A comparative study of three different assimilable organic carbon (AOC) methods: results of a round-robin test


ABSTRACT

Easily assimilable organic carbon (AOC) is frequently used for the assessment of the biological stability of drinking water, which is an important consideration in the control of bacterial growth in distribution networks. The first AOC bioassay was developed in 1982 and is based on growth of two bacterial strains (Pseudomonas fluorescens P17 and Spirillum spp. NOX) in drinking water relative to their growth on acetate. Much research was subsequently conducted to modify, simplify and increase the speed of the assay which resulted in a number of alternative AOC assays. Application of these assays raises legitimate questions about the comparison of AOC data from different studies. In the present study, a round-robin test was performed to evaluate the correlation between three established AOC methods. A total of 14 water samples, covering a wide range of AOC concentrations, were analyzed with the original ‘van der Kooij’ method, the ‘Werner & Hambsch’ method and ‘Eawag’ method. Good correlations were found between AOC concentrations measured with the various methods. The data suggest an acceptable compatibility between different AOC methods, although deviations between the methods call for careful interpretation and reporting of AOC data.

Key words | assimilable organic carbon (AOC), biological stability, drinking water, growth potential, round robin

INTRODUCTION

Production of biologically stable drinking water is important to control bacterial growth and the deterioration of drinking water quality in distribution systems that are either not chlorinated or chlorinated at low doses. Microbial growth requires carbon (C), nitrogen (N) and phosphorus (P) in a typical ratio of 100:10:1 (C:N:P), and therefore it is assumed that in most waters organic carbon is the growth limiting nutrient for bacteria (Escobar et al. 2001b; Liu et al. 2002). The readily available carbon for cell growth is also referred to as easily assimilable organic carbon (AOC). The first assay for AOC determination was developed by van der Kooij et al. (1982) and was based on the growth of Pseudomonas fluorescens strain P17 on endogenous substrate in a sample. In this assay, the AOC concentration, expressed as ‘acetate-carbon equivalents’, is calculated from the maximum concentration of P. fluorescens strain P17 and the growth yield of this bacterium on sodium acetate as a sole carbon source. Pseudomonas fluorescens strain P17 is capable of utilizing many of the easily biodegradable compounds: amino acids, carboxylic acids, hydrocarboxylic acids, alcohols and carbohydrates (excluding polysaccharides) (Stanier et al. 1966). However, some compounds, such as oxalic acid, which is produced during ozonation, cannot be degraded by P17, and therefore Spirillum spp. strain NOX was later added to the assay (van der Kooij & Hijnen 1984). Since the development of the first AOC bioassay, much research has been conducted to modify, simplify and increase the speed of the assay. A schematic overview of the important steps of an AOC assay and some of the proposed modifications to the ‘van der Kooij’ method by different studies is given in Table 1.

The essential modifications concern sample pretreatment, type of inoculum, growth measurement,
Table 1 | Overview modifications made to the original AOC method by different researchers

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</thead>
<tbody>
<tr>
<td>Sample preparation</td>
<td>Pasteurization at 60 °C for 0.5 hours</td>
<td>Filter sterilization</td>
<td>Filter sterilization</td>
<td>Filter sterilization</td>
<td>Pasteurization at 70 °C for 0.5 hours</td>
<td>Pasteurization at 70 °C for 0.5 hours</td>
<td>N/C**</td>
<td>Filter sterilization</td>
</tr>
<tr>
<td>Inoculation</td>
<td>2 known strains, PI7 and NOX</td>
<td>Inoculum retrieved from filter</td>
<td>Inoculum retrieved from filter</td>
<td>4 species; <em>Pseudomonas fluorescens</em>, <em>Curtobacterium</em> sp., <em>Corynebacterium</em> sp., Unidentified coryneform</td>
<td>N/C</td>
<td>N/C</td>
<td>Bioluminescent derivatives of AOC test bacteria; PI 715, NOX 13</td>
<td>Natural inoculum</td>
</tr>
<tr>
<td>Incubation</td>
<td>15 °C for 5–25 days</td>
<td>20 °C for 3–5 days</td>
<td>22 °C</td>
<td>20 °C for 6 days</td>
<td>N/C</td>
<td>22 °C for 2–4 days</td>
<td>N/C</td>
<td>30 °C for 3–4 days</td>
</tr>
<tr>
<td>Growth measurement</td>
<td>Plating</td>
<td>Turbidity at 12 °C forward scattering</td>
<td>ATP</td>
<td>N/C</td>
<td>N/C</td>
<td>ATP</td>
<td>ATP luminescence</td>
<td>Flow cytometry TCC</td>
</tr>
<tr>
<td>Conversion/ yield values</td>
<td>4.6 × 10^6 P17, 1.2 × 10^7 NOX</td>
<td>2.3 ppm per 1 mg/L Acetate – C</td>
<td>N/A*</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>1 × 10^7 cells</td>
</tr>
</tbody>
</table>

*N/A indicates that the information is not available.
**N/C indicates that no changes were made in the method compared with the ‘van der Kooij’ method.
incubation temperature and factors for the conversion of cell numbers into acetate-C equivalents as illustrated by Table 1. It is evident that the differences between assays are substantial and would not only alter the use (or practical aspects) of the assay, but also potentially the outcome of the assay.

In the present study a round-robin test with 14 water samples, taken from drinking water treatment plants, covering a wide range of AOC concentrations (4–130 \(\mu g/L\) acetate-C equivalents) was performed using three well-established AOC methods to elucidate the differences between results obtained with different assays. The assays applied were (1) the original ‘van der Kooij’ assay (van der Kooij et al. 1982; van der Kooij & Hijnen 1984), (2) the ‘Werner & Hambsch’ assay (Werner & Hambsch 1986; Hambsch & Werner 1990) and (3) the ‘Eawag’ assay (Hammes & Egli 2005; Hammes et al. 2010a). By analyzing the same samples with different methods, the study also investigated whether the various methods provided comparable AOC concentrations.

### MATERIAL AND METHODS

#### Sampling locations

All samples were taken at two WaterNet drinking water production locations, Leiduin (LDN) and Weesperkarspel (WPK). WaterNet is the water cycle company of Amsterdam and surrounding areas, in the Netherlands. In total 14 samples were taken at different locations in the full-scale and pilot-scale treatment trains, summarized in Table 2. The treatment schemes of both locations are similar, however the raw water source and pre-treatment are different resulting in different water qualities. Location LDN receives water from the river Rhine, pre-treated by coagulation, sedimentation and rapid sand filtration. The water is further treated by dune infiltration and abstracted after a mean residence time of about 90 days. WPK raw water originates from seepage water from the Bethune polder, a peat-rich area that gives the water a relatively high natural organic matter (NOM) concentration. The water is pre-treated by coagulation and sedimentation, followed by about 100 days retention in a lake reservoir. Subsequently it is filtered over rapid sand filters (sample 2). Both treatments consist of ozonation (\(O_3\)), pellet softening (PS) (samples 9 and 10 for WPK and sample 6 for LDN), biological activated carbon filtration (BAC) (sample 5 for WPK and sample 3 for LDN) and slow sand filtration, with the difference that the post treatment at LDN is preceded by rapid sand filtration (sample 7). At the WPK production location, a pilot plant (WPK-PP) consisting of the same treatment steps as the full scale plant was also sampled. One of the experiments in this pilot plant involved increasing ozone dosage above standard operational values, providing highly oxidized samples (samples 12, 13 and 14). Additionally, at the pilot plant, a fluidized ion exchange (FIX) system (samples 1 and 8) was being tested to reduce the organic matter loading before ozonation (Grefte et al. 2011).

The samples covered a broad range of AOC concentrations present in drinking water treatment, with high and low NOM concentrations. In addition, the samples were also taken after different types of treatment, representing oxidized, non-oxidized, biodegraded, non-biodegraded samples and thus different organic carbon compositions.

#### Sampling

The samples were taken according to the protocol of the relevant method. In short, samples for analyses according to the ‘van der Kooij’ method were taken in 1 L Erlenmeyer flasks with glass stoppers. Flasks were rinsed overnight in acid and subsequently heated at 550 °C for 6 hours. Flasks were filled to 600 mL, without pre-washing. Samples for the analyses according to the ‘Werner & Hambsch’ method were taken in 1 L Schott bottles with plastic caps. The bottles were soaked for 12 hours in detergent followed by 10 rinses with hot water and 3 rinses with de-ionized water. The bottles were pre-washed 2 times after which they were filled to 2 cm under the cap. The samples that were analyzed according the ‘Eawag’ method were taken in 100 mL Schott bottles with plastic caps. Bottles were rinsed in acid (HCl, 1 M, overnight) and subsequently heated at 500 °C for 4 hours. The caps were rinsed in a 10% sodium persulfate solution at 60 °C for at least 1 hour. The bottles were filled to 2 cm under the cap without pre-washing.

No addition of thio-sulfate (or similar compounds) was necessary to quench ozone after the ozonation step, since
the contact times in the ozonation chambers were sufficiently long that no residual ozone was present at the sampling points. All samples were taken from stainless steel taps with continuous water flow. Samples were transported at a temperature below 4 °C to the laboratories and were analyzed within 24 hours. All samples were taken in duplicate.

AOC analysis: the ‘van der Kooij’ method

Analysis was carried out as described by van der Kooij et al. (1982), van der Kooij & Hijnen (1984) and Standard Methods number 9217 (Eaton et al. 2005). In short, after pasteurization, samples (600 mL) were inoculated with an AOC-free suspension (ca. 500 cfu/mL) of pre-cultured cells of P17 and NOX. In contrast to the protocol described in Standard Methods, no nutrient salts were added, since it generally assumed the water is not limited in nutrients other than carbon (personal communication with the laboratory). Inoculated samples were incubated at 15 °C without shaking until the maximum number of colony forming units per milliliter (cfu/mL) was attained (analysis of cfu/mL was done once every 2 days over a period of 15–30 days). The AOC concentration was calculated from the maximum concentration of each of the two strains in a sample and the conversion factors (growth yield) of 4.6 × 10⁶ cfu/μg-C for P. fluorescens strain P17 and 1.2 × 10⁷ cfu/μg-C for Spirillum spp. NOX. AOC concentration was expressed as μg acetate-C equivalents/L. The detection limit was 1 μg acetate-C equivalents/L.

AOC analysis: the ‘Werner & Hambsch’ method

Analysis was carried out as described by Werner & Hambsch (1986) and Hambsch & Werner (1990). The method is based on correlation between turbidity and total bacterial cell number after growth of an undefined indigenous inoculum in a water sample. In short, samples were filtered (0.2 μm), placed into a cuvette, and a sterile carbon-free nutrient salt solution was added. To minimize the potential addition of organic carbon from the filters, these were rinsed prior to use with 1 L of ultra-pure water. Moreover, in every run of samples, a negative control (ultra-pure water) was analyzed in parallel. The sample (250 mL) was inoculated to about 5 × 10⁴ cells/mL with a suspension of bacteria collected from the filter used for pre-treatment. The cuvette was incubated in a specially modified turbidimeter at approximately 22 °C. Turbidity was measured every 30 minutes for 2–4 days by applying 12-degrees forward scattering on a specifically designed instrument, until stationary phase was reached. Acetate-C-equivalents were calculated from the turbidity increase using the turbidity yield on

<table>
<thead>
<tr>
<th>Sample #</th>
<th>Location</th>
<th>O₃-dose [mg/L]</th>
<th>DOC [mg/L]</th>
<th>Temp °C</th>
<th>AOC Vdk [μg-C/L]</th>
<th>AOC W&amp;H [μg-C/L]</th>
<th>AOC Eawag [μg-C/L]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>After FIX (WPK-PP)</td>
<td>0.0</td>
<td>1.2</td>
<td>21</td>
<td>4</td>
<td>13</td>
<td>26</td>
</tr>
<tr>
<td>2</td>
<td>Before O₃ (WPK)</td>
<td>0.0</td>
<td>6.0</td>
<td>21</td>
<td>8</td>
<td>13</td>
<td>28</td>
</tr>
<tr>
<td>3</td>
<td>After O₃-PS-BAC (LDN)</td>
<td>0.7</td>
<td>1.0</td>
<td>17</td>
<td>9</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>Before O₃ (WPK)</td>
<td>0.0</td>
<td>6.3</td>
<td>20</td>
<td>10</td>
<td>16</td>
<td>16</td>
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<tr>
<td>5</td>
<td>After O₃-PS-BAC (WPK)</td>
<td>2.4</td>
<td>4.8</td>
<td>20</td>
<td>17</td>
<td>16</td>
<td>27</td>
</tr>
<tr>
<td>6</td>
<td>After O₃-PS (LDN)</td>
<td>0.7</td>
<td>1.7</td>
<td>17</td>
<td>30</td>
<td>24</td>
<td>35</td>
</tr>
<tr>
<td>7</td>
<td>Before O₃ (LDN)</td>
<td>0.0</td>
<td>2.6</td>
<td>17</td>
<td>34</td>
<td>73</td>
<td>57</td>
</tr>
<tr>
<td>8</td>
<td>After FIX-O₃ (WPK-PP)</td>
<td>0.9</td>
<td>1.4</td>
<td>21</td>
<td>48</td>
<td>66</td>
<td>63</td>
</tr>
<tr>
<td>9</td>
<td>After O₃-PS (WPK)</td>
<td>2.3</td>
<td>2.1</td>
<td>21</td>
<td>51</td>
<td>70</td>
<td>77</td>
</tr>
<tr>
<td>10</td>
<td>After O₃-PS (WPK)</td>
<td>2.4</td>
<td>5.2</td>
<td>20</td>
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<td>After O₃ (WPK)</td>
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<td>64</td>
<td>140</td>
<td>211</td>
</tr>
<tr>
<td>12</td>
<td>After O₃ (WPK-PP)</td>
<td>4.0</td>
<td>5.4</td>
<td>21</td>
<td>97</td>
<td>267</td>
<td>82</td>
</tr>
<tr>
<td>13</td>
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<td>2.5</td>
<td>6.2</td>
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<td>120</td>
<td>218</td>
<td>160</td>
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<tr>
<td>14</td>
<td>After O₃ (WPK-PP)</td>
<td>4.0</td>
<td>5.7</td>
<td>20</td>
<td>130</td>
<td>269</td>
<td>140</td>
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acetate-C (1 mg/L acetate-C: 2.3 ppm turbidity increase). The detection limit was 10 μg/L acetate-C-equivalents. Also, DOC-removal and total cell number increase were determined by analyzing DOC and total cell number (epifluorescence microscopy after staining with acridine orange (Hobbie et al. 1977)), of the samples in the cuvettes at the start and at the end of the experiments.

**AOC analysis: the ‘Eawag’ method**

Analysis was carried out according to the method described by Hammes & Egli (2009). In short, water samples were filtered (0.2 μm), after which the filtered sample (20 mL) was inoculated with an undefined indigenous bacterial community obtained from non-chlorinated drinking water. The filters were rinsed with 1 L nano-pure water prior to use to minimize potential carbon contamination during filtration. The samples were incubated at 30 °C until the bacteria reached stationary phase (2–4 days). Bacterial cells were counted by flow cytometry as described elsewhere (Hammes et al. 2008). The AOC concentration (expressed as μg C) was calculated based on the maximum cell concentrations (cells/mL) using a conversion value of $1 \times 10^7$ cells/(μg C) (Vital et al. 2007; Hammes et al. 2010a). The detection limit of the assay was about 10 μg/L.

**Statistical methods**

In this research a total of 14 ($n = 14$) sampling locations were selected across the treatment train in order to compare the performance of the three different methods for a wide range of AOC values. At each sampling location, triplicate samples were taken to check for variation of each method at the same AOC-value. As the triplicate samples taken at each sampling location were statistically interdependent of each other, the paired $t$-test should be applied to determine the statistical difference between the method results. The use of the paired $t$-test is only legitimate if the sample distribution of different methods was normal, which was affirmed by applying the Kolmogorov–Smirnov test (Drezner et al. 2010). The datasets were checked for outliers by applying the analysis of variance (ANOVA) test. If the $p$-value, determined by the tests, was larger than 0.05 it could be concluded that, with a confidence interval of 95%, the tested hypotheses were not significantly different. All statistical analyses were performed with the statistical toolbox available within Matlab® (Matlab 2009). Quality control was performed using blank controls.

**RESULTS**

**AOC concentrations**

In Table 2, AOC concentrations measured according to the three AOC assays are provided together with other water quality parameters of the samples. The samples are organized from the lowest (4 μg C/L) to the highest (130 μg C/L) AOC concentration determined with the conventional ‘van der Kooij’ method. This range of values is considered typical for drinking water treatment and distribution systems (Escober & Randall 2002a).

Statistical analysis of the dataset indicated that AOC values determined by the ‘Eawag’ method contained one outlier. This outlier (value = 211 μg C/L, indicated in bold in Table 2) was ignored in the comparison made below.

A statistical comparison of the datasets obtained by the ‘van der Kooij’ method and ‘Eawag’ and ‘Werner & Hambsch’ methods resulted in $p$-values of 0.01 and 0.04, respectively, indicating a significant difference between the van der Kooij method and the other two methods. Comparison between the datasets for the ‘Eawag’ and ‘Werner & Hambsch’ methods resulted in a $p$-value of 0.28, indicating that the results of the methods did not significantly differ.

The AOC concentrations for the ‘Eawag’ and ‘Werner & Hambsch’ methods showed a good correlation with the ‘van der Kooij’ method (Figure 1). However, both the ‘Werner & Hambsch’ method and the ‘Eawag’ method showed higher AOC results than the ‘van der Kooij’ method (2.0 times and 1.2 times, respectively; Figure 1).

In Figure 2 the increase in cell numbers during the ‘Werner & Hambsch’ and ‘Eawag’ assays versus the ‘van der Kooij’ assay is given. As illustrated, the samples with the highest AOC concentrations measured by ‘Werner & Hambsch’ were left out of this dataset. In this way only the effect of the use of a natural inoculum is illustrated, which is discussed in more detail below. The observed/ found correlations were significant.

It can be seen that the application of natural inoculum leads to an increase in cell numbers of approximately 20%
(slopes of the linear regression lines are 1.20 and 1.25). In both assays, culture-independent measurement of growth is applied, and viable but not culturable cells will also contribute to the final outcome. This is also reflected in the lower sensitivity of the two methods in the low range of AOC concentration beneath 10 μg/L.

**DISCUSSION**

Because AOC assays are based on bacterial growth, variations in the methodology (e.g. inoculum, incubation temperature, measurement technique, conversion factors) can have a profound impact on the outcome. Below we
discuss a number of critical steps in detail, being pre-
treatment, incubation volume, inoculum, and yield and conversion values.

Pre-treatment

Pre-treatment is applied to remove or collect indigenous bacteria. The use of pasteurization versus filtration is dis-
cussed. A possible disadvantage of pasteurization is that it can change the organic carbon quality of the sample (denaturation of proteins) as well as the inorganic quality (precipitation of minerals in the form of carbonates and phosphates). A disadvantage of filtration is possible contamination with organic carbon from the filters and materials used in the filtration process. Therefore, in the ‘Werner & Hambsch’ method, a negative control is ana-
alyzed in parallel. Filtration is also not a sterilization step (Wang et al. 2007), and can therefore only be used in assays where a natural microbial community is used for the inoculum (some natural contamination would not matter). Depending on the method used for growth detec-
tion, either pasteurization or filtration should be used. Filtration is required in the methods of ‘Werner & Hambsch’ and ‘Eawag’, in order to remove particles/bac-
teria that can interfere with their detection methods. For the ‘van der Kooij’ method pasteurization is needed since filtration is not sterilization. It was not possible to identify if any differences in the results of the round-robin test were introduced because of the different pre-treatment methods. Higher cell yields in the ‘Werner & Hambsch’ and ‘Eawag’ methods than in the ‘van der Kooij’ method may be partly related to the pre-treatment, but other factors such as difference in the type of inoculum and addition of inorganic nutrient salts seem to be more relevant.

Incubation volume

The different assays prescribe the use of differently sized and shaped bottles to incubate the water samples in. According to Krammer et al. (2008) the volume for batch growth should be at least 800 mL to decrease the possibility of the ‘bottle effect’, which could influence the bacterial growth. However, Hammes et al. (2010b) showed through several experiments that the size and shape of the bottle does not influence the bacterial growth such that it should be taken into account. Therefore, it was assumed that incubation in different bottles and volumes did not interfere with the retrieved results.

Inoculum

The inoculum (composition and quantity) can affect the outcome of the AOC assay profoundly. This is because different bacteria consume a different range of substrates (Vital et al. 2010), thus the range of targeted AOC compounds will be determined by the inoculum (Sack et al. 2011). Moreover, as discussed below, different bacteria have profoundly different yields when consuming organic carbon, even for exactly the same substrate. The type of inoculum used in the three assays varied from a known set of bacteria (‘van der Kooij’ method) to undefined indigenous bacteria (‘Eawag’ and ‘Werner & Hambsch’ methods). The advantage of the ‘van der Kooij’ method is that it allows standardization of the assay. How-
ever, Huck (1990) and Servais et al. (1987) have noted that the application of a known set of two species might not cover the full growth potential, since these species do not represent the whole spectrum that a natural community covers. In this respect, recent research in the ‘van der Kooij group’ suggested the additional use of Flavobacterium johnsoniae strain A3 that utilizes oligo-
and polysaccharides (Sack et al. 2011). The ‘Werner & Hambsch’ and ‘Eawag’ methods used a natural inoculum, of which the first assay retrieved the indigenous bacteria from the filtered raw water sample taken at WPK treatment plant, thus containing a natural occurring inoculum. The ‘Eawag’ method used a natural microbial consortium consisting of a mixture of non-chlorinated tap water and bottled drinking water. While the use of indigenous drinking water communities has the advantage that it potentially broadens the substrate spectrum range, it has the disadvantage of being undefined, and thus more difficult to standardize. This could introduce a deviation in the values obtained, with the expectation that the results of AOC assays with a natural inoculum will be higher. Both the ‘Werner & Hambsch’ and ‘Eawag’ assays resulted in higher final cell numbers (25 and 20%, respectively; Figure 2), which con-
irms the idea that when a natural inoculum is applied, a
broader range of substrates can be utilized, and consequently the AOC concentrations found would be higher.

**Incubation temperature**

Effect of incubation temperature on yield could not be clearly derived from the obtained data. Although the ‘Eawag’ method applied the highest incubation temperature, highest cell yields were observed in the ‘Werner & Hambsch’ method suggesting that incubation temperature, for this range, is not affecting maximal cell yield.

**Yield and conversion values**

AOC assays convert measurements of cell numbers to equivalent carbon concentrations. This is an indirect approach, firstly since organic carbon itself is not measured, and secondly because the yield of different bacteria on different organic carbon substrates varies. As a result, some AOC assays convert growth relative to the growth on a pure AOC substrate such as acetate or oxalate. In the three assays compared in the present study, the conversion to (acetate-) C equivalents is retrieved by different yield values. The conversion values of the ‘van der Kooij’ method are $4.6 \times 10^6$ cfu/$\mu$g acetate-C for P17 and $1.2 \times 10^7$ cfu/$\mu$g acetate-C for NOX. Depending on the type of substrate the species P17 and NOX reach different levels of maximum growth. The conversion value of the ‘Werner & Hambsch’ method was determined with the turbidity yield on acetate: 1 mg/L acetate-C equal to 2.3 ppm turbidity increase. The conversion value of the ‘Eawag’ method is constant with $1 \times 10^7$ cells/$\mu$g C). Hence, it is evident that conversion of cell concentrations (Figure 2) can potentially lead to considerable differences in final AOC concentrations.

It was shown that a natural inoculum resulted in higher increase in cell numbers. A difference in results between the AOC concentration determined by the ‘van der Kooij’ method and the ‘Eawag’ and ‘Werner & Hambsch’ methods of 18 and 101% was found (Figure 1). Based on the inoculum it was shown that a difference in cell yield of respectively 20 and 25% was introduced. When comparing the conversion values of the ‘van der Kooij’ method with the ‘Eawag’ method it is seen that for P17 the conversion values of Eawag are 117% higher, while for NOX these values are 17% lower than ‘van der Kooij’ conversion values. The conversion of the cell numbers into equivalent carbon concentration resulted in a decrease in difference between the ‘van der Kooij’ method and the ‘Eawag’ method of 10%, indicating that NOX was predominant in the samples determined by the ‘van der Kooij’ method. Since the conversion value of the ‘Werner & Hambsch’ method is related to ppm instead of cell numbers it is difficult to quantify the effect of the conversion value. However the indicated difference between AOC concentrations determined by ‘van der Kooij’ and ‘Werner & Hambsch’ of 101% in relation to 25% (introduced by natural inoculum) does show that the applied conversion values might introduce a high difference. However, there is one other aspect that might play an important role in the difference between the cell numbers and AOC concentration, which is the addition of nutrient salts applied solely in the ‘Werner & Hambsch’ method, discussed in detail below. When taking this effect into consideration and discarding the highest AOC concentrations it was found that the conversion values account for an increase from 25 to 66% (data not shown).

**AOC versus growth potential**

Multiple factors aside from the organic carbon content of a water sample determine the rate and extent of bacterial growth. The ‘Werner & Hambsch’ method was the only assay in which a carbon free nutrient salt solution was added, which would ensure that AOC was indeed the limiting nutrient. If the water samples were limited in a nutrient other than carbon this could result in higher results that represent the actual AOC concentration. The other two methods would represent the actual growth potential of the water if no additional source of nutrients were introduced into the system. Several researchers have shown that humic rich surface waters pretreated with coagulation were limited in phosphate instead of carbon (Sathasivan et al. 1997; Juhna & Rubulis 2004), since during coagulation and sedimentation most of the phosphate was removed from the water. Without the addition of carbon free nutrient salt solution, it can no longer be assumed that the AOC is the limiting nutrient.

In Figure 1 it can be seen that the ‘Werner & Hambsch’ method differs mostly in the higher ranges of AOC.
concentrations. The samples in these ranges originate from humic rich surface water that was pre-treated with coagulation-sedimentation, removing organic compounds as well as phosphate, and then ozonated, increasing the amount of readily available carbon. The phosphate levels in these waters were below detection limits, indicating a possible limitation in phosphate in the samples instead of organic carbon.

CONCLUSIONS

A round-robin test was performed to evaluate correlation between three established AOC assays: ‘Werner & Hambsch’, ‘Eawag’ and ‘van der Kooij’ methods. An AOC assay consists of several steps, such as sample pre-treatment, incubation volume and temperature, inoculum, growth measurement, yield and conversion values, which may all contribute to variation in measured values. The use of indigenous bacteria instead of a set of two known strains resulted in an increase in cell growth of approximately 20%. In the application of culture-independent measurement of growth, viable but non-culturability cells may contribute to the final outcome. The sensitivity of these assays is lower (detection limit is 10 µg/L) than for the ‘van der Kooij’ method in which plating is used to determine growth (detection limit is 1 µg/L). The addition of a carbon free nutrient salt solution resulted in higher concentrations in some samples, since samples seemed to be limited in nutrients other than organic carbon. The overall results of the round-robin test suggest an acceptable compatibility between the different AOC methods, although deviations between the methods call for careful interpretation and reporting of AOC data.

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