Virulence genes of *Aeromonas* isolates, bacterial endotoxins and cyanobacterial toxins from recreational water samples associated with human health symptoms

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ABSTRACT

Exposure to cyanobacterial water blooms has been associated with various kinds of adverse health effects. In addition to cyanobacteria and their toxins, the bacteria associated with cyanobacteria could also be the etiological agents. We isolated *Aeromonas* strains ($n = 176$) from water samples ($n = 38$) taken from sites where cyanobacteria were suspected to have caused human health symptoms, of which fever and gastrointestinal symptoms were the most common. The isolates were screened by PCR for six virulence gene types (12 genes). The majority (90%) of the strains contained at least one of the virulence genes. Most common amplification products were those of genes (*act*/*aerA*/*hlyA*) that encode cytotoxic enterotoxin and haemolytic products. The genes encoding cytotonic enterotoxins (*ast* and *alt*), phospholipase (*lip*/*pla*/*lipH3*/*alp-1*), elastase (*ahyB*) and flagellin subunits (*flaA*/*flaB*) were also present in 5–37% of the *Aeromonas* strains. Analysed toxins (cyanobacterial hepatotoxins and neurotoxins, and bacterial endotoxins) were not detectable or were present in only low concentrations in the majority of the samples. The results indicated that the toxins were unlikely to be the main cause of the reported adverse health effects, whereas more attention should be paid to bacteria associated with cyanobacteria as a source of health effects.

Key words | *Aeromonas*, cyanobacteria, health effect, toxin, virulence gene, water bloom

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INTRODUCTION

Cyanobacteria produce several secondary metabolites, such as hepatotoxins and neurotoxins, and they contain endotoxic lipopolysaccharides as a part of their cell structure (e.g. Sivonen 2009). These compounds can cause mild to fatal health effects in humans and animals (Kuiper-Goodman *et al.* 1999; Sivonen 2009). Risk analysis concerning mass occurrences of cyanobacteria, the blooms, in water bodies used for recreation has generally focused on the toxins (e.g. Chorus *et al.* 2000). However, the majority of the reported health effects associated with exposure to cyanobacteria do not resemble the characteristic effects of the known toxins (Kuiper-Goodman *et al.* 1999; Stewart *et al.* 2006).

Cyanobacterial blooms foster diverse communities of accompanying heterotrophic bacteria (Eiler & Bertilsson 2004; Kolmonen *et al.* 2004; Berg *et al.* 2009) that may be the source of negative health effects, such as skin rash, flu-like symptoms, eye and ear irritation, gastrointestinal symptoms and pneumonia (Kuiper-Goodman *et al.* 1999; Stewart *et al.* 2006). However, the role of these bacteria as the etiologic agents of the symptoms observed after exposure to the blooms is still unclear.

In our previous study more than 400 heterotrophic bacterial strains associated with cyanobacteria were isolated (Berg *et al.* 2009). The strains represented several genera with putative pathogens, of which the most common was the genus *Aeromonas*. Members of this genus are indigenous to aquatic environments (Martin-Carnahan & Joseph 2005). They can function as pathogens of poikilothermic animals, and their role as a primary cause of illness in humans has also become increasingly evident (Figueras 2005; Janda & Abbott 2010). Suspected human illnesses caused by *Aeromonas* include wound, ocular and respiratory tract infections, septicaemia and meningitis, and *Aeromonas* are also increasingly accepted as a cause of fever and gastroenteric symptoms such as diarrhoea, nausea and vomiting (Figueras 2005; von Graevenitz 2007; Schlenker & Surawicz 2009; Janda & Abbott 2010). In two recent studies, comparison of pulse-field gel electrophoresis patterns and virulence gene patterns between clinical and environmental *Aeromonas* strains indicated that exposure to aeromonads in water may lead to gastroenteritis (Khajanchi *et al.* 2010; Pablos *et al.* 2010).

The infective dose of *Aeromonas* in humans has not been established, but based on studies in animal models by intraperitoneal or intramuscular exposure and limited studies on humans by oral exposure it seems to vary from 10^3 to 10^9 CFU (Carnahan *et al.* 1991; Janda 1991; Han *et al.* 2008; Janda & Abbott 2010). Many of the *Aeromonas* species are resistant to several antibiotics, and the mortality from *Aeromonas* infections in risk groups such as immunocompromised persons, children and the elderly can be high, up to 75% (Janda & Abbott 2010). The infection can progress rapidly and have serious consequences, even among persons with no underlying diseases (Figueras 2005; Martin-Carnahan & Joseph 2005). Furthermore, factors such as the hepatotoxins produced by cyanobacteria can lower the immunity and increase the susceptibility to infection of persons exposed to the cyanobacterial blooms.

The virulence of *Aeromonas* has been suggested to be multifactorial and to include both structural parts of the cell, for example flagella and pili, and several toxins and enzymes (Martin-Carnahan & Joseph 2005; Galindo *et al.* 2006). Various virulence genes have been screened from both clinical and environmental isolates of *Aeromonas*

(e.g. Kingombe *et al.* 1999; Albert *et al.* 2000; Khajanchi *et al.* 2010; Pablos *et al.* 2010). However, surveys of *Aeromonas* strains associated with adverse health effects connected with cyanobacterial blooms have not been carried out previously.

The aim of this study was to investigate the virulence gene contents of *Aeromonas* strains isolated from cyanobacterial blooms that had been connected with adverse human health effects, and to assess the possible role of *Aeromonas* bacteria as the agent causing the adverse health effects.

MATERIALS AND METHODS

Water samples and health symptoms

The water samples (25 fresh water and 13 brackish water samples) were collected from sites where a cyanobacterial bloom was surmised to have caused human health symptoms associated with recreational use of water. The information on adverse human health effects was collected by medical experts through telephone interviews of the exposed persons. The first interview was performed immediately when the symptomatic persons contacted the telephone service. Another interview was performed one or two weeks later in order to acquire information on the duration of the symptoms and to exclude other explanatory causes of the symptoms than exposure to cyanobacteria. The water samples were taken from the exposure sites as soon as possible, usually within a day, after the first interview.

Toxin analyses

The known cyanobacterial toxins were analysed from the water samples. The hepatotoxins (microcystins and nodularins) were measured with enzyme-linked immunosorbent assay (ELISA) test (Envirogard Microcystins Plate Kit, Strategic Diagnostics Inc, Newark, USA) as described by Rapala *et al.* (2002a). The neurotoxins were analysed from the samples that contained on the basis of microscopical analyses putative neurotoxin producers *Anabaena* spp., *Aphanizomenon* spp. or *Planktothrix* spp. The concentrations of the neurotoxins anatoxin-a, homoanatoxin-a, and their epoxy and dihydro degradation products were

analysed with high performance liquid chromatography (HPLC) as described by James *et al.* (1997). The presence of neurotoxic saxitoxins was analysed with the commercial Jellett rapid PSP test strips (Jellett Rapid Testing Ltd, Chester Basin, Canada) as described by Rapala *et al.* (2005) and the putative presence of anatoxin-a(S) with acetylcholine esterase inhibition assay as described by Ellman *et al.* (1961).

The bacterial endotoxin concentrations were analysed with the kinetic chromogenic LAL assay (Bio Whittaker, Walkersville, USA) as described by Rapala *et al.* (2002b). The aerosol exposure at the measured endotoxin concentrations was estimated with the formula for aerosol exposure in showers as described by Anderson *et al.* (2007) using the empirical constants of 0.03 and 0.2.

Heterotrophic colony count and isolation of *Aeromonas* strains

For estimation of total amounts of cultivable heterotrophic bacteria in the samples, the R2A agar medium (Difco, Kansas City, USA) plates and 7 days incubation at $20 \pm 2\,°C$ in the dark were used. Starch ampicillin agar (SAA) was used as the selective medium for isolation of *Aeromonas* strains (Nordic Committee on Food Analysis 2004). The plates were incubated in the dark at $36 \pm 2\,°C$ for 21 ± 3 h or at $20 \pm 2\,°C$ for 72 ± 3 h. In order to obtain pure strains, colonies were picked from SAA plates and subcultured on R2A plates (Difco) in the dark at $20 \pm 2\,°C$. The purity of the strains was assessed by microscopic examination of Gram-stained cells. The pure strains ($n = 170$) were stored at $-70\,°C$ in skimmed milk (Difco) tubes (100 ml of purified water and 20% skim milk, autoclaved at $115\,°C$, for 15 min). In addition, 15 *Aeromonas* strains (AM989203, AM989213–15, AM989230–31, AM989236–38, AM989240–44, AM989247) isolated previously from water samples taken from places where cyanobacteria had been surmised to have caused human health symptoms (Berg *et al.* 2009) were included in the study.

The isolated strains were tested for oxidase and catalase production, and their motility, H_2S and indole production and acid and alkaline reactions caused by mannitol or inositol fermentation, or by ornithine decarboxylation, were tested with AH-tubes as described by Kaper *et al.* (1979).

PCR for the *Aeromonas* virulence genes and 16S rRNA gene sequencing

For the PCR template, cells of each of the 185 isolated bacterial strains were collected from the R2A plates and mixed with 10 µl of sterile de-ionized water. The samples were frozen at $-20\,°C$, thawed and diluted in sterile de-ionized water. For the PCR, 5 µl of the template, 12.5 µl of the Ampli*Taq*™ Gold PCR Master mix ($2\times$, Applied Biosystems, Branchburg, USA) and 0.5 µl of each primer (50 pmol µl^{-1}, 1 µM) were mixed. The volume was adjusted to 25 µl with sterile de-ionized water.

Three primer sets (Thermo Electron Corporation, Ulm, Germany) were used in the *Aeromonas* virulence gene PCR, and one primer set (number 4) was used to control the success of the PCR reaction (Table 1). The PCR was run as described by Sen & Rodgers (2004). The size and yield of the virulence gene PCR products were analysed on 3% agarose gel by electrophoresis, with the MassRuler SM0383 1031-80 bp DNA ladder (Fermentas, Burlington, Canada) as the marker, and visualized by ethidium bromide staining.

The primers used for the amplification of 16S rRNA gene for sequencing were f27 (Sait *et al.* 2003) and r1404 (ACGGGCGGTGTGTA), modified from the universal

Table 1 │ The primer sets used in the *Aeromonas* virulence gene PCR

Primers	Targeted *Aeromonas* gene/virulence factor	Reference
Set 1		
ActF, ActR	*act, aerA, hlyA*/cytotoxic enterotoxin, haemolytic toxins	Kingombe *et al.* (1999)
AstF, AstR	*ast*/cytotonic enterotoxin	Sen & Rodgers (2004)
Set 2		
FlaF, FlaR	*flaA, flaB*/flagellin subunits FlaA and B	Sen & Rodgers (2004)
AltF, AltR	*alt*/cytotonic enterotoxin	
Set 3		
LipF, LipR	*lip, pla, lipH3, alp-1*/phospholipase	Sen & Rodgers (2004)
ElaF, ElaR	*ahyB*/elastase	
Set 4		
A16SF, A16SR	16S rRNA gene	Wang *et al.* (2003)

primer 1392–1406R (Edwards et al. 1989). The PCR thermal-cycling protocol was denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 10 min (modified from Blackwood et al. 2003). The partial 16S rRNA gene sequencing of the strains isolated in this study was done at the Institute of Biotechnology of the University of Helsinki. The partial 16S rRNA gene sequences were deposited in the National Center for Biotechnology Information (GenBank) database under accession numbers EU723887–EU724059.

Taxonomic assignment of the strains

The 16S rRNA gene sequences of the 185 isolated strains were used to assign the strains taxonomically using the Ribosomal Database Project II (RDPII, Cole et al. 2009) taxonomic classifier tool (Naïve Bayesian rRNA Classifier) with annotated sequences (Wang et al. 2007). This tool provides rapid, accurate taxonomic placement down to the genus level of 16S rRNA gene sequences of 400 bp and above. The strains were assigned to the lowest taxonomic hierarchy above a confidence threshold of 80%.

The Sequence Match tool (Cole et al. 2005) of the the RDPII (Cole et al. 2009) was used to search for the closest type strains to the isolates. The 16S rRNA gene sequences of the isolated strains and their closest type strains were further compared using the BioEdit Sequence Alignment Editor, version 7.0.5.3 (Hall 1999), pairwise alignment and sequence similarity calculation tools.

Statistical analysis of the data

The associations between the Aeromonas strains and the detected health symptoms, virulence genes, physiology and origin of the strains were studied using a multivariate multiple regression analysis with a forward selection (McArdle & Anderson 2001). The analysis was done with the distance-based multivariate analysis for a linear model with forward selection (DISTLM forward) program (Anderson 2003a). The 141 strains with no missing values were included in the analysis. The number of permutations was 9,999 and the chosen integer 12. The distance matrix of the aligned 16S rRNA gene sequences was based on the F84 model and calculated by the Phylip dnadist program (Felsenstein 1993)

on the Mobyle portal of the Pasteur Institute. The 16S rRNA gene sequence alignment was done with the BioEdit Sequence Alignment Editor, version 7.0.5.3 (Hall 1999).

The possible grouping which could be use as a priori groups in statistical canonical discriminant analysis (CAP, Anderson & Robinson 2003; Anderson 2004) of the strains was first visualized by principal coordinate analysis (PCO) using PCO program (Anderson 2003b). The grouping was based on the 16S rRNA gene sequence distances by the F84 model used also in the multivariate multiple regression analysis. The 176 strains assigned as Aeromonas based on the taxonomic classifier tool analysis were included in the PCO analysis.

Differences in the a priori groups of multivariate observations were tested by canonical discriminant analysis using the canonical analysis of principal coordinates (CAP) program (Anderson 2004) which allows groups of different size to be analysed. The same 141 strains with no missing values that were included in the multivariate multiple regression analysis, were included in CAP analysis. The chosen distance measure was chi-squared distance, the number of permutations 9,999 and the integer 12.

RESULTS

The bacterial strains isolated in this study originated from aquatic samples that were associated with various human disease symptoms after recreational contact with cyanobacteria. The most common symptoms were fever and gastrointestinal symptoms (Figure 1, Appendix A, available online at http://www.iwaponline.com/jwh/009/206.pdf).
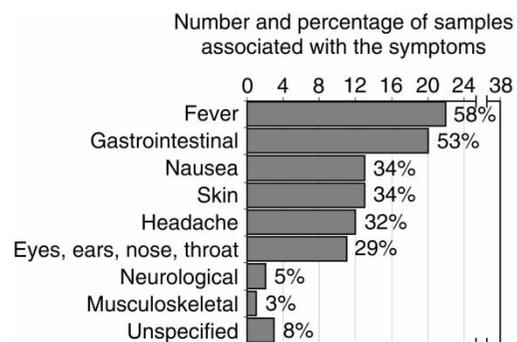

Figure 1 | Human health symptoms associated with the water samples.

Of the analysed toxins, the median concentrations of the measured hepatotoxins (microcystins and nodularin) and neurotoxins (anatoxin-a, homoanatoxin-a, and their dihydro and epoxy degradation products) were below the detection limits (Table 2, Appendix A, available online at http://www.iwaponline.com/jwh/009/206.pdf). Signs of the presence of anatoxin-a(S) were detected in one sample and all samples were negative for saxitoxins.

The bacterial endotoxin concentrations (including also cyanobacterial endotoxins) were between 10 and 300 EU ml^{-1} in the majority (89%) of the samples, with a median of 58 EU ml^{-1} (Table 2, Appendix A, available online at http://www.iwaponline.com/jwh/009/206.pdf). The estimated aerosol concentrations with the majority (89%) of the samples varied between 0.3 and 60.4 EU m^{-3}, with total range of 0.3–2,108 EU m^{-3} (data not shown). The median calculated with the empirical constants 0.03 and 0.2 were 2 EU m^{-3} and 12 EU m^{-3}, respectively.

Based on the results, the bacterial endotoxins concentrations were low and the concentrations of cyanobacterial hepatotoxins and neurotoxins remained under the detection limit in the majority of the samples.

The concentration of presumptive *Aeromonas* spp. in the water samples varied between 5 and 30,000 CFU ml^{-1}, with a median of 68 CFU ml^{-1} (see Appendix A: http://www.iwaponline.com/jwh/009/206.pdf). The concentration of heterotrophic bacteria varied between 770 and 3,727,000 CFU ml^{-1}, with a median of 8,000 CFU ml^{-1}. The proportion of the *Aeromonas* CFU ml^{-1} to the heterotrophic CFU ml^{-1} ranged between <0.1 and 19.2%, with a median of 0.9%.

The 16S rRNA genes were partially (397–481 bp) sequenced from 170 of the isolated strains, originating from 38 water samples. Of these, 161 (95%) were assigned to the genus *Aeromonas* with a threshold value of 100%, based on RDPII taxonomic classifier analysis. In addition, six strains were assigned to the genus *Chryseobacterium*, one strain to the genus *Pantoea* and two to the family Enterobacteriaceae (see Appendix B online at http://www.iwaponline.com/jwh/009/206.pdf). In the principal coordinate analysis the studied *Aeromonas* strains formed three genogroups, based on their 16S rRNA gene sequence distances (Table 3). The majority

Table 2 │ Cyanobacterial toxins and bacterial endotoxins in the 38 water samples associated with adverse human health effects

Toxins	Toxin concentration or presence	Number of samples	Median of the concentrations
	Concentration		
Hepatotoxins[a]	<0.1 µg l^{-1}[b]	21	<0.1 µg l^{-1}
	0.1–12.3 µg l^{-1}	13	
	3,050 µg l^{-1}	1	
	na	3	
Anatoxin-a[c]	<dl[d]	18	<dl
	1.9–10.3 µg l^{-1}	4	
	na	16	
Endotoxin	10–300 EU ml^{-1}	34	58 EU ml^{-1}
	1,240–3,179 EU ml^{-1}	3	
	10,540 EU ml^{-1}	1	
	Presence		
Anatoxin-a(S)	Negative	30	
	Weak positive	1	
	na	7	
Saxitoxins	Negative	27	
	na	11	

na, sample not analysed; dl, detection limit.
[a]Microcystins and nodularin.
[b]0.1 µg ml^{-1} is the detection limit of the ELISA test.
[c]Anatoxin-a, homoanatoxin-a, and their epoxy and dihydro degradation products.
[d]Detection limit dependent on the volume of the concentrated sample.

Table 3 │ Genogroups and the closest type strains of the *Aeromonas* strains

Genogroup/ No. of strains[a]	Closest type strains (accession number)[b]	Number and percentage (%) of the strains
I/86	*A. veronii*[T] (X60414)/ *A. ichthiosmia*[T] (X71120)	77 (44)
	A. jandaei[T] (X60413)	10 (6)
II/80	*A. salmonicida* sp. *salmonicida*[T] (AY987751)/sp. *masoucida*[T] (AB027542)	42 (24)
	A. popoffii[T] (AJ224308)	25 (14)
	A. sobria[T] (X60412)	12 (7)
	A. encheleia[T] (AJ224309)	2 (1)
III/8	*A. hydrophila* sp. *hydrophila*[T] (DQ207728)	7 (4)
	A. media[T] (X60410)	1 (1)

[a]Of the total 176 *Aeromonas* strains, two strains were not assigned to genogroups because they were placed separately from the rest of the strains in the principal coordinate analysis.
[b]The 16S rRNA gene sequences of *A. veronii*[T] (X60414) and *A. ichthiosmia*[T] (X71120) as well as *A. salmonicida* sp. *salmonicida*[T] (AY987751) and *A. salmonicida* sp. *masoucida*[T] (AB027542) were identical along the segment used in this study.

of the *Aeromonas* strains had either *Aeromonas veronii/ Aeromonas ichthiosmia* or *A. salmonicida* as their closest type strain, based on their partial 16S rRNA gene sequences (Table 3, Appendix B, available online at http:// www.iwaponline.com/jwh/009/206.pdf).

The majority (90%) of the *Aeromonas* strains contained one or more of the screened virulence gene types (Figure 2(a) and (b), Appendix B, available online at http:// www.iwaponline.com/jwh/009/206.pdf). The *act* was present in 77% and the gene segments for *fla, ahyB* and *lip* in 30–37% of the strains (Figure 2(a)). In the genogroup III, 76% of the strains contained 4–6 of the screened virulence gene types (Figure 2(b)). In the genogroup II, 91% of the strains contained 2–4 of the virulence gene types and 96% of them at least one type, while in the genogroup I, 65% of the strains contained only one of the gene types. The highest number of the gene types in the genogroup I was three. This implied that the strains in genogroups II and III were potentially more virulent than the strains in the genogroup I.

The three *Aeromonas a priori* genogroups derived from the principal coordinate analysis diverged from each other because of differences in the presence of the virulence gene types ($p = 0.001$) in the canonical discriminant analysis (Figure 3, Table 4). The *lip* and *fla* genes were the explanatory variables associated the most strongly with the genogroup II, whereas the *ast* gene had a strong association with the genogroup III. The indole reaction and mannitol fermentation had the strongest association with the genogroup I. The results confirmed that the potentially most virulent *Aeromonas* strains belonged to the genogroups II and III.

DISCUSSION

Virulence genes were detected in the majority of the 176 *Aeromonas* strains isolated from the samples that were associated with disease symptoms surmised to be caused by exposure to cyanobacteria. The high frequency of virulence genes in the
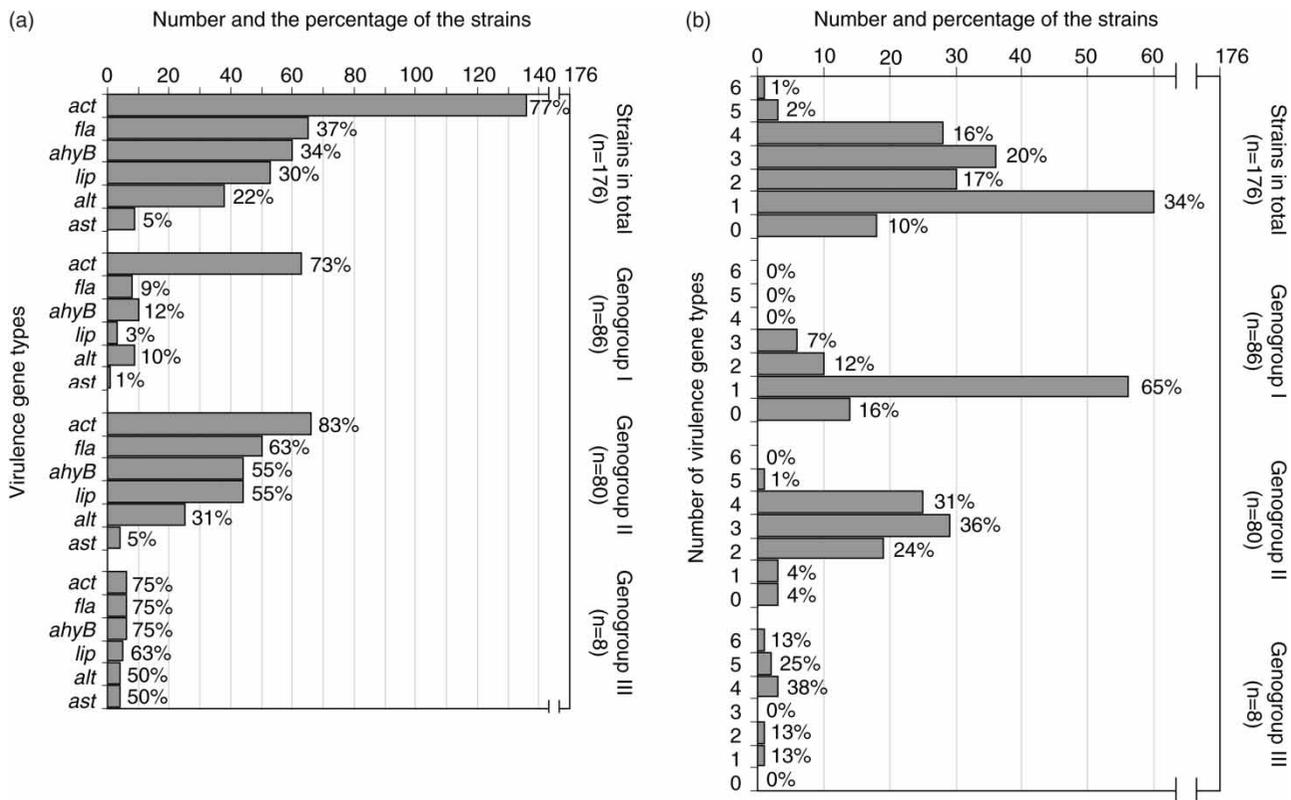

Figure 2 │ Virulence genes of *Aeromonas* strains in total and in different genogroups. (a) Type of detected virulence genes. (b) Number of detected virulence gene types. The *act* refers to the cytotoxic enterotoxin and haemolytic genes (*act/aerA/hlyA*), *ast* and *alt* to cytotonic enterotoxin genes, *ahyB* to the elastase gene, *lip* to phospholipase genes (*lip/pla/lipH3/alp-1*) and *fla* to flagellin subunit A and B genes (*flaA/flaB*).
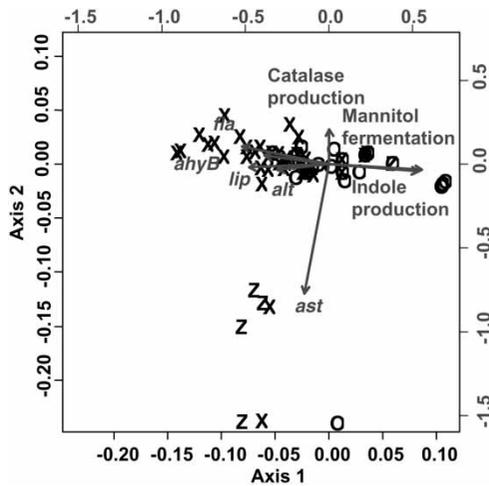
Figure 3 | The canonical correlation plot showing the grouping of the *Aeromonas* strains and their association with the eight explanatory variables with the highest discrimination values. The O refers to genogroup I (76 strains), the X to genogroup II (59 strains) and the Z to genogroup III (6 strains). The misclassification error of the analysis was 15%.

Table 4 | Explanatory values from multivariate multiple regression and correlation values from canonical discriminant analysis

Variable[a]	Explanatory value[b] Percentage (%) explained	p-value	Correlation value[c] To axis 1	To axis 2
lip/pla/lipH3/alp-1 genes	24.55	0.0001	0.610	0.002
alt gene	15.84	0.0001	0.407	0.077
ast gene	4.91	0.0004	0.218	0.923
flaA/flaB genes	2.82	0.0014	0.709	0.049
ahyB gene	2.18	0.0034	0.429	0.037
Mannitol fermentation	8.63	0.0001	−0.559	0.229
Indole production	1.42	0.0125	−0.581	0.196
Catalase production	1.11	0.0379	−0.054	−0.032

[a]Variables that had a *p*-value of <0.05 and explained >1% of the proportion of 16S rRNA gene sequence variability in the multivariate multiple regression analysis are included in the table.
[b]Refers to the proportion of 16S rRNA gene sequence variability explained.
[c]The *p*-value of the analysis was 0.0001 and the misclassification error 13%.

Aeromonas strains implies their pathogenic potential and suggests that *Aeromonas* may play a role in the adverse health effects associated with cyanobacterial blooms.

Of the virulence gene types, the cytotoxic enterotoxin and haemolytic gene products encoding *act* (*act/aerA/hlyA*) was present in the majority of the studied *Aeromonas* strains. It was common among strains of all three genogroups. Fever and gastrointestinal symptoms were the most commonly detected health problems. The cytotoxic enterotoxin encoded by the *act* gene induces an inflammatory response in host cells (Galindo *et al.* 2006) that can contribute to systemic effects, such as fever. The presence of the *act* gene has also been associated with bloody diarrhoea, and in mouse tests deletion of the *act* gene reduced fluid secretion by 64% compared with secretion induced by wild type *Aeromonas* (Albert *et al.* 2000; Sha *et al.* 2002). The haemolytic toxins encoding *aerA* and *hlyA* genes have also been shown to contribute to the virulence of *A. hydrophila* (Heuzenroeder *et al.* 1999; McCoy *et al.* 2010).

The *alt* gene was also common in the *Aeromonas*, especially among the strains of the genogroup II and III. This cytotonic enterotoxin-encoding gene has previously been found to be associated with gastrointestinal symptoms (Albert *et al.* 2000; Sha *et al.* 2002; Khajanchi *et al.* 2010; Pablos *et al.* 2010). Therefore, the abundance of the *act* and *alt* genes in the strains isolated from the cyanobacterial water bloom samples supports the conclusion that *Aeromonas*, not the cyanobacterial toxins, may play the key role as an explanatory factor of the detected gastrointestinal and fever symptoms. Moreover, synergism of different *Aeromonas* types that contain distinct virulence gene sets, may contribute to the adverse health effects associated with the blooms. However, as the virulence gene profiles of the three genogroups were clearly different, specific *Aeromonas* types are likely to be of different health importance.

The cyanobacterial hepatotoxins and neurotoxins were not detected in the majority of the samples. The endotoxin concentrations detected in this study (median 58 EU ml^{-1}) were also low. They were lower than those detected previously by Rapala *et al.* (2002b) from cyanobacterial bloom samples (median 2.9×10^2 EU ml^{-1}). The concentrations were more consistent with endotoxin concentrations detected previously from pristine and agriculturally impacted surface waters and in several occasions also from treated drinking water (Anderson *et al.* 2002; Rapala *et al.* 2002b). Very little information about the effects of endotoxin exposure by ingestion is available (Anderson *et al.* 2002, 2007). A guideline of 10 ng m^{-3} (~100 EU m^{-3}) for no observed effects of airway inflammations and 100 ng m^{-3} (~1,000 EU m^{-3}) for no observed systemic effects have been proposed for occupational exposure

(Anderson *et al.* 2007). With the endotoxin activity detected from the majority of the water samples in our study, the estimated aerosol concentrations ($<$1–60 EU m^{-3}) were well below the 100 EU m^{-3}. Microcystin concentrations of 10 µg l^{-1} or below are considered to present a relatively low probability of adverse health effects (Falconer *et al.* 1999). Based on the rarity of the detected cyanobacterial hepatotoxins and neurotoxins and the low concentrations of the bacterial endotoxins in this study, they were very unlikely to be the cause of the reported gastrointestinal and fever symptoms of the exposed persons. However, when present, the toxins and their possible adverse health effects may assist the development of harmful health effects caused by other factors, such as the associated heterotrophic bacteria.

The assignment of the isolated strains to the genus *Aeromonas* was reliable with high threshold values. The distribution of the strains into the three genogroups in relation to their closest *Aeromonas* type strains was consistent with previously observed, 16S rRNA gene sequence based, phenotypic relationships of the *Aeromonas* species (Martin-Carnahan & Joseph 2005; Martínez-Murcia *et al.* 2005). However, the *Aeromonas* species exhibit high 16S rRNA gene sequence similarity within the genus (Martin-Carnahan & Joseph 2005; Martínez-Murcia *et al.* 2005). For example, the 16S rRNA gene sequences of the *A. salmonicida* and *A. bestiarum* type strains differ only by two nucleotides (Martínez-Murcia *et al.* 2005). More detailed species assignment would require comparison of additional genes such as the housekeeping genes *gyrB* and *rpoD* (Martínez-Murcia *et al.* 2005; Janda & Abbott 2010). Therefore, the taxonomic identification of the isolated strains in this study is reliable only to genus level.

The detected *Aeromonas* colony counts were similar to those measured previously from clean rivers and lakes (1–10^2 CFU ml^{-1}) and rivers receiving sewage discharges (10–10^4 CFU ml^{-1}; World Health Organization 2002). They were slightly elevated compared with *Aeromonas* counts measured by Rapala *et al.* (2002b) from cyanobacterial bloom samples collected previously from Finnish freshwaters (median 7 CFU ml^{-1}). The results are in accordance with our previous study, where *Aeromonas* was the most common genus with putative pathogens isolated from cyanobacterial blooms (Berg *et al.* 2009), and support the prevalence of *Aeromonas* in association with cyanobacteria.

CONCLUSIONS

The cyanobacterial hepatotoxins and neurotoxins that are often suspected to be the causative agents of adverse health effects associated with cyanobacterial water blooms were rarely detected in significant concentrations from the water samples of this study. The measured bacterial endotoxin concentrations were also low. Therefore the studied toxins were unlikely to be the cause of the most commonly reported gastrointestinal and fever symptoms. Instead, virulence genes, such as the *act* and *alt* genes that have been associated with gastrointestinal symptoms, were abundant among the *Aeromonas* strains isolated from the water samples, indicating that *Aeromonas* bacteria may be the causative agent of some the reported health symptoms. The study supports the importance of taking the bacterial community into consideration when assessing the possible health impacts of cyanobacterial water blooms and the precautions taken against these impacts. This is also important for evaluation of the possible co-operative actions that cyanobacterial toxins, bacterial endotoxins and bacterial activity may have for the adverse health effects.

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