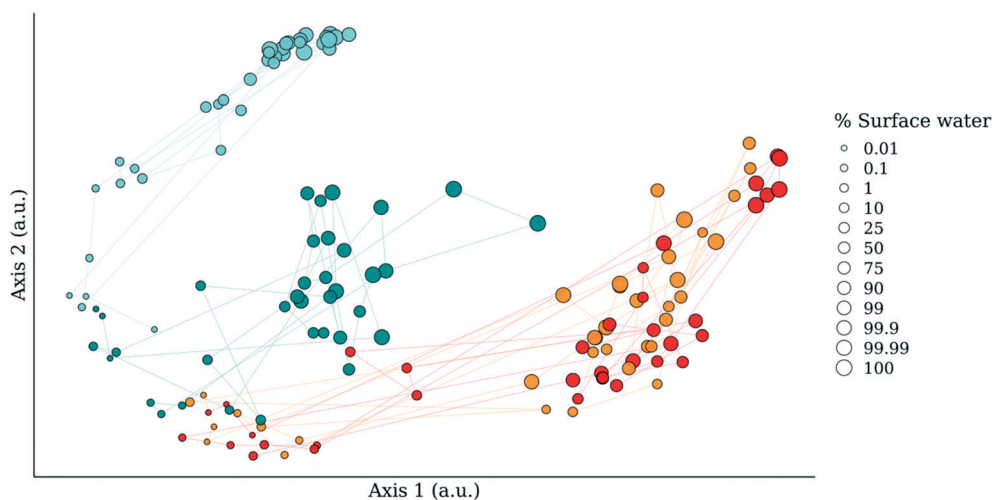

Fig. 3 PCoA ordination of the cytometric fingerprints of all samples. All samples were prepared as biological triplicates and are shown individually. The marker size indicates the degree of dilution (% surface water). A distinction is visible between samples before and after incubation. Also, incubated blank samples are distinguished from samples incubated with a complex or defined substrate. The cytometric fingerprint of control samples changed to a lesser extent than when substrate was added. The effect of dilution is visible for all conditions.

samples containing high complex substrate concentrations, and after two days for the other samples spiked with the complex or defined substrate (Fig. 4). For samples enriched with a sole P-source, growth was visible after three days of incubation at 28 °C. Growth was limited for blank samples and for samples with carbon or nitrogen source addition. The normalized cytometric diversity D_2 fluctuated greatly compared to the respective surface water samples. D_2 of tap water samples fluctuated similarly over time for all conditions. Even in samples with limited growth (C, N), changes in D_2 were observed. A similarity based fingerprinting approach on the other hand showed no clear distinction between the different conditions at any time point unlike surface water (data not shown).

3.3 Demineralized water

Higher nutrient concentrations yielded stronger regrowth but no difference between the complex and the defined substrate could be noticed based on the cell concentration (Fig. S2†). The normalized cytometric diversity index D_2 in function of time showed important deviations during the first two days of the experiment. This is in contrast with the cell concentration, where measurable regrowth was only observed after two days. No effect of the substrate concentration on the cytometric diversity was observed (Fig. S2†). The addition of biocides to demineralized water prevented growth in general, except for the lowest biocide concentration that induced regrowth (Fig. S3†). The lowest biocide concentration was ten times lower than the industrially required concentration (100 μL L^{-1}). The corrosion inhibitor also induced some regrowth, which was higher when low concentrations of corrosion inhibitor were added. For the highest concentration of corrosion inhibitor, heavy background fluorescence was observed and cell concentrations are therefore less reliable. A comparison between the blanks and the water to which biocide or corrosion inhibitor was added reveals that both products, but especially the corrosion inhibitor have a strong impact on the cytometric diversity index D_2 (Fig. S3†). The effect of both the biocide and the corrosion inhibitor could not be related to the product concentration although a higher concentration of biocide showed to induce less differences in the cytometric diversity. An overall comparison of the samples was done by using the similarity between fingerprints as an alternative for the diversity indices and could provide complementary information. A principal coordinate analysis (PCoA) shows that all fingerprints changed over time (Fig. 5). No clear distinction can be made between the different treatments or concentrations, except for the samples that contained high concentrations of corrosion inhibitor.

4 Discussion

Nutrients are the most important factor inducing microbial regrowth and more specifically the presence of the elements C, N, and P are vital for microbial life. By adding nutrients to microbial communities in several types of water we attempted to simulate changing process conditions and to determine the effect of these changes on the dynamics in the flow cytometry data and cytometric diversity indices by using our in-house fingerprinting algorithm.[25,33] Regrowth of the microbial community in the surface water was partially due to nutrients already present in the water and the favorable incubation conditions as also blank samples showed regrowth (Fig. 1). The addition of the defined substrate did not induce more regrowth than the control samples although both added substrates contained the same concentration of TOC. Certain elements or compounds (e.g. sulfur, magnesium) may lack in the defined substrate as compared to the defined substrate. Also the type of carbon, nitrogen, or phosphorus sources can be an explanation as not all molecules promote optimal growth for all species present.[34] An in-depth comparison of the cytometric diversity index D_2 between different dilutions shows that D_2 is generally lower for more diluted samples (Fig. 2) because the cytometric index D_2 is mainly determined by the most abundant conceptual phenotypes as defined by the gating strategy in the fingerprinting algorithm. By diluting the surface water, the microbial community of the most diluted samples was reduced to the most abundant species which resulted in a taxonomically less rich community and subsequently in less conceptual phenotypes in the fingerprints. After incubation, the cytometric index D_2 increased together with the cell concentration for all samples, yet a distinction could still be made between the more and less diluted samples because the community richness could not increase. This also suggests that different microbial communities react differently to the changed environmental factors. A comparison between both cell density, fingerprinting with cytometric diversity indices, and fingerprinting based on the similarity of the fingerprints (Fig. 1–3) shows that the combination of cell concentration and cytometric fingerprinting indices is informative to discern differences between samples.[26]

The different types of substrates were tested individually in different concentrations to estimate how sensitive the flow cytometric fingerprinting method was to detect specific environmental changes. Microbial regrowth was observed to be higher for higher substrate concentrations as expected but samples spiked with a defined substrate at low concentrations (<8.15 mg L^{-1} TOC addition) showed faster regrowth than samples spiked with higher concentrations of defined substrate. This may be attributed to a "nutrient shock" effect of the substrate on the microbial community, i.e. that the microbial community needs time to adapt to relatively high nutrient concentrations in their environment.[35] For samples with sole phosphate addition, regrowth occurred inversely proportional to the added concentration. During the experiment, white flocks were formed in high-phosphate containing samples. These may have been phosphate, which precipitated with the available calcium thus rendering the phosphorous unavailable for microbial growth which could explain the inversed proportional effect.[36,37] The lowest concentration of complex substrate or acetate increased the TOC

This journal is © The Royal Society of Chemistry 2019

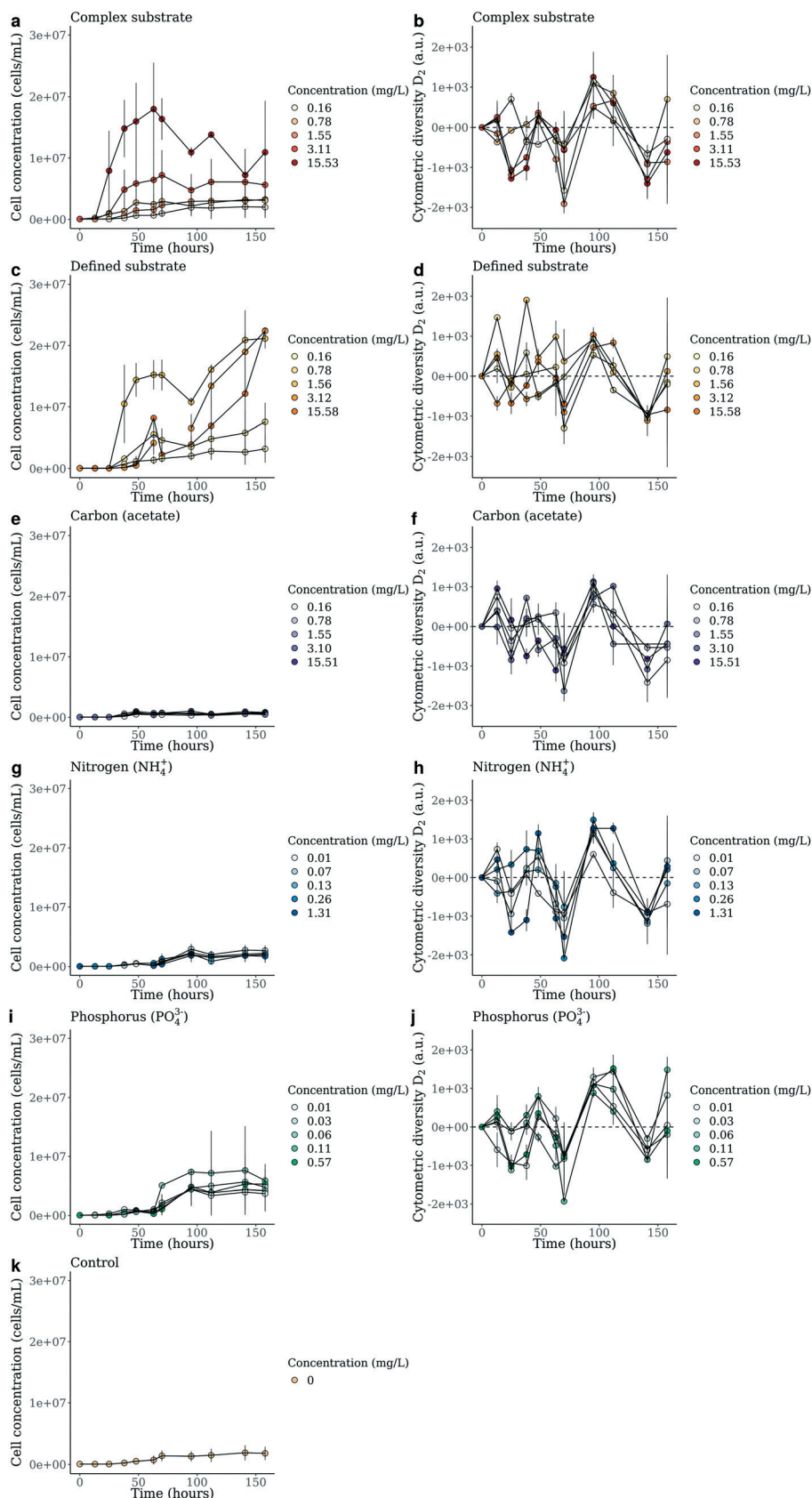Environ. Sci.: Water Res. Technol., 2019, 5, 1672–1682 | 1677

Fig. 4 Average cell concentration (cells per mL) (a, c, e, g, i, and k) and average flow cytometric indices D_2 (b, d, f, h, and j) of tap water, with their respective standard deviation in function of time for each substrate and concentration ($n = 3$). The cytometric diversity indices were normalized by subtracting the cytometric diversity indices of the blank samples. The dashed line represents the blank samples as reference. After 95 hours, measurements were performed on another instrument of the same brand and model.
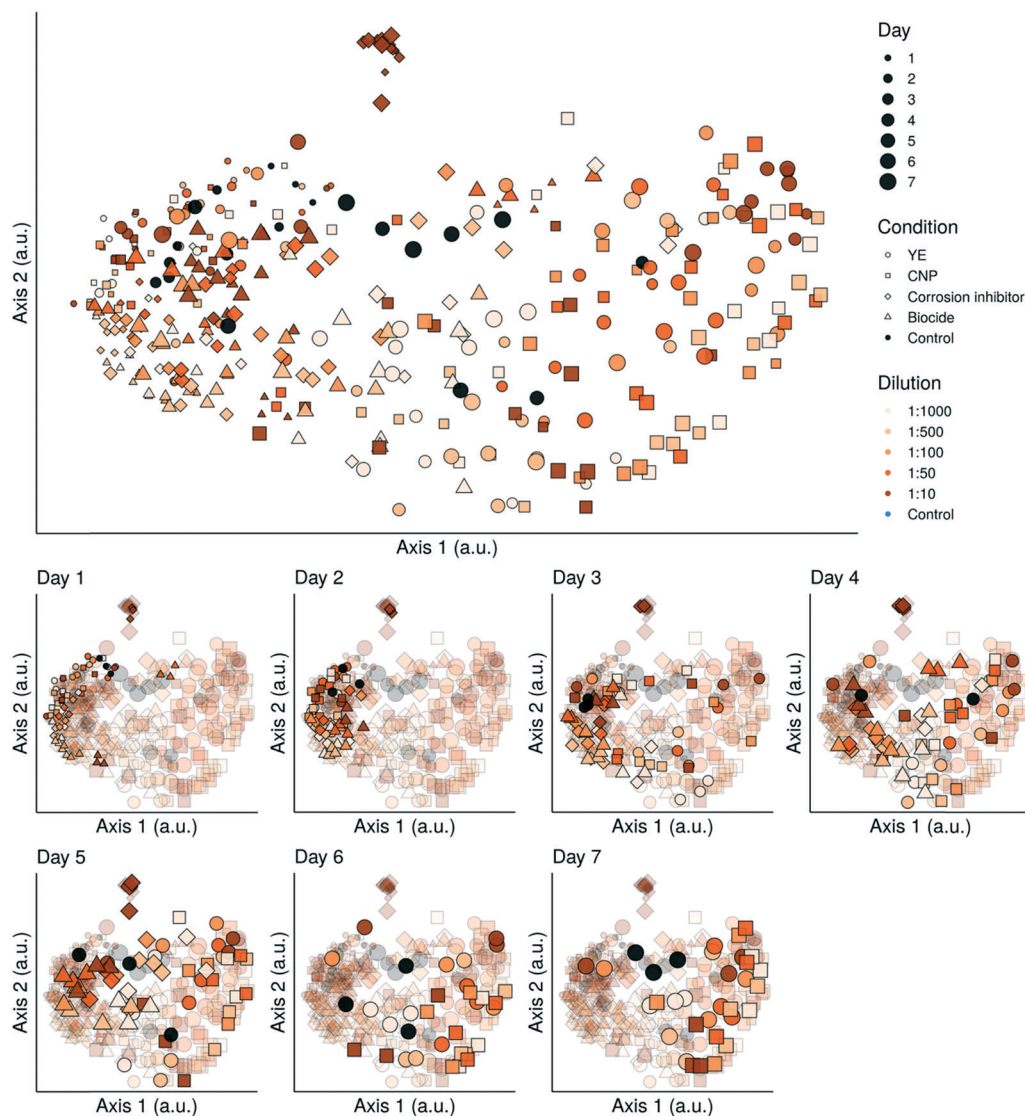
Fig. 5 Principal coordinates analysis (PCoA) of demineralized water samples incubated with a complex substrate, a defined substrate, biocide, and corrosion inhibitor. All samples were prepared as biological triplicates and are shown individually. The concentration of the added compound is visualized in different colors, the effect of time by the size of the shapes. Separate plots were made for each measured time point with the samples of that time point highlighting against all other samples.

concentration with 0.8 mg L^{-1} in water which already contained 8.15 ± 0.005 mg L^{-1} TOC. This shows that even small increases in TOC concentrations (±10%) can lead to microbial growth in a period of 24 hours and that flow cytometry can be used to detect microbial regrowth after minor organic contamination of a water sample, which makes it useful for water treatment monitoring.

Tap water is frequently used in industrial applications but differs markedly from surface water in terms of its physical–chemical properties, and as a consequence, its microbial community.[38] An experimental design similar to that for surface water shows that the bacterial community of tap water reacted slower than the microbial community of surface water to the addition of proportionally identical amount of the same nutrients (Fig. 4). As the tap water matrix is more oligotrophic than surface water, regrowth in tap water was influenced to a bigger extent by the type of added nutrients and their concentration. The addition of individual nutrients induced no regrowth apart from phosphorous which suggests that phosphorus was a nutrient limiting factor in this water. This could be expected as this has been demonstrated before in drinking water.[39,40] Consequently, the addition of a combination of nutrients is required for bacterial regrowth in tap water.[41] The cytometric diversity (D_2) in function of time showed more fluctuations over the course of the experiment as compared to surface water. Based on the findings of Props, Monsieurs,[25] Koch and Müller,[42] and Prest, El-Chakhtoura[43] changes in the cytometric fingerprints would suggest a change in the composition of the microbial communities. Assuming that the microbial community composition changed with similar dynamics, this would indicate that the communities reacted in a deterministic manner

This journal is © The Royal Society of Chemistry 2019

Environ. Sci.: Water Res. Technol., 2019, **5**, 1672–1682 | **1679**

regardless of the different substrates added albeit differently for both types of water as the microbial community in surface water showed to remain more stable in comparison to the microbial community in the tap water. This observation can be explained by the hypothesis that deterministic mechanisms of community structure are found in communities that are shaped by selective parameters such as the addition of substrate.[44–48] The noticeable effect of deterministic mechanisms however does not necessarily result in community stability as can be seen in our results as well.[44]

In many applications high quality demineralized water is preferred. Due to substantial treatment, demineralized water is chemically and biologically different than the surface water it often originates from. Despite the low concentration of nutrients, demineralized water contains bacteria that can cause problems by fast regrowth as consequence of contaminations with additional bacteria or nutrients.[6,49–51] To maintain the water quality and to mitigate corrosion of the treated water, biocides and corrosion inhibitors are added, especially in recirculating water circuits. Our results confirm that bacteria are present in fresh demineralized water and that the addition of a carbon source induces growth (Fig. S2†). While regrowth in surface water was observed after a day and in tap water after two days, regrowth in demineralized water was only observed after three days which demonstrates that the microbial communities reacted slower in more oligotrophic environments. This is either a consequence of the low initial concentration of bacteria or an increased lag time as consequence of the changed environment or a combination of both elements. In practice, demineralized water is often buffered for multiple days which may allow microbial regrowth, even when only very small amount of nutrients are available. The addition of corrosion inhibitor and a suboptimal concentration of biocide also induced some regrowth (Fig. S3†) which shows that incorrect dosing of biocides or the use of additives may worsen microbiology related problems.[52] The addition of biocide and especially the corrosion inhibitor also influenced the flow cytometric fingerprinting indices immediately after addition which suggest an effect on the fingerprints or on the physiology of the microbial community (Fig. S3†).[53] The effect induced by the corrosion inhibitor can be attributed to the observed increase in background fluorescence and illustrates that also non-biological contaminations could be observed directly with flow cytometry though only in a qualitative way. These findings were confirmed with a PCoA analysis of all samples (Fig. 5). Samples with high concentrations of corrosion inhibitor showed to be markedly different from the other samples while all other samples changed in function of time. These observations, in combination with the observations regarding the effect of different nutrients on surface water, show that cytometric fingerprints of the aquatic microbial communities are influenced by the type and concentrations of chemicals added. This suggests that cytometric fingerprints can provide indirect information about the changed environmental conditions. However, it is also clear that not all chemicals can be distinguished from one another and that our findings are different for each type of water.

5 Conclusions

By exposing the aquatic microbiota of different types of industrially relevant water to several types of substrates and additives with different concentrations, we assessed how cytometric fingerprints changed in response to experimental manipulations. We found that the microbial community in surface water reacted quickly to the addition of nutrients but also that the fingerprints were different for different substrates. The aquatic bacteria in tap water and demineralized water reacted much slower to the addition of nutrients but the environmental change could be observed with flow cytometric fingerprinting before regrowth was measured. Our findings showed that cytometric fingerprinting can be a telltale of water quality changes regardless of total cell counts, particularly when these changes affect microbial community physiology or composition.

Contributions

B. B., N. B., and B. D. G. conceived the experimental design, J. F., L. V., V. B., and A. N. performed the experiments and all measurements, B. B. analyzed the data and B. B. and J. F. wrote the manuscript in consultation with N. B and B. D. G.

Conflicts of interest

The authors declare that there was no conflict of interests.

Acknowledgements

References

1 A. N. Angelakis, T. Asano, A. Bahri, B. E. Jimenez and G. Tchobanoglous, Water Reuse: From Ancient to Modern Times and the Future, *Front. Environ. Sci.*, 2018, **6**, 26.

2 T. R. Bott, *Fouling in heat exchangers*, Elservier Science B.V., Amsterdam, The Netherlands, 1995.

3 T. J. Dondero, R. C. Rendtorff, G. F. Mallison, R. M. Weeks, J. S. Levy and E. W. Wong, *et al.* An Outbreak of Legionnaires' Disease Associated with a Contaminated Air-Conditioning Cooling Tower, *N. Engl. J. Med.*, 1980, **302**(7), 365–370.

4   D. W. Keller, R. Hajjeh, A. DeMaria, B. S. Fields, J. M. Pruckler and R. S. Benson, *et al.* Community outbreak of legionnaires' disease: An investigation confirming the potential for cooling towers to transmit Legionella species, *Clin. Infect. Dis.*, 1996, **22**(2), 257–261.

5   K. P. Meesters, J. W. Van Groenestijn and J. Gerritse, Biofouling reduction in recirculating cooling systems through biofiltration of process water, *Water Res.*, 2003, **37**(3), 525–532.

6   S. B. S. Ghayeni, P. J. Beatson, A. J. Fane and R. P. Schneider, Bacterial passage through microfiltration membranes in wastewater applications, *J. Membr. Sci.*, 1999, **153**(1), 71–82.

7   D. Hou, Z. Huang, S. Zeng, J. Liu, D. Wei and X. Deng, *et al.* Environmental Factors Shape Water Microbial Community Structure and Function in Shrimp Cultural Enclosure Ecosystems, *Front. Microbiol.*, 2017, **8**, 2359.

8   R. E. Carlson, A trophic state index for lakes, *Limnol. Oceanogr.*, 1977, **22**(2), 361–369.

9   J. A. Fuhrman, J. A. Cram and D. M. Needham, Marine microbial community dynamics and their ecological interpretation, *Nat. Rev. Microbiol.*, 2015, **13**, 133.

10 W. Köster, T. Egli, N. Ashbolt, K. Botzenhart, N. Burlion and T. Endo, *et al.* Analytical methods for microbiological water quality testing, *Assessing microbial safety of drinking water*, 2003, p. 237.

11 K. De Roy, L. Clement, O. Thas, Y. Wang and N. Boon, Flow cytometry for fast microbial community fingerprinting, *Water Res.*, 2012, **46**(3), 907–919.

12 F. Hammes, M. Berney, Y. Y. Wang, M. Vital, O. Koster and T. Egli, Flow-cytometric total bacterial cell counts as a descriptive microbiological parameter for drinking water treatment processes, *Water Res.*, 2008, **42**(1–2), 269–277.

13 A. Carducci, M. Verani and R. Battistini, Legionella in industrial cooling towers: monitoring and control strategies, *Lett. Appl. Microbiol.*, 2010, **50**(1), 24–29.

14 M. Tachikawa, M. Tezuka, M. Morita, K. Isogai and S. Okada, Evaluation of some halogen biocides using a microbial biofilm system, *Water Res.*, 2005, **39**(17), 4126–4132.

15 H. A. Videla and L. K. Herrera, Microbiologically influenced corrosion: looking to the future, *Int. Microbiol.*, 2005, **8**(3), 169–180.

16 L. Fiksdall and I. Tryland, Application of rapid enzyme assay techniques for monitoring of microbial water quality, *Curr. Opin. Biotechnol.*, 2008, **19**(3), 289–294.

17 C. A. Stedmon, B. Seredynska-Sobecka, R. Boe-Hansen, N. Le Tallec, C. K. Waul and E. Arvin, A potential approach for monitoring drinking water quality from groundwater systems using organic matter fluorescence as an early warning for contamination events, *Water Res.*, 2011, **45**(18), 6030–6038.

18 F. Hammes, M. Berney and T. Egli, Cultivation-independent assessment of bacterial viability, *Adv. Biochem. Eng./Biotechnol.*, 2011, **124**, 123–150.

19 B. De Graaf, F. Williamson, M. K. Koerkamp, J. Verhoef, R. Wuestman and B. Bajema, *et al.* Implementation of an innovative sensor technology for effective online water quality monitoring in the distribution network, *Water Practice and Technology.*, 2012, 7(4), wpt2012099.

20 B. Højris, S. C. B. Christensen, H.-J. Albrechtsen, C. Smith and M. Dahlqvist, A novel, optical, on-line bacteria sensor for monitoring drinking water quality, *Sci. Rep.*, 2016, **6**, 23935.

21 F. Hammes and T. Egli, Cytometric methods for measuring bacteria in water: advantages, pitfalls and applications, *Anal. Bioanal. Chem.*, 2010, **397**(3), 1083–1095.

22 Y. Wang, F. Hammes, K. De Roy, W. Verstraete and N. Boon, Past, present and future applications of flow cytometry in aquatic microbiology, *Trends Biotechnol.*, 2010, **28**(8), 416–424.

23 E. I. Prest, F. Hammes, S. Kotzsch, M. C. van Loosdrecht and J. S. Vrouwenvelder, Monitoring microbiological changes in drinking water systems using a fast and reproducible flow cytometric method, *Water Res.*, 2013, **47**(19), 7131–7142.

24 C. Koch, F. Harnisch, U. Schroder and S. Muller, Cytometric fingerprints: evaluation of new tools for analyzing microbial community dynamics, *Front. Microbiol.*, 2014, **5**, 273.

25 R. Props, P. Monsieurs, M. Mysara, L. Clement and N. Boon, Measuring the biodiversity of microbial communities by flow cytometry, *Methods Ecol. Evol.*, 2016, 7, 1376–1385.

26 M. D. Besmer, J. A. Sigrist, R. Props, B. Buysschaert, G. N. Mao and N. Boon, *et al.* Laboratory-Scale Simulation and Real-Time Tracking of a Microbial Contamination Event and Subsequent Shock-Chlorination in Drinking Water, *Front. Microbiol.*, 2017, **8**, 1900.

27 F. A. Hammes and T. Egli, New Method for Assimilable Organic Carbon Determination Using Flow-Cytometric Enumeration and a Natural Microbial Consortium as Inoculum, *Environ. Sci. Technol.*, 2005, **39**(9), 3289–3294.

28 S. Van Nevel, S. Kotzsch, H. U. Weilenmann, N. Boon and F. Hammes, Routine bacterial analysis with automated flow cytometry, *J. Microbiol. Methods*, 2013, **94**(2), 73–76.

29 R Core Team, *R: A language and environment for statistical computing*, R foundation for Statistical Computing, Vienna, Austria, 2015.

30 B. Ellis, P. Haaland, F. Hahne, N. Le Meur, N. Gopalakrishnan and J. Spidlen, *et al.* flowCore: Basic structures for flow cytometry data. r package version 13410, *R package version 1.34.10 ed: Bioconductor*, 2017.

31 M. Wand, KernSmooth: functions for kernel smoothing supporting Wand & Jones (1995), *R package version 2.23-15 ed*, 2015.

32 J. Oksanen, F. G. Blanchet, R. Kindt, P. Legendre, P. R. Minchin and R. B. O'Hara, *et al.* vegan: Community Ecology Package, *R package version 2.3-4 ed*, 2016.

33 B. Buysschaert, F. M. Kerckhof, P. Vandamme, B. De Baets and N. Boon, Flow cytometric fingerprinting for microbial strain discrimination and physiological characterization, *Cytometry, Part A*, 2017, **93**(2), 201–212.

34 B. Wawrik, L. Kerkhof, J. Kukor and G. Zylstra, Effect of different carbon sources on community composition of

This journal is © The Royal Society of Chemistry 2019

Environ. Sci.: Water Res. Technol., 2019, **5**, 1672–1682 | 1681

bacterial enrichments from soil, *Appl. Environ. Microbiol.*, 2005, **71**(11), 6776–6783.

35 N. F. Azevedo, S. M. Bragança, L. C. Simões, L. Cerqueira, C. Almeida and C. W. Keevil, *et al.* Proposal for a method to estimate nutrient shock effects in bacteria, *BMC Res. Notes*, 2012, **5**(1), 422.

36 W. A. House, The Physico-Chemical Conditions for the Precipitation of Phosphate with Calcium, *Environ. Technol.*, 1999, **20**(7), 727–733.

37 H. Carlsson, H. Aspegren, N. Lee and A. Hilmer, Calcium phosphate precipitation in biological phosphorus removal systems, *Water Res.*, 1997, **31**(5), 1047–1055.

38 E. Santofimia, E. González-Toril, E. López-Pamo, M. Gomariz, R. Amils and A. Aguilera, Microbial Diversity and Its Relationship to Physicochemical Characteristics of the Water in Two Extreme Acidic Pit Lakes from the Iberian Pyrite Belt (SW Spain), *PLoS One*, 2013, **8**(6), e66746.

39 D. L. Correll, Phosphorus: a rate limiting nutrient in surface waters, *Poult. Sci.*, 1999, **78**(5), 674–682.

40 I. T. Miettinen, T. Vartiainen and P. J. Martikainen, Phosphorus and bacterial growth in drinking water, *Appl. Environ. Microbiol.*, 1997, **63**(8), 3242–3245.

41 T. Egli, How to live at very low substrate concentration, *Water Res.*, 2010, **44**(17), 4826–4837.

42 C. Koch and S. Müller, Personalized microbiome dynamics – Cytometric fingerprints for routine diagnostics, *Mol. Aspects Med.*, 2018, **59**, 123–134.

43 E. I. Prest, J. El-Chakhtoura, F. Hammes, P. E. Saikaly, M. C. M. van Loosdrecht and J. S. Vrouwenvelder, Combining flow cytometry and 16S rRNA gene pyrosequencing: A promising approach for drinking water monitoring and characterization, *Water Res.*, 2014, **63**, 179–189.

44 Z. Liu, N. Cichocki, T. Hubschmann, C. Suring, I. D. Ofiteru and W. T. Sloan, *et al.* Neutral mechanisms and niche differentiation in steady-state insular microbial communities revealed by single cell analysis, *Environ. Microbiol.*, 2019, **21**(1), 164–181.

45 M. E. Hibbing, C. Fuqua, M. R. Parsek and S. B. Peterson, Bacterial competition: surviving and thriving in the microbial jungle, *Nat. Rev. Microbiol.*, 2010, **8**(1), 15–25.

46 M. Vellend, Conceptual synthesis in community ecology, *Q. Rev. Biol.*, 2010, **85**(2), 183–206.

47 D. R. Nemergut, S. K. Schmidt, T. Fukami, S. P. O'Neill, T. M. Bilinski and L. F. Stanish, *et al.* Patterns and processes of microbial community assembly, *Microbiol. Mol. Biol. Rev.*, 2013, **77**(3), 342–356.

48 K. Faust and J. Raes, Microbial interactions: from networks to models, *Nat. Rev. Microbiol.*, 2012, **10**(8), 538–550.

49 A. Rozej, A. Cydzik-Kwiatkowska, B. Kowalska and D. Kowalski, Structure and microbial diversity of biofilms on different pipe materials of a model drinking water distribution systems, *World J. Microbiol. Biotechnol.*, 2015, **31**(1), 37–47.

50 C. R. Proctor, M. Gachter, S. Kotzsch, F. Rolli, R. Sigrist and J. C. Walser, *et al.* Biofilms in shower hoses - choice of pipe material influences bacterial growth and communities, *Environ. Sci.: Water Res. Technol.*, 2016, **2**(4), 670–682.

51 L. A. Kulakov, M. B. McAlister, K. L. Ogden, M. J. Larkin and J. F. O'Hanlon, Analysis of bacteria contaminating ultrapure water in industrial systems, *Appl. Environ. Microbiol.*, 2002, **68**(4), 1548–1555.

52 D. M. Grant and T. R. Bott, Biocide Dosing Strategies for Biofilm Control, *Heat Transfer Eng.*, 2005, **26**(1), 44–50.

53 T. Song, M. Duperthuy and S. N. Wai, Sub-Optimal Treatment of Bacterial Biofilms, *Antibiotics*, 2016, **5**(2), 23.