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Algal toxicity of the alternative disinfectants peracetic acid (PAA), performic acid (PFA), chlorine dioxide (ClO₂) and their by-products hydrogen peroxide (H₂O₂) and chlorite (ClO₂⁻)

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Highlights
- Algal toxicity of disinfectants PAA, PFA and ClO₂ and by-products determined
- Chlorine dioxide more toxic to microalgae than performic acid and peracetic acid
- By-products chlorite and hydrogen peroxide least toxic
- Chlorine dioxide and performic acid degraded completely during the test
- Chlorine dioxide most toxic to algae but also degraded fastest in the test

Abstract
Environmental effect evaluation of disinfection of combined sewer overflow events with alternative chemical disinfectants requires that the environmental toxicity of the disinfectants and the main by-products of their use are known. Many disinfectants degrade quickly in water which should be included in the evaluation of both their toxicity as determined in standardized tests and their possible negative effect in the water environment. Here we evaluated according to the standardized ISO 8692 test the toxicity towards the green microalgae, Pseudokirchneriella subcapitata, of three disinfectants: performic acid (PFA), peracetic acid (PAA) and chlorine dioxide (ClO₂) as well as two by-products of their use: hydrogen peroxide (H₂O₂) and chlorite. All of the five chemicals investigated showed clear toxicity to the algae with well-defined dose response curves. The EC50 values ranged from 0.16 to 2.9 mg/L based on nominal concentrations leading to the labeling of the chemicals as either toxic or very toxic. The five investigated chemicals decreased in toxicity in the order chlorine dioxide, performic acid, peracetic acid, chlorite and hydrogen peroxide. The stability of the chemicals increased in the same order as the toxicity decrease. This indicates that even though ClO₂ has the highest environmental hazard potential, it may still be suitable as an alternative disinfectant due to its rapid degradation in water.

Keywords: Peracetic acid, performic acid, chlorine dioxide, hydrogen peroxide, chlorite, algal toxicity, Pseudokirchneriella subcapitata

1 Introduction
Combined sewer systems, in which wastewater is mixed with rain water and transported to the wastewater treatment plant for processing, is common in many cities. The design capacity of combined sewer systems can be exceeded when significant rainfall events occur which results in the discharge of untreated combined sewer overflows (CSO) to nearby surface waters. In recent years, disinfection of inflowing CSO water in the CSO discharge structures has been studied using alternative disinfectants to minimize the impact from the discharge of untreated CSO to the surface waters (DesiCSO, 2014; FRODO, 2014).

Various disinfectants are used in the water industry to limit the number of pathogenic organisms and to prohibit the spread of diseases. Chlorine is the best known disinfectants used in the water industry (White, 2010), which could be used to reduce contamination by microorganisms. However, the toxic chlorination by-products are of environmental concern (Bayo et al., 2009; Boczek et al., 2010; Emmanuel et al., 2004; Hrudey and Charrois, 2012; Nurizzo et al., 2005; Watson et al., 2012; White, 2010). Chlorine dioxide (ClO₂) has been used as alternative to chlorine, since it does not form chloramines and chlororganic compounds as toxic by-products (Hofmann et al., 1999). Moreover, ClO₂ has higher oxidation capacity compare to chlorine and it effectively inactivates microorganisms within a wide range of pH (Junli et al., 1997). ClO₂ is e.g. synthesized by the reaction of chlorine with strong acid:

\[ 5\text{ClO}_2^- + 4H^+ \rightarrow 4\text{ClO}_2 + \text{Cl}^- + 2\text{H}_2\text{O} \]  
Eq. 1A
During disinfection, ClO$_2$ is reduced to chlorite as a degradation product as shown in Eq. 1B by oxidizing bacteria and other matter in the treated water thus forming chlorite as a significant by-product of the treatment (Korn et al., 2002; Lee et al., 2004; Svecevicius et al., 2005):

$$\text{ClO}_2 + e^- \rightarrow \text{ClO}_2^- \quad \text{Eq. 1B}$$

Peroxyacetic acids such as peracetic acid (PAA) and performic acid (PFA) are increasingly used for water disinfection. They do not generate toxic by-products (Chhetri et al., 2014; Liberti and Notarnicola, 1999). Commercially available peroxyacetic acids are always available as a mixture of the peroxyacetic acids, hydrogen peroxide and corresponding carboxylic acid.

PFA emerged as a common disinfectant in the medical field and food industry (Gehr et al., 2009). In recent years, PFA has been used to disinfect primary and secondary WWTP effluents (Gehr et al., 2009; Ragazzo et al., 2013) and to disinfect combined sewer overflows (Chhetri et al., 2015). PFA is unstable and needs to be generated on-site, when needed, as a quaternary equilibrium mixture of performic acid (PFA), formic acid, hydrogen peroxide and water:

$$\text{CHO} - \text{OH} + \text{H}_2\text{O}_2 \rightleftharpoons \text{CHO} - \text{OOH} + \text{H}_2\text{O} \quad \text{Eq. 2A}$$

PFA degrades into formic acid by oxidation of matter in water (Eq. 2B) and formic acid and hydrogen peroxide water will also come from their presence the mixture of the disinfectant.

$$\text{CHO} - \text{OOH} + 2e^- \rightarrow \text{CHO} - \text{O}^- + \text{HO}^- \quad \text{Eq. 2B}$$

Formic acid is not toxic to aquatic fauna and is readily biodegradable (Gehr et al., 2009; USEPA, 2001). Hydrogen peroxide is itself a weak disinfectant which trend to degrade slower than peroxyacetic acids (Wagner et al., 2002). Moreover, there is a stringent discharge limit of hydrogen peroxide to the surface water.

PAA is a well-known disinfectant with a wide spectrum of antimicrobial activity. It was introduced to wastewater treatment approximately 30 years ago (Antonelli et al., 2006; Baldry, 1983; Falsanisi et al., 2006; Kitis, 2004; Koivunen and Heinonen-Tanski, 2005; Luukkonen et al., 2015) and recently has been used to disinfect combined sewer overflows (Chhetri et al., 2016, 2014). Commercial PAA is available as an acidic quaternary equilibrium mixture of PAA, hydrogen peroxide, acetic acid, and water (Eq. 3A) and as it oxidizes matter acetic acid remains (reaction 3B):

$$\text{CH}_3\text{COOH} + \text{H}_2\text{O}_2 \rightleftharpoons \text{CH}_3\text{COOOH} + \text{H}_2\text{O} \quad \text{Eq. 3A}$$

$$\text{CH}_3\text{CO} - \text{OOH} + 2e^- \rightarrow \text{CH}_3\text{CO} - \text{O}^- + \text{HO}^- \quad \text{Eq. 3B}$$

The residues after PAA treatment use are thus acetic acid, hydrogen peroxide, and water.

To minimize the impact of the discharged disinfected effluents in the receiving waters and aquatic ecosystem, it is important to evaluate the ecotoxic effect of residual disinfectant. Algal growth inhibition tests are among the ecotoxicity tests, the most commonly used test for ecotoxicity screening. Microalgae constitute the base of the food web in aquatic ecosystems and growth inhibition tests employing unicellular algae are routinely used to test chronic toxicity of pollutants. The most commonly used species is the freshwater microalgae *Pseudokirchneriella subcapitata* (*P. subcapitata*) formerly known as *Selenastrum capricornutum*. This species and the standard test setup was chosen as test organism to increase the regulatory relevance of the results and to ensure that they can be compared to other studies in future. At present, only few studies report the ecotoxicity of PAA, PFA, hydrogen peroxide, ClO$_2$ and chlorite and there is no consistent information regarding toxic effect of disinfected effluents (Antonelli et al., 2009; Liu et al., 2015; Meinertz et al., 2008; Svecevicius et al., 2005). Ecotoxicity data of disinfectants are important as input to environmental risk assessments of possible disinfection systems using these chemicals and eventually to obtain permit from the authorities for their use in this application. Here a typical discharge permit will define that a predicted no-effect concentration cannot be exceed outside of a defined dilution zone in the receiving waters. In order to define such predicted no-effect concentrations, data for standardized ecotoxicity tests, like the algal growth test, are needed. For evaluation of the ecotoxicity tests results generated even in simple test
systems, the degradation kinetics of disinfectant must be considered. Degradation of the compounds during testing will alter the effect concentration (EC) result of the disinfectant. In general, nominal concentration of chemical compounds are used for interpretation of toxicity results. However, PAA, PFA, ClO₂ and hydrogen peroxide degrade significantly over the time frame of ecotoxicological tests as well as then they are applied for disinfection of CSO water. To our knowledge, the currently available results of toxicity test with disinfectants are based on nominal concentrations and the degradation kinetics of disinfectant in the test medium has not been reported.

The objective of this study was therefore to comprehensively evaluate the toxicity of the disinfectants: PAA, PFA, ClO₂ and their degradation products: hydrogen peroxide and chlorite by using the standardized algal growth rate inhibition test. Concurrent to the toxicity testing, degradation kinetics of disinfectants and degradation products on the test medium was measured, to account for the fact that the disinfectant concentration decreases over time.

2 Materials and methods

2.1 Chemicals

ABTS (2,200-azino-bis [3-ethylbenzothiazoline-6-sulfonic acid] diammonium salt), sodium chlorite and hydrogen peroxide (35% w/w) were all purchased from Sigma–Aldrich (Brøndby, Denmark). All chemicals were of reagent grade. PAA solution containing 30–40% (w/w) of technical grade disinfectant was supplied by Sigma–Aldrich (Brøndby, Denmark). Chlorine dioxide was synthesized as described by Hey et al., (2012). In short, 400 mL of demineralized water was mixed with 25 mL of 9% HCl and 7.5% NaClO₂. The reaction mixture was allowed to react overnight and was then diluted to 1000 mL with demineralized water. This resulted in an approximately 1 g/L chlorine dioxide solution which was quantified using the method described below.

PFA was prepared in two steps as described by Chhetri et al., (2014) before an experiment was performed. In the first step 11 mL of formic acid (85% w/w) was mixed with 1.0 mL sulfuric acid (95%) in a glass test tube. In the second step 0.9 mL of this mixture was added to 1.1 mL of hydrogen peroxide (50% w/w) in a 5 mL test tube, immersed in a water bath controlled at 20 °C and the reactants were allowed to react for 10 min. The PFA solution was immediately used for experiments and quantified, in parallel, by dilution (125 μL to 100 mL) of a subsample in demineralized water, to yield a solution of 2 mg/L which was analyzed as described below.

2.2 Chemical analysis

PAA and PFA concentration was analyzed using the colorimetric method described by Chhetri et al. (2014) based on selective oxidation of ABTS by PAA or PFA without interference from hydrogen peroxide. Hydrogen peroxide was analyzed using the titanium oxide-oxalate colorimetric assay (Antoniou and Andersen, 2015), chlorite dioxide was measured using Hach Lange test kit LCK 310 and chlorine concentration was measured using IonPac AS14 analytical column (4 mm × 250 mm, Dionex) and an IonPac G14 guard column (4 mm × 50 mm, Dionex). The eluent phase consisted of 8 mM Na₂CO₃ and 1 mM NaHCO₃. Chlorite was quantified by a Jasco 870-UV (Japan) UV-detector at λ = 340 nm.

2.3 Algal growth inhibition test

The toxicity towards the freshwater microalgae *P. subcapitata* was determined using modified ISO 8692(2012). Laboratory culture of *Pseudokirchneriella subcapitata* was obtained from the Norwegian Institute for Water Research, Oslo, Norway (NIVA). The ISO algal medium was used to prepare a range of concentrations of the disinfectants and degradation products, which were then inoculated with exponentially growing algae to a density of 10⁴ cells/mL. For disinfectants inhibition tests, five concentrations were tested using triplicates in two experiments: range finding test and a final test. For range finding experiment, disinfectants concentration PFA (0.1-10 mg/L), PAA (0.3-30 mg/L) and ClO₂ (0.1-10 mg/L) and degradation products H₂O₂ (1-1000 mg/L) and ClO₂⁻ (1-1000 mg/L) were used for growth inhibition test. For final experiment, disinfectants concentration PFA (0.03-2 mg/L), PAA (0.1-3 mg/L) and ClO₂ (0.1-10 mg/L) and degradation products H₂O₂ (0.1-30 mg/L) and ClO₂⁻ (0.1-100 mg/L) were used for growth inhibition test. A mini-scale test with 4 mL test solutions in 30 mL polystyrene containers (Nunc) was applied in this study (Arensberg et al., 1995). The containers were placed on a shaker (200 rpm) at 20 ± 1 °C to allow for mixing and CO₂ diffusion and continuously illuminated at 80–105 E/m²/s (measured under the test vessel). The light source in the tests was a cold light fluorescent tube emitting light in the visible spectrum. Light intensity in
the test setup was measured using a LI-COR light meter (model LI-189) with an attached quantum sensor, measuring light within the wavelength range 400–700 nm. The tests were conducted at a pH of 7.8–8.0 with typical control growth rates of 1.4–1.6 d⁻¹ during the 72 h incubation. Samples of 0.4 mL were taken at times 0, 24, 48 and 72 h and the algal growth rates were calculated on the basis of total algal biomass in each sample quantified by acetone extractions of chlorophyll, as described by Mayer et al. (1997). The fluorescence of the samples was subsequently measured on a fluorescence spectrophotometer (Hitachi F-2000) using an excitation wavelength of 430 nm and emission wavelength of 671 ± 20 nm. Growth rates and concentration–response curves were estimated by use of a nonlinear-regression program with control variance weighting (Christensen et al., 2009) assuming lognormal distribution. By use of logistic curve fitting and inverse estimation EC-values were determined with corresponding 95% confidence limits.

3 Result and discussion

Concentration-response curves of the three disinfectants and their degradation products obtained in 72 h growth rate inhibition tests with P. subcapitata is presented in Fig 1. Effect concentration (EC₅₀ and EC₉₀) of the disinfectants and degradation products obtained from algal growth inhibition test is presented in table 1. Among three disinfectants ClO₂ was the most toxic to P. subcapitata with an EC₅₀-value of 0.16 mg/L followed by PFA and PAA (Table 1). It was also evident that disinfectants were more toxic than their by-products.

Figure 1: Concentration-response curves from 72 h growth rate inhibition tests of P. subcapitata from disinfectants performic acid (PFA), peracetic acid (PAA), chlorine dioxide (ClO₂) and their degradation products hydrogen peroxide (H₂O₂) and chlorite (ClO⁻₂⁻). Error bars indicate standard deviation (n=3) of result at each tested concentration. Note: Different scales on the primary axes.

Table 1: Effect concentration (EC₅₀ and EC₉₀) of disinfectants and their degradation products in 72 h algal growth rate inhibition tests with P. subcapitata. Effect concentrations are based on nominal concentration. 95% confidence interval indicated in parenthesis.

<table>
<thead>
<tr>
<th>Effect concentration</th>
<th>PFA (mg/L)</th>
<th>PAA (mg/L)</th>
<th>ClO₂ (mg/L)</th>
<th>H₂O₂ (mg/L)</th>
<th>Chlorite (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC₅₀</td>
<td>0.19</td>
<td>0.23</td>
<td>0.06</td>
<td>1.78</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>(0.12-0.32)</td>
<td>(0.10-0.53)</td>
<td>(0.05-0.07)</td>
<td>(1.63-1.94)</td>
<td>(0.59-0.60)</td>
</tr>
<tr>
<td>EC₉₀</td>
<td>0.34</td>
<td>1.38</td>
<td>0.16</td>
<td>2.90</td>
<td>1.10</td>
</tr>
<tr>
<td></td>
<td>(0.29-0.39)</td>
<td>(0.96-1.99)</td>
<td>(0.15-0.17)</td>
<td>(2.87-2.92)</td>
<td>(1.10-1.11)</td>
</tr>
</tbody>
</table>

The data on algal toxicity of disinfectants and their degradation products are limited and contradictory. Thus, Antonelli et al., (2009) have reported EC₅₀ value of PAA 8.89 mg/L. This contrasts with the EC₅₀ (<1 mg PAA/L) reported by, ECETOC, (2001). For hydrogen peroxide Drábková et al., (2007) have reported EC₅₀ value 21.26 mg/L which is eight times higher than we have obtained. Similarly, van Wijk et al., (1998) has reported EC₅₀ value of chlorite 0.67 mg/L which is closer to the EC₅₀ value we obtained. The difference in the EC₅₀ might be due to the test method that has been used for algal toxicity test and different test conditions.

According to the CLP regulation EC₅₀ values less than 1 mg/L gives a classification as “Acute toxic 1” i.e. very toxic to the aquatic organisms (EU Commission, 2011). This means that the alternative disinfectants PFA and
ClO₂ will be considered as very toxic for aquatic organisms whereas this is not the case for PAA or the degradation products hydrogen peroxide and chlorite.

Concentration profiles were obtained by measuring concentration of disinfectants and degradation products over time in the media used for toxicity analysis of P. subcapitata (Fig 2). Fig 2 shows concentrations of disinfectants and degradation products measured in samples taken for at the same time as samples for biomass quantification of P. subcapitata, i.e. at 0, 24, 48 and 72 h of contact time. However, due to the limit of quantification of colorimetric assay for concentration profiles of very low concentration of PAA, PFA and ClO₂ in test media was not possible to obtain.

![Concentration profiles of disinfectants (PFA, PAA and ClO₂) and by-products (H₂O₂ and chlorite) in an algal media.](image)

Figure 2: Concentration profiles of disinfectants (PFA, PAA and ClO₂) and by-products (H₂O₂ and chlorite) in an algal media.

A first order degradation kinetics model was used for curve fitting. Fitted curves generated using the first order expression are shown in Fig 2 along with derived parameters presented in Table 2. Fast degradation of PFA and chlorine dioxide was observed and complete degradation of both PFA and chlorine dioxide was observed after 24 h whilst hydrogen peroxide and chlorite were stable in the test media. Moreover, higher concentration of PAA (~30 mg/L and ~10 mg/L) was not completely degraded after 72 h. PFA and chlorine dioxide are unstable disinfectants. Faster degradation of PFA was observed than PAA when it was used to disinfect combined sewer overflows (Chhetri et al., 2015, 2014) and similarly, faster degradation chlorine dioxide was observed when it was used to remove pharmaceuticals in biologically treated wastewater (Hey et al., 2012).

Even though all three alternative disinfectants were almost completely degraded during the first 24 hours of the algal test, clear concentration-response patterns were obtained during the 72 hours incubation (Fig 1). This appears to be due to an acute toxic effect of the disinfectants during the first few hours of contact with the algae. As shown in Fig 2 the disappearance of the degradation products is slower and the algal responses obtained after 24 hours may be influence by the toxicity of residual H₂O₂ or chlorite in the tests. These type of mixed effects are however difficult to quantify using the standard algal testing setup. For PFA and ClO₂ the fast degradation will however not change the fact that they should be considered very toxic to aquatic organisms. Since PAA showed half-lives of 6-19 hours in the algal medium and the lower 95% confidence interval for the EC50 of PAA is <1 mg/L, this compound may also be considered as a very toxic compound to aquatic organisms.
Table 2: Fitted parameters of concentration curves for concentration profiles in algae media shown in Figure 2.

<table>
<thead>
<tr>
<th>Disinfectants</th>
<th>Nominal dose (mg/L)</th>
<th>C&lt;sub&gt;initial&lt;/sub&gt; (mg/L)</th>
<th>k (h&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>R&lt;sup&gt;2&lt;/sup&gt;</th>
<th>T&lt;sub&gt;½&lt;/sub&gt; (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFA</td>
<td>1</td>
<td>1.5</td>
<td>5.0 x 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.91</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.1</td>
<td>4.9 x 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.90</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3.7</td>
<td>1.2 x 10&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.99</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>10.4</td>
<td>1.0 x 10&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.99</td>
<td>6.8</td>
</tr>
<tr>
<td>PAA</td>
<td>1</td>
<td>1.2</td>
<td>9.9 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>0.98</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.5</td>
<td>1.2 x 10&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>0.99</td>
<td>5.8</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>12.2</td>
<td>3.7 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>0.99</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>26.1</td>
<td>3.6 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>0.99</td>
<td>19</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>10</td>
<td>7.8</td>
<td>1.8 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>0.94</td>
<td>394</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>29.1</td>
<td>3.4 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>0.71</td>
<td>2012</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100.1</td>
<td>5.6 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>0.83</td>
<td>122</td>
</tr>
<tr>
<td>ClO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>3</td>
<td>3.6</td>
<td>~1.6 x 10&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>1</td>
<td>~0.04</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>7.3</td>
<td>1.4 x 10&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>1</td>
<td>4.8</td>
</tr>
<tr>
<td>Chlorite</td>
<td>1</td>
<td>0.7</td>
<td>2.8 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>0.84</td>
<td>246</td>
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<td></td>
<td>10</td>
<td>7.6</td>
<td>1.8 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>0.96</td>
<td>394</td>
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<tr>
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<tr>
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<td>100</td>
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<td>286</td>
<td>1.6 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>0.87</td>
<td>44</td>
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<tr>
<td></td>
<td>1000</td>
<td>1416</td>
<td>1.5 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>0.89</td>
<td>45</td>
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</table>

4 Conclusion
All of the five chemicals investigated showed clear toxicity to the alga, P. subcapitata, according to the standardized ISO 8692 test with well-defined dose response curves. The EC<sub>50</sub> values ranged from 0.16 to 2.9 mg/L based on nominal concentrations leading to the labeling of the chemicals as either toxic or very toxic. The five investigated chemicals decreased in toxicity in the order chlorine dioxide, performic acid, peracetic acid, chlorite and hydrogen peroxide.

The stability of the chemicals increased in the same order as the toxicity decrease which could be considered in the environmental evaluation in the way that the exposure of the algae to the most toxic chemicals was less that indicated by the nominal concentration.

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