The impact of cyanobacteria on growth and death of opportunistic pathogenic bacteria
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ABSTRACT
Climate change may cause increased microbial growth in water sources and more knowledge is required on how this may affect the hygienic water quality, i.e., whether increased occurrence of cyanobacteria and algae may stimulate the growth rate of opportunistic pathogenic bacteria. Laboratory experiments were performed to investigate if the presence of the cyanobacteria Anabaena lemmermannii and Microcystis aeruginosa affected the survival and growth rate of the opportunistic pathogenic bacteria Aeromonas hydrophila and Pseudomonas aeruginosa, and the faecal indicators Escherichia coli and coliforms. Cyanobacteria were cultured in bottles containing the nutrient-poor medium O2. Sewage, A. hydrophila or P. aeruginosa was added to cyanobacterial cultures and the bacterial growth and survival was followed. E. coli and coliforms from sewage died within few days and the decay rate was not affected by the presence of cyanobacteria. The presence of Anabaena stimulated the growth rate of P. aeruginosa, but had no effect on the growth rate of A. hydrophila. Microcystis had no effect on the growth rate of P. aeruginosa and an inhibiting effect on the growth rate of A. hydrophila.

Key words | bacterial growth, climate change, cyanobacteria, drinking water, hygienic water quality, opportunistic pathogenic bacteria

INTRODUCTION
Cyanobacterial blooms are well known to cause ecological, economic and health related issues worldwide and the problems cyanobacteria cause are often associated with the toxins they produce (Chorus & Bartram 1999). Little is known, however, about impacts of other secondary metabolites in the water source, such as the effect on growth of opportunistic pathogenic bacteria in association with cyanobacterial blooms.

Cyanobacteria have the ability to float on the water surface where they can establish a biofilm. A biofilm represents a nutrient-rich environment that promotes growth and survival of bacteria, and it is known that cyanobacterial blooms includes a variety of heterotrophic bacteria that uses organic substances as their energy source (Eiler & Bertilsson 2004; Utkilen et al. 2008). Berg et al. (2009) showed that a wide range of heterotrophic bacteria was found in different waters (lakes, rivers, brackish water, drinking water sources) with frequent occurrences of cyanobacterial blooms. Of most importance was the detection of opportunistic pathogenic bacteria, such as Aeromonas and Pseudomonas species, that may cause adverse health effects in animals and humans. Other studies have also showed that cyanobacteria or cyanobacterial extracts have a positive impact on the growth of pathogenic bacteria such as Legionella pneumophila and Vibrio cholera (Tison et al. 1980; Eiler et al. 2007). In contrast, cyanobacteria have also been identified as a source of biologically active compounds with antibacterial properties (Østensvik et al. 1998; Abed et al. 2009).

There is a need to provide more information on how cyanobacterial blooms may affect the survival and potential growth of pathogenic bacteria and if this represents a risk to human health, both in relation to drinking water and for recreational purposes (Utkilen et al. 2008). Climate changes may aggravate the situation since higher temperatures may increase the frequency and intensity of cyanobacterial blooms (Paerl & Huisman 2008). More heavy rainfalls and runoff from the catchments to surface waters may increase the concentration of nutrients for cyanobacterial growth.

Aeromonas hydrophila and Pseudomonas aeruginosa were chosen as representatives of two opportunistic pathogenic bacteria in this study. They are both emerging waterborne pathogens with the ability to take advantage of changing growth conditions in a future climate, such as increased temperature and higher organic content in surface waters (Sharma et al. 2003). A. hydrophila is a species within the Aeromonad genus, which are known to include opportunistic pathogens of humans and animals. Aeromonads are considered primarily as aquatic organisms and the infections the genus causes are often associated with water contact. Aeromonads are widespread in surface, fresh and marine waters. Their presence in water may be indicative of faecal pollution as well as eutrophication in general. P. aeruginosa is typically found in natural habitats that has been exposed to faecal pollution, such as surface waters influenced by wastewater discharges, and in soil. P. aeruginosa is among the most important opportunistic pathogens causing nosocomial infections in immunocompromised people and people with underlying diseases. It has been shown that water is one source of infection (Mena & Gerba 2009).

Escherichia coli was used as an indicator of faecal pollution. Coliforms are no longer used as indication of faecal pollution since the methods used for their detection also includes species that can grow in the environment. In recent years, unusually high numbers of coliforms have been detected in late summertime in some drinking water sources in the eastern part of Norway, most probably due to growth in the water source (Bjerke et al. 2006). Although much information on die-off of E. coli in the aquatic environment is available, some studies also support their ability to grow in the environment if the temperature is high. Vital et al. (2008) showed that E. coli O157 was able to grow in sterile freshwater at low carbon concentrations. An early study by McFeters et al. (1978) also reported growth of coliforms (including E. coli) on low carbon sources, specifically on excreted products of algae.

The aim of the present study was to gain initial knowledge on the possible impact of cyanobacteria, i.e. Anabaena and Microcystis, on growth or death of P. aeruginosa, A. hydrophila, E. coli, coliforms and heterotrophic bacteria. Both Anabaena and Microcystis are present in Norwegian surface waters, they are potential toxin producing cyanobacteria and can cause problems in surface waters used both as drinking water and for recreational purposes (Skulberg 2004; Haande & Rohlack 2005; Halstvedt et al. 2007; Haande et al. 2010).

METHODS

Preparation of bacterial inoculum

Cultures of A. hydrophila (ATCC 14715) and P. aeruginosa (ATCC 27853) were grown overnight in nutrient broth (37 °C). One milliliter of the respective cultures was centrifuged, washed twice in O2 medium (Van Liere & Mur 1978) and resuspended in O2 medium. Fresh sewage was collected from Bekkelaget wastewater treatment plant (Oslo, Norway) and was used as a natural inoculum of E. coli and coliforms.

Preparation of cyanobacterial cultures

The two strains, Anabaena lemmanmannii (NIVA-CYA 266/1) and Microcystis aeruginosa (NIVA-CYA 118/2) were chosen from the algae culture collection at the Norwegian Institute for Water Research (NIVA). The Anabaena and the Microcystis cultures were not axenic; i.e., they were accompanied with heterotrophic bacteria in an approximate concentration of 10^5 CFU mL^-1. Prior to the experiment, the Anabaena and Microcystis strains were inoculated in glass flasks with O2 medium and kept at 20 °C with 24 h light conditions. The two strains grew with highly different growth rates in the O2 medium. When starting up the experiment, the biovolume of Anabaena and Microcystis were 11,160 and 1,035 mm³ mL⁻¹, respectively.

Enumeration of cyanobacteria

Biovolume of cyanobacteria was measured with a cell counter (CASY 1 model TTC, Schärfe System, Germany) fitted with a 60 μm capillary for Microcystis and with a 150 μm capillary for Anabaena. The CASYtome electrolyte (Schärfe System, Germany) was used, and 1 mL samples were counted for both organisms in order to determine the biovolume.

Enumeration of bacteria

Aeromonas hydrophila were enumerated by spread plate on the Aeromonas selective medium (Aeromonas medium base (Ryan medium), CMO 0835, Oxoid) supplemented with ampicillin (SR155). The plates were incubated at 37 °C and characteristic colonies were counted after 24 h. P. aeruginosa were
enumerated by spread plate on the selective growth medium *Pseudomonas Agar Base* (CM 0559, Oxoid) supplemented with *Pseudomonas* C-N Supplement SR 102. The plates were incubated at 37 °C for 24 h. Fluorescent colonies were counted by the use of a UV lamp (Spectroline Model EA-160/FE, Spectronic Cooperation, USA). Twenty fluorescent colonies were confirmed by the API 20E/API 20NE identification kit (bioMérieux, France) and were all identified as *P. aeruginosa*. Heterotrophic bacteria were enumerated by spread plate on R2A agar (Difco™ R2A agar). The plates were incubated at 22 °C and colonies were counted after 7 days. Coliforms and *E. coli* were quantified using Colilert® Quantitray (IDEXX Laboratories) after 18–24 h incubation at 37 °C.

**Experimental set up**

Experiments were performed at laboratory scale in 100 mL glass bottles representing microcosms. Prior to commencing the experiment, glass bottles were acid washed and rinsed with distilled water to remove any carbon substances that could impact on bacterial growth. The glass bottles were then autoclaved for 20 min at 121 °C. The experimental set up consisted of 27 glass bottles containing 30 mL of O2 medium. Nine of the glass bottles were inoculated with *Anabaena*, nine of the bottles were inoculated with *Microcystis* and the final nine bottles were not inoculated with any cyanobacteria, serving as controls (O2-controls) to investigate if the opportunistic pathogenic bacteria were able to grow in pure O2 medium. Three parallels of the *Anabaena*, *Microcystis* and O2-control microcosms were further inoculated with *A. hydrophila* and *P. aeruginosa*, respectively to a final concentration of approximately $10^3$ CFU mL$^{-1}$, or with 0.3 mL fresh sewage.

Inoculated glass bottles were kept on a table with continuous shaking, at constant temperature (20 °C) and continuous light regime (24 h). Samples were withdrawn from the glass bottles at frequent intervals (days 0, 2, 5) in the beginning of the experiment and then more seldom (once a week). Sampling times were set in order to identify differences in growth and/or die-off of the different bacterial strains. The experiment was finished after 42 days. On each sampling day, one sample was withdrawn from each of three parallel microcosms, and analyzed for the biovolume of cyanobacteria and the concentration of heterotrophic bacteria, *A. hydrophila*, *P. aeruginosa*, coliforms and *E. coli* in the respective bottles. All results are presented as the arithmetic mean of the three parallels with standard deviation (SD). All calculations were performed in MS Excel.

**RESULTS AND DISCUSSION**

**Impact of *Anabaena* and *Microcystis* on growth of *P. aeruginosa***

Growth of *P. aeruginosa* was observed in all microcosms during the experimental period of 42 days (Figure 1). Overall, the concentration of *P. aeruginosa* in *Anabaena* microcosms was significantly and in average one log unit

![Figure 1](http://iwaponline.com/wst/article-pdf/64/2/384/444178/384.pdf)
higher than what was observed in the presence of Microcystis and in the O2-control. There was no significant difference in growth rate in Microcystis microcosms and in O2-control. The results suggest that the presence of Anabaena stimulated the growth rate of P. aeruginosa, whereas no such stimulation could be observed in the presence of Microcystis. Anabaena is known to stimulate bacterial growth, and heterotrophic bacteria seem to cluster around the heterocyst (Whitton 1973). Anabaena is also a rich source of organic nitrogen. This may indicate that P. aeruginosa, which can utilize a wide variety of organic compounds as their nutrient and energy source (Frimmersdorf et al. 2010), are further stimulated by nitrogen containing compounds and can be the reason for the positive effects of Anabaena in this study. P. aeruginosa also grew well in the sterile O2-control. This was not surprising since P. aeruginosa is able to grow at very low nutrient levels such as in tap water and as pure culture in distilled water (Mena & Gerba 2009). The approximate similar growth rate of P. aeruginosa in the Microcystis microcosms compared to the O2 control, suggest that there were no compounds in the Microcystis microcosms that stimulated further growth. The significantly lower biovolume of Microcystis cells compared to Anabaena cells (Table 1) may also have caused a too low concentration of compounds stimulating additional growth. Microcystis may also produce compounds that inhibit further growth.

\textit{Pseudomonas aeruginosa} also grew well in the presence of competitive heterotrophic bacteria. High numbers of heterotrophic bacteria were associated with both the Anabaena and the Microcystis culture, being 1–2 log units higher than the added amount of P. aeruginosa. An increase in heterotrophic bacteria was observed during the experiment, but the growth of heterotrophic bacteria did not outnumber or suppress the growth of P. aeruginosa (Figure 1 and Table 1). Consistent with the observations for P. aeruginosa, the highest growth rate of heterotrophic bacteria was observed in the microcosms with Anabaena.

**The impact of Anabaena and Microcystis on growth of A. hydrophila**

A similar growth pattern was observed in both the O2-control and Anabaena microcosms, suggesting that the growth rate of A. hydrophila was not stimulated by the presence of Anabaena (Figure 2). In contrast, no growth of

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### Table 1 | Concentration of heterotrophic bacteria (CFU mL$^{-1}$) and cyanobacterial biovolume (mm$^3$ L$^{-1}$) in control, Anabaena and Microcystis microcosms inoculated with P. aeruginosa

<table>
<thead>
<tr>
<th>Sampling day</th>
<th>Heterotrophic bacteria (CFU mL$^{-1}$)</th>
<th>Biovolume (mm$^3$ L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anabaena microcosms</td>
<td>Microcystis microcosms</td>
</tr>
<tr>
<td>0</td>
<td>$3 \times 10^4$</td>
<td>$1 \times 10^5$</td>
</tr>
<tr>
<td>2</td>
<td>$4 \times 10^5$</td>
<td>$1 \times 10^6$</td>
</tr>
<tr>
<td>5</td>
<td>$3 \times 10^6$</td>
<td>$3 \times 10^6$</td>
</tr>
<tr>
<td>12</td>
<td>$2 \times 10^7$</td>
<td>$4 \times 10^6$</td>
</tr>
<tr>
<td>20</td>
<td>$2 \times 10^7$</td>
<td>$4 \times 10^6$</td>
</tr>
<tr>
<td>27</td>
<td>$7 \times 10^6$</td>
<td>$7 \times 10^6$</td>
</tr>
<tr>
<td>33</td>
<td>$4 \times 10^7$</td>
<td>$2 \times 10^7$</td>
</tr>
<tr>
<td>42</td>
<td>$3 \times 10^7$</td>
<td>$5 \times 10^6$</td>
</tr>
</tbody>
</table>

Each number is the average of three replicates ($n = 3$).
A. hydrophila was observed in the presence of Microcystis, which may indicate growth inhibition of A. hydrophila in association with Microcystis. It is well known that some cyanobacteria may inhibit growth of bacteria (Whitton 1973) and antibacterial properties of Microcystis towards A. hydrophila have been observed by Østensvik et al. (1998).

High numbers of heterotrophic bacteria were associated with both the Anabaena and the Microcystis culture and a further growth of heterotrophic bacteria was observed during the experimental period (Table 2). Consistent with the findings in the microcosms inoculated with P. aeruginosa, the highest growth rate of heterotrophic bacteria was observed in the microcosms with Anabaena, which also contained the highest cyanobacterial biovolume (Table 2).

### Table 2: Concentration of heterotrophic bacteria (CFU mL⁻¹) and cyanobacterial biovolume (mm³ L⁻¹) in control, Anabaena and Microcystis microcosms inoculated with A. hydrophila

<table>
<thead>
<tr>
<th>Sampling day</th>
<th>Anabaena microcosms</th>
<th>Microcystis microcosms</th>
<th>Biovolume (mm³ L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3 × 10⁴</td>
<td>1 × 10³</td>
<td>11,160</td>
</tr>
<tr>
<td>2</td>
<td>3 × 10⁶</td>
<td>1 × 10⁶</td>
<td>417,620</td>
</tr>
<tr>
<td>5</td>
<td>6 × 10⁶</td>
<td>3 × 10⁶</td>
<td>880,256</td>
</tr>
<tr>
<td>12</td>
<td>4 × 10⁷</td>
<td>4 × 10⁶</td>
<td>509,438</td>
</tr>
<tr>
<td>20</td>
<td>5 × 10⁷</td>
<td>1 × 10⁷</td>
<td>576,643</td>
</tr>
<tr>
<td>27</td>
<td>7 × 10⁷</td>
<td>2 × 10⁷</td>
<td>603,070</td>
</tr>
</tbody>
</table>

Each number is the average of three replicates (n = 3).

Sewage was added to simulate an episode of faecal contamination and to observe whether the presence of cyanobacteria affected the growth or survival of E. coli and coliforms. No growth of coliforms or E. coli was observed in the presence of Anabaena or Microcystis, or in the O₂-controls. A rapid die-off was observed in all microcosms. After 5 days, no E. coli (i.e. <10 CFU mL⁻¹) was detected (results not shown) and after 12 days, no coliforms (i.e. <1 CFU mL⁻¹) were detected in any of the microcosms (Figure 3). In contrast, the concentration of heterotrophic bacteria remained relatively stable throughout the experimental period in control and Microcystis microcosms, whereas an approximate increase of one log was observed in the Anabaena microcosms (Table 3).

### CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK

The presence of Anabaena and Microcystis did not affect either growth or survival of E. coli and coliforms. Overall, a higher growth rate of heterotrophic bacteria was observed in microcosms with Anabaena compared to microcosms with Microcystis. The presence of Anabaena also stimulated the growth rate of P. aeruginosa, but had no effect on the growth rate of A. hydrophila. Microcystis did not stimulate the growth rate of P. aeruginosa relative to the control and seemed to have a growth-inhibiting effect on A. hydrophila.
Our findings suggest that cyanobacteria seem to create an environment where heterotrophic and opportunistic pathogenic bacteria thrive and growth is stimulated, although it was also observed that this was not always the case. The present study focused on documentation of growth or death of bacterial species in the cyanobacterial microcosms. More elaborate studies should be undertaken to more closely investigate the mechanisms causing the trends observed. Studies in the natural environment should also be performed to confirm laboratory observations.

The growth stimulating effect of cyanobacteria on some heterotrophs, indicate that lakes with a cyanobacterial bloom could contain higher numbers of opportunistic pathogens than lakes with no blooms and should be taken into consideration during risk assessment of cyanobacterial blooms. So far, risk assessments of blooms have generally focused on the cyanobacteria and their toxins. As seen in this study, heterotrophic bacteria including opportunistic pathogens can grow in association with cyanobacteria and may therefore be responsible for many adverse health affects associated with cyanobacterial blooms. Acute illness resulting from exposure to cyanobacteria or cyanotoxins in drinking water as well as recreational waters could therefore be misdiagnosed.

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