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Bacterial communities' response to microcystins exposure and nutrient availability: Linking degradation capacity to community structure

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ABSTRACT

Eutrophication of freshwater bodies followed by cyanobacterial bloom and toxin production is an important issue in freshwater supply in both developed and developing countries. The primary mechanism for microcystins (MCs) (the main class of cyanobacterial toxins) dissipation is microbial degradation. Repeated exposure of freshwater bodies to cyanobacterial toxins MCs may affect indigenous microbial communities and may also enhance biodegradation of MCs, but the factors driving this relationship remain unclear. Six Scottish freshwater bodies with different histories of natural exposure to MCs and ability to degrade MC-LR (the most common microcystin) were chosen as case study. Terminal Restriction Fragment Length Polymorphism (T-RFLP) and Biolog EcoPlate™ were used to study the structure and physiology of the bacterial communities. Previous exposure to MCs significantly contributed to the bacterial communities shape and microbial physiology of the water bodies under study. Other factors that significantly affected the bacterial communities were dissolved organic carbon and concentration of nitrogen compounds as well as temperature. Moreover a significant relationship was found between bacterial communities' structure and MC-LR half-life. These data suggest that exposure to MCs drives changes in structure and physiology of bacterial communities and in turn those communities differentially perform degradation of MC-LR.

1. Introduction

Freshwater is one of the most precious natural resource on the planet. Natural waters have very low concentrations of nitrates and phosphorous. Runoff from farm lands, along with wastewater deriving from urban and industrial activities increase nutrient loads. Eutrophication, a higher nutrient (e.g. nitrates and phosphorus) concentration, and high temperatures stimulates cyanobacterial blooms of inland water bodies (Chorus and Bartram, 1999; Sharpley et al., 2003). The blooms represent an overgrowth of cyanobacteria, which are a diverse group of ancient autotrophs that occur globally. The increased incidence of toxic cyanobacterial blooms represent a hazard for human and animal health (Chorus and Bartram, 1999; Chorus et al., 2000). The toxicity of the cyanobacterial bloom is due to the presence of a wide range of toxins produced by cyanobacteria: microcystins and nodularins (hepatotoxins and carcinogens), saxitoxins and anatoxins (neurotoxin), and cylindrospermopsin (protein synthesis inhibitor) (Edwards and Lawton, 2009). Microcystins (MCs) are the most common cyanotoxins and may be expected wherever blooms of cyanobacteria occur in surface water. Their occurrence is highly likely when these blooms consist of the taxa *Microcystis*, *Anabaena*, or *Planktothrix* (Chorus and Bartram, 1999). MCs are chemically stable in water (Jones and Orr, 1994; Harada et al., 1996) and resistant to eukaryotic and many bacterial peptidases (Dierstein et al., 2001), but susceptible to breakdown by some aquatic bacteria found naturally in diverse water bodies (Jones et al., 1994). More than 70 different MCs have been characterized and MC-LR has been the

Abbreviations: MCs, microcystins; MC-LR, microcystin-LR; T-RFLP, terminal restriction fragment length polymorphism; DOC, dissolved organic carbon; HPLC, high performance liquid chromatography; BSA, bovine serum albumin; TRF, terminal restriction fragment; OTU, operational taxonomic unit; AWCD, average well colour development; PCA, principal components analysis; ANOVA, analysis of variance; CVA, canonical variate analysis; RDA, redundancy analysis; MANOVA, multivariate analysis of variance.

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most studied due to its high toxicity and frequent production. Toxicity of MCs have been described for animals (Milutinović et al., 2003; Žegura et al., 2008) and plants (Mcelhiney et al., 2001), while their ability to affect microbial communities structure was shown by Christoffersen et al. (2002). The detrimental effects of MCs on a broad spectrum of living organisms and their effect on ecosystem functioning (Codd et al., 2005) requires adequate ways for screening toxicity and for evaluating water quality of exposed water bodies. A number of works has been published investigating biodegradation of MCs in freshwater (Jones and Orr, 1994; Cousins et al., 1996) and some reported a link between previous exposure to MCs and rate of degradation (Christoffersen et al., 2002; Edwards et al., 2008). However the mechanisms that dictate the relation between past exposure and degradation rate have not been elucidated. Microorganisms and in particular bacteria have been studied in a number of ways through history starting from observation with magnifying glass by Antony van Leeuwenhoek back in the 17th century (Van leeuwenhoek, 1702) to arrive to newly-developed high-output DNA sequencing (Gobet et al., 2012). Different methods measure different parameters (e.g. morphology, physiology, biochemistry, molecular biological structure and diversity) of the bacterial communities and some authors showed how different methods can lead to diverse results (Grayston et al., 2004; Singh et al., 2006). Here we used T-RFLP to evaluate bacterial communities' structure and Biolog EcoPlate to determine their physiology. We investigated the relation between bacterioplankton communities structure and physiology of six Scottish water bodies previously studied by Edwards et al. (2008) and: a) past exposure to MCs; b) half-life of MC-LR; c) water chemical and physical parameters.

2. Material and methods

2.1. Sites and water sampling

Six Scottish water bodies were selected to include various cyanobacterial bloom exposure histories and MC-LR exposure. The water bodies chosen were Loch Rescobie (NO 525 515) and Loch Balgavies (NO 523 516) two closely located lakes with the outflow from the former flowing into the later via a small stream (approximately 600 m long) with both waterbodies supporting populations of microcystin producing cyanobacteria. Loch Forfar (NO 450 507) approximately 7 km to west of these lakes annually supports cyanobacterial blooms however microcystins have never been detected here. There is no direct water flow between any of these lakes. Loch Leven (NO 132 018) which is located 60 km south west of Forfar Loch and often supports cyanobacterial growth but microcystins have only been detected on a couple of occasions over a 20 year sampling period. River Carron (NO 877 857) and River Cowie (NO 876 864) are closely located rivers around 50 km north east of Forfar Loch and as fast-flowing rivers have no previous history of significant cyanobacterial growth and no microcystin occurrence. All water sources were also selected since they have been involved in a number of previous studies and their history of supporting cyanobacteria and microcystin was known (Personal observations). Details about cyanobacterial bloom history, MC-LR exposure and half-life of MC-LR in these water bodies are outlined in Table 1. Surface water samples were collected in triplicate on 26 September 2007 from the selected water bodies in sterile 1 l Duran glass bottles and stored at 4 °C over night until analysed. Surface water temperature and pH were measured at the site using a thermometer and pH metre (Jenway, Essex, UK). Water samples were filtered (0.45 μm cellulose acetate: Whaan, Kent, UK) and dissolved nitrogen (NO_3, NO_2, NH_4, and TN) and phosphate (PO_4 and TP) were determined colourimetrically using a San++ analyser (Skalar, Breda, the Netherlands). Dissolved organic nitrogen and phosphate were calculated as the difference between total and inorganic values. Dissolved organic carbon (DOC) was determined automatically at 550 nm following persulphate/UV digestion (Schreurs, 1978). Samples for T-RFLP were immediately filtered in aliquots of 1 l onto 0.22 μm pore size membrane filters (Millipore Stericup). The filters were removed in sterile conditions from the disposable filter units and stored in sterile Petri dishes at −20° C until DNA extraction.

2.2. Biodegradation of MC-LR

The half-life data of MC-LR were derived from the work of Edwards et al. (2008) for the same waterbodies and were analysed for statistical linkage between the microbial community structure and rate of degradation. In brief, water samples were prepared by placing 50 ml of freshly collected water in 100 ml sterile Erlenmeyer flasks stoppered with cotton wool bungs. MC-LR (final concentration of 1 μg ml^{-1}) was added aseptically in triplicate, to water samples and sterile control water samples (i.e. autoclaved). Incubation was at 29 °C shaking at 100 rpm. Aliquots (500 μl) for analysis were taken aseptically every 3–4 days, frozen, freeze-dried, reconstituted in 80% aqueous methanol and centrifuged at 15 000 × g then the supernatant analysed by HPLC (Edwards et al., 2008). The ability of microbial communities from the water bodies studied in this work to degrade MC-LR has been further investigated in other studies (Ghimire, 2007; Manage et al., 2009a) giving consistent results.

2.3. T-RFLP analysis

DNA was extracted from half of the polyethersulfone filter (Millipore Stericup) stored at −20 °C using the Power Soil DNA Extraction Kit (Mo Bio, Carlsbad, California) following the manufacturer's

Table 1
Water chemistry, MCs natural exposure and MC-LR half life for Scottish freshwater bodies under study. Water chemistry analysis done within 24 h of sampling.

	Loch Rescobie	Loch Forfar	Loch Balgavies	Loch Leven	River Carron	River Cowie
NH_4-N (μg ml^{-1})	0.46 ± 0.01	0.65 ± 0.04	0.17 ± 0.03	0.05 ± 0.00	0.08 ± 0.04	0.12 ± 0.06
NO_3-N (μg ml^{-1})	0.74 ± 0.00	2.22 ± 0.01	0.57 ± 0.00	0.07 ± 0.01	6.14 ± 0.08	1.12 ± 0.83
Total-N (μg ml^{-1})	1.75 ± 0.04	3.25 ± 0.06	1.36 ± 0.02	0.59 ± 0.03	6.33 ± 0.04	2.26 ± 0.13
Org-N (μg ml^{-1})	0.55 ± 0.09	0.37 ± 0.06	0.62 ± 0.01	0.48 ± 0.03	0.11 ± 0.10	0.53 ± 0.12
PO_4-P (μg ml^{-1})	0.13 ± 0.01	0.29 ± 0.02	0.22 ± 0.01	0.04 ± 0.06	0.04 ± 0.00	0.02 ± 0.01
Tot-P (μg ml^{-1})	0.15 ± 0.03	0.38 ± 0.05	0.29 ± 0.02	0.06 ± 0.06	0.04 ± 0.00	0.05 ± 0.03
Org-P (μg ml^{-1})	0.02 ± 0.01	0.09 ± 0.04	0.08 ± 0.02	0.02 ± 0.00	0.01 ± 0.01	0.03 ± 0.02
DOC (μg ml^{-1})	7.68 ± 0.28	5.83 ± 0.04	8.52 ± 0.33	6.76 ± 0.37	3.78 ± 0.53	9.09 ± 0.52
NO_2–N (μg ml^{-1})	0.03 ± 0.00	0.06 ± 0.00	0.03 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
MCs exposure[a]	Regular	No	Regular	Occasional	No	No
pH	11.9 ± 0.00	7.4 ± 0.00	7.8 ± 0.00	8.5 ± 0.00	7.9 ± 0.00	7.8 ± 0.00
Temperature	11.1 ± 0.00	10.5 ± 0.00	11.6 ± 0.00	9.5 ± 0.00	8.6 ± 0.00	8.2 ± 0.00
MC-LR half-life (days) (source Edwards et al., 2008)	4	9	4	5	13	14

[a] Unpublished data from authors sampling over a period of >20 years. Regular = microcystins always found during typical bloom season (June–September), Occasional = microcystins only detected twice in >20 years, No = microcystins never detected in these locations (Lawton, Personal observations).

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instructions. PCR reaction was performed on extracted DNA for each sample to amplify the universal bacterial 16S rRNA genes. Briefly, PCR was performed in a final volume of 50 μl containing: 1 × NH_4 reaction buffer, 2 mM $MgCl_2$, 400 μM of each deoxynucleoside triphosphate, and 2.5 U of Biotaq DNA polymerase (all reagents from BIOLINE, UK), 20 μg bovine serum albumin (BSA, Roche Diagnostics, UK) and 5 μl of template DNA. Bacterial primers used were 63F-VIC (Marchesi et al., 1998) and 1087R (Hauben et al., 1997) were used at a concentration of 200 nM. PCR reactions were performed with a DYAD DNA Engine Peltier thermal cycler (MJ Research, Waltham, MA). The cycle consisted of 5 min at 95 °C, followed by 30 cycles of denaturing at 94 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 1 min, and a last cycle of 10 min extension period at 72 °C. PCR products were visualised with ethidium bromide staining on a 1% (w/v) agarose gel using UV radiation. PCR products were then purified using ChargeSwitch® PCR Clean-Up Kit (Invitrogen) following manufacturer instructions.

For the restriction digestion, 250 ng of purified PCR product were digested with 20 U of Hha I and 2 μl of buffer in a final volume of 20 μl containing 0.1 mg ml^{-1} of acetylated BSA (all reagents from Promega, UK). Samples were incubated at 37 °C for 3 h followed by 15 min at 95 °C to inactivate the enzyme. After digestion, 2 ml of each sample were mixed with 0.3 ml of LIZ-Labelled GS500(-250) internal size standard and 12 ml of formamide (Applied Biosystems, UK) and denatured at 95 °C for 5 min, then chilled on ice for 5 min. Fragment size analysis was carried out with an ABI PRISM3130xl genetic analyser (Applied Biosystems, UK).

Bacterial T-RFLP profiles were produced using GeneMapper software (version 3.7; Applied Biosystems, UK) and fragments quantified using the advanced mode and second-order algorithm. Selected fragments ranged from 35 to 500 basepair (bp): these were within the linear range of the internal size standard used while excluding primer dimers and probable undigested products. All TRFs with fluorescence units less than 35 were discarded from the data analysis to minimize the effect of artefacts. The relative abundance of a terminal restriction fragment (TRFs) in a profile was calculated as a proportion of the total peak height of all the TRFs in a profile. Any peak that was less than 0.5% of the total fluorescence unit was removed from the data before statistical analysis.

2.4. Biolog EcoPlate analysis

Physiological bacterial communities profiles were detected by the Biolog EcoPlates (Catalog no.1506, Biolog Inc., Hayward, CA 94545, USA) which contain 96 wells with 31 different carbon sources in triplicate and the other 3 micro wells do not have any source of carbon and are used as control. All water samples were processed under sterile conditions within 24 h of collection from the field. Water samples (160 μl) were inoculated into each well of EcoPlate (Biolog) and wrapped with wet paper towel to maintain humidity and incubated at 25 ± 1 °C in dark. The metabolism of each carbon source was measured spectrophotometrically (A_{590}) by reduction of tetrazolium violet to formazan. Readings were performed every 24 h for a period of 14 days using of a microplate reader (V_{max}, Molecular Devices, Oxford, UK) subtracting the absorbance of the control well (without carbon source).

2.5. Statistical methods

For T-RFLP profile data (relative abundance), principal components analysis (PCA) using a co-variance similarity matrix was used. ANOVA was carried out on the principal component (PC) scores for the first five dimensions to examine the effects of sampling site and previous exposure to MC-LR on the bacterial communities. Data were also explored using canonical variate analysis (CVA) on the first 5 PC scores. Linear regression analysis was done to test the relation between the degradation rate (i.e. half-life) of MC-LR reported by Edwards et al. (2008) and structure (T-RFLP principal components) of the bacterial communities present in the water bodies under study. In order to reveal relationships between bacterial communities and environmental variables, a redundancy analysis (RDA) was used with the CANOCO software 4.5 (Microcomputer Power, Ithaca). RDA was used to examine a number of physico-chemical factors (i.e. pH, temperature, DOC, total-N, Org-N, NH_4-N, NO_3-N, NO_2-N, Tot-P, Org-P, PO_4-P) affecting the bacterial population (T-RFs). T-RFs relative abundance data were log transformed before the analysis. The environmental variables that significantly influenced the bacterial population were identified by forward selection (Braak and Verdonschot, 1995), eliminating factors which failed to improve significantly ($P < 0.05$) the explanatory model. This was achieved using a Monte Carlo permutation test was carried out (999 random permutations).

For Biolog EcoPlate™ the average well colour development (AWCD) of all 31 carbon sources for each sample was calculated and used to transform individual well values to eliminate variation in AWCD caused by different cell densities (Garland, 1996). The AWCD of different substrate groups (i.e. polymers, phenolic compounds, carboxylic acids, carbohydrate, amino acids and amines) was calculated and treatment effects assessed by a one way ANOVA (site). The Biolog data for utilisation of 31 carbon sources were also analysed using two forms of multivariate analysis, firstly by principal components analysis (PCA) to reduce the dimensionality in the data arising from having more variates than samples and then by canonical variate analysis (CVA) on the first 5 PC scores. All analyses were carried out using GenStat version 11 (VSN International Ltd., Hempstead, UK).

3. Results

3.1. Chemistry and properties of freshwater bodies

The water chemical and physical properties along with natural exposure to MCs and previously determined half-life of MC-LR, are reported in Table 1.

3.2. T-RFLP

For bacterial T-RFLP data, the first five PC accounted for 88.97% of the variance. ANOVA of the PC scores revealed that the bacterial community was significantly affected by sampling site on the first ($P = 0.023$), third ($P < 0.001$), fourth ($P < 0.001$) and fifth dimensions ($P = 0.014$). The CVA analysis on the PC scores (Fig. 1A) revealed a significant separation between bacterial communities associated with all the lakes under study while the communities associated with River Carron and River Cowie clustered together. Non-overlapping circles in CVA biplot shows significantly different bacterial communities ($P < 0.05$), the major axis (i.e. CV1) accounts for most of the variability of the system and shifts along this axis are to be considered more relevant than shift on the second axis (i.e. CV2). Shannon diversity calculated on T-RFs relative abundance did not show significant differences amongst the different sampling sites. ANOVA on the PC scores revealed that bacterial communities were also significantly affected by natural exposure to MC-LR on the first ($P = 0.04$), fourth ($P = 0.002$) and fifth dimension ($P = 0.033$). MANOVA analysis on the 5 PC scores showed a significant ($P < 0.001$) effect of natural exposure of MC-LR on bacterioplankton communities. CVA biplot (Fig. 1B) revealed significant shifts in the structure of the bacterial community due to natural exposure to MC-LR.

Linear regression analysis carried out to explore whether bacterial community structure had impact on rate of MC-LR degradation.

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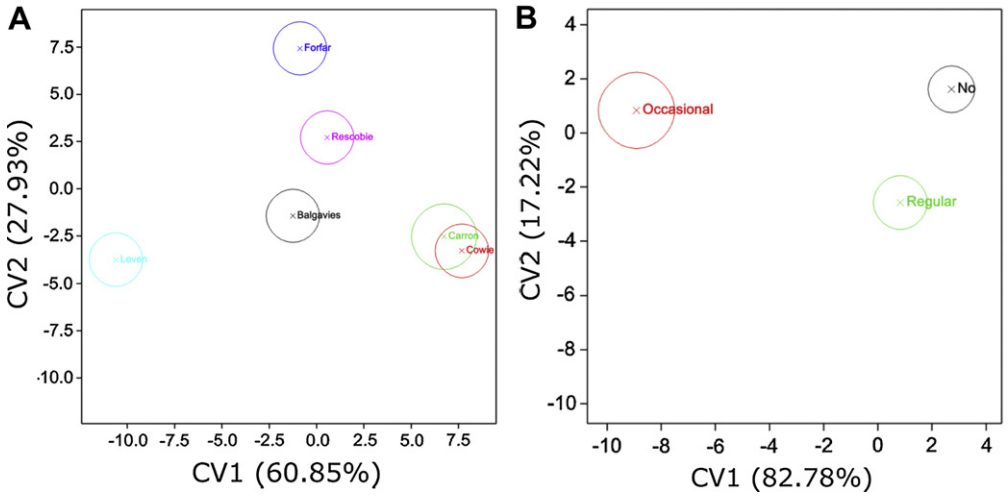

Fig. 1. A) Effect of location on bacterioplankton (T-RFLP data). CVA on 5 PC scores, circles represent 95% confidence (CV1 = 60.85% - CV2 = 27.93%); B) Effect of natural exposure to microcystins on freshwater bacterioplankton structure (T-RFLP data). CVA on 5 PC scores, circles represent 95% confidence (CV1 = 82.78% - CV2 = 17.22%). Regular = regular exposure to MCs; No = no previous exposure to MCs; Occasional = occasional exposure to MC-LR.

It showed a significant correlation between the bacterial communities' structure and the half-life of MC-LR (see Table 2), as well as a significant correlation ($P < 0.001$) between previous exposure to MC-LR and half-life of MC-LR.

To identify the main factors affecting the bacterial communities, RDA was carried out with physico-chemical water parameters as environmental variables and bacterial T-RFs as species (see triplot Fig. 2). Only variables that significantly influenced the bacterial population were included in the graph (i.e. DOC, NO_3-N, NH_4-N and Temperature) (refer to Statistical, Section 2.5). Plots can be interpreted qualitatively, where the length of the arrow indicates how much variance was explained by that factor and the direction of the arrows for individual environmental factors indicates an increasing concentration of that factor. The TRF arrows pointing in approximately the same direction as the environmental factor arrows indicate a high positive correlation (the longer the TRF arrow, the stronger the relationship) (Macdonald et al., 2008). Samples are indicated as small circles along with an identity number created from the statistical software; the wider circles including the replicates of a site and site names were manually drawn to facilitate the reading of the RDA triplot. RDA results show that only DOC, NO_3-N, NH_4-N and Temperature had a significant effect ($P < 0.05$) on the bacterial communities. Nitrate-N concentration showed a positive correlation with the bacterial communities of River Carron. Similarly ammonium-N was positively correlated to the communities of Loch Leven and Forfar. Temperature showed to be positively correlated with the bacterioplankton of Loch Rescobie.

Terminal Restriction Fragments (TRFs) with length 154, 174, 310 and 417 base pairs were found to be solely associated with water bodies regularly exposed to MC-LR. Sequences of microcystin degrading bacteria listed by Manage et al. (2009b) were screened using the online application REMA (i.e. Restriction Enzyme Mapping Application, http://bioperl.macaulay.ac.uk/) in the attempt identify the TRFs uniquely found in water bodies regularly exposed to MC-LR: no matches were found.

Table 2
Linear regression analysis of the principal components scores (T-RFLP data) versus half life of MC-LR. (*95% significance).

Principal component (% variation)	*P* value
PC1 (33.35)	0.015*
PC2 (25.24)	0.789
PC3 (13.08)	0.863
PC4 (10.97)	0.001*
PC5 (6.33)	0.349

3.3. Biolog EcoPlate

For Biolog EcoPlate data, the first five PC accounted for more that 60% of the variance. ANOVA carried out on the data regarding the bacterial physiology of each carbon source category supplied (i.e. polymers, phenolic compounds, carboxylic acids, carbohydrate, amino acids and amines) revealed that there was a significant difference in the metabolism of polymers ($P < 0.001$), phenolic compounds ($P < 0.001$), carboxylic acids ($P < 0.001$) and carbohydrate ($P < 0.001$) amongst the freshwater bodies tested. CVA revealed shifts in the metabolism of the microbial communities due to sampling site (Fig. 3A). Biolog EcoPlate data after 48 h incubation were used in this analysis because they showed the greatest

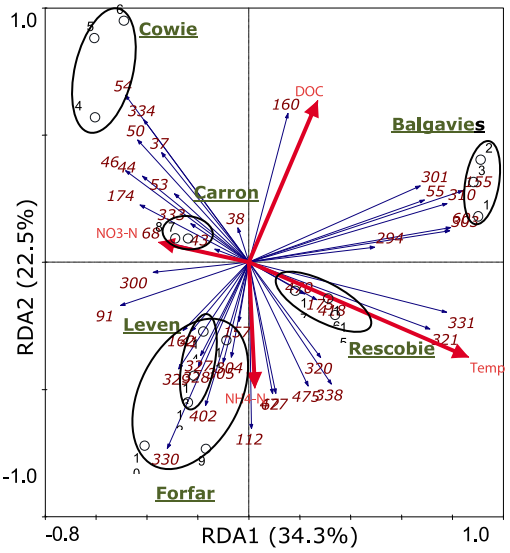

Fig. 2. Effect of water chemistry on bacterioplankton communities. Redundancy analysis: T-RFs as species and chemistry as environmental variables. Only significant ($P < 0.05$) environmental variables are showed. Variance explained by the variables selected: 56%.

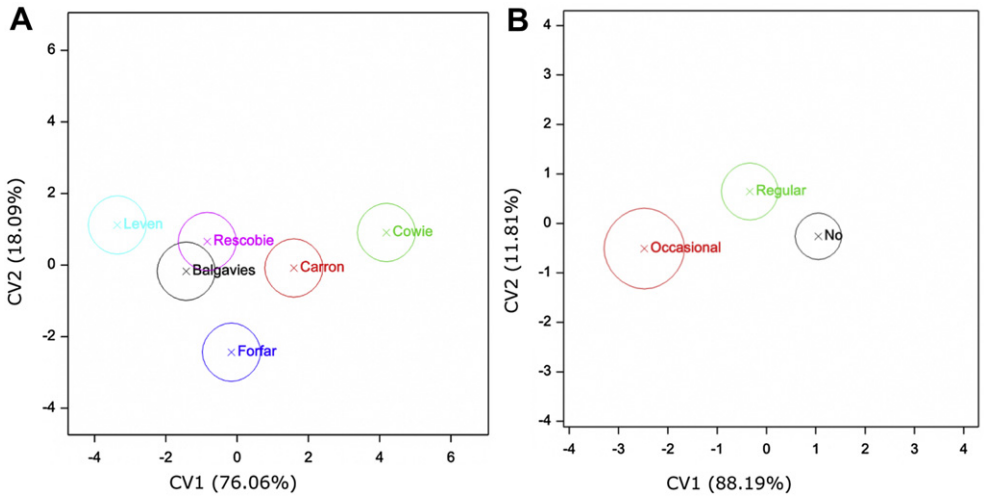

Fig. 3. A) Effect of location on living community physiology (Biolog EcoPlate™ data after 48 h). CVA on 5 PC scores, circles represent 95% confidence. B) Effect of natural exposure to microcystins on living community physiology (Biolog EcoPlate™ data after 48 h data). CVA on 5 PC scores, circles represent 95% confidence. Regular = regular exposure to MCs; No = no previous exposure to MCs; Occasional = occasional exposure to MC-LR.

discrimination between samples. The first three canonical variants (CVs) accounted for 98.5% of the variance within the first five PC dimensions. There was a clear separation between all the freshwater bodies under study with exception of Loch Balgavies and Loch Rescobie, which did not show significant separation in the CVA biplot. CVA revealed shifts in physiology of the microbial communities due to natural exposure to MC-LR (Fig. 3B). No significant correlation was found between living community physiology profiles and MC-LR half-life.

4. Discussion

4.1. T-RFLP

The T-RFLP results indicate that bacterioplankton communities from the lakes included in this work are significantly different while the communities from the rivers cluster together (Fig. 1A). The magnitude in difference between the lakes investigated in this work observed in Fig. 1A reflects their geographical position. Loch Leven, which is geographically more distant, was separated from the other lakes along the major axis which accounted for most of the variation of the system (i.e. 60.85%). On the other hand, the three lakes closely located geographically (i.e. Rescobie, Balgavies and Forfar) were separated only along the second axis of the CVA biplot which accounted for 27.93% of the variation of the system. For T-RFLP data (Fig. 1A), the fact that Loch Rescobie drains into Balgavies (Stewart et al., 1982) does not further contribute to the communities' structure of the two lakes beyond their geographical position. Similar results were found by Crump et al. (2007), who showed that lakes and rivers host different bacterial communities, and that the two systems interact and are influenced by spatial patterns.

Another significant driver for the freshwater bacterioplankton communities' structure was natural exposure to MCs (Fig. 1A). Chemical signalling, inhibitor/stimulator of growth activities along with toxicity to microorganisms (Christoffersen, 1996; Kearns and Hunter, 2000, 2001; Babica et al., 2006) are the most plausible direct effect of MCs on bacterial communities structure. On the other hand MCs can be toxic to macroorganisms (Mackintosh et al., 1990; Christoffersen, 1996), and their toxicity cause death or differential feeding patterns at different level in the food web (Kaebernick and Neilan, 2001) and that in turn affect the bacterial communities structure. MCs are therefore able to affect both directly and indirectly bacterial communities structure.

It has to be considered however that MCs are commonly produced during cyanobacterial bloom and the blooms change the structure of bacterial population (Riemann and Winding, 2001; Worm et al., 2001; Eiler and Bertilsson, 2007) mainly due to increased organic carbon that boosts the growth of heterotrophic microorganisms and lead to oxygen depletion.

Previous studies (Rapala et al., 1994; Christoffersen et al., 2002; Edwards et al., 2008) have shown a clear relation between past natural exposure to MCs and the ability of freshwater bacterioplankton communities to degrade these natural toxins. In this work we report a correlation between bacterial community structure and half-life of MC-LR (Table 2). This finding, linked with the correlation between past natural exposures to MCs and MC-LR half-life ($P < 0.001$) of the present dataset, suggests that the exposure to MCs is able to shape the whole bacterial community structure and not only to select for few bacteria able to degrade these toxins as suggested from previous authors (Rapala et al., 1994).

T-RFLP analysis also showed the selective presence of few OTUs (i.e. 154, 174, 310 and 417 base pairs) in water bodies with regular exposure to MCs. Those OTUs should be further investigated in order to assess whether they selective appear in water bodies with regular exposure to MCs also in other systems as they could be used as bioindicators for water quality. If such bioindicators would be confirmed, they could additionally be employed to predict the likelihood of MCs production based on microbial data. That would be a very useful tool for policy makers and regulatory agencies.

4.2. Water chemistry and communities structure

Dissolved organic carbon, ammonia, nitrate and temperature had a significant effect in shaping the bacterial communities structure for the freshwater bodies studied in this work (Fig. 2). Previous studies (Lindström et al., 2005; Yannarell and Triplett, 2005) have shown that pH is one of the key factors in shaping bacterial communities in freshwater lakes. Here, two nitrogen compounds (i.e. ammonium and nitrate) significantly influenced bacterial communities. Nitrogen is a key nutrient in aquatic ecosystems essential for productivity (Frette et al., 2009); it is considered to be the primary limiting nutrient for phytoplankton biomass accumulation (Rabalais, 2002) and to be linked with harmful algal bloom (Paerl, 1997). Nitrogen affects cyanobacterial bloom and the production of cyanotoxins differentially in nitrogen-oxidizing and non-nitrogen-oxidizing cyanobacteria (Kaebernick and Neilan, 2001). Some of

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the water bodies studied in this work have history of eutrophication, cyanobacterial bloom and cyanotoxins detection (Edwards et al., 2008), consequently the relation between nitrogen compounds and the structure of the bacterioplankton communities is expected. Temperature is a key factor in regulating freshwater bacterial growth (White et al., 1991; Felip et al., 1996) communities structure (Yannarell and Triplett, 2004, 2005). Temperature is one of the key factors that dictates the occurrence of cyanobacterial bloom and consequent release of cyanotoxins (Chorus and Bartram, 1999; Kaebernick and Neilan, 2001). In this work, the two water bodies with higher temperature (i.e. Rescobie and Balgavies) are the ones with the most frequent exposure to MCs. Also in this work temperature appear to be a parameter closely related with bacterial communities structure (Fig. 3).

4.3. Biolog EcoPlate

Bacterial carbon substrate utilization profiles, determined with Biolog Ecoplate, were used to establish differences amongst bacterial communities' physiology.

Biolog EcoPlates has been used in various studies to study aquatic microbial communities (Comte and Del giorgio, 2009) and their ability to distinguish amongst communities was shown by Choi and Dobbs (1999). A clear separation between all the water bodies with the exception of Rescobie and Balgavies was observed (Fig. 3A). The similarity in physiology between bacterioplankton communities of Loch Rescobie and Loch Balgavies may be driven by their close spatial position and from overflow of water that goes from Rescobie to Balgavies (Stewart et al., 1982). The link between the two lakes certainly facilitates exchange in nutrients as well as in bacterial communities, and that explains the similar metabolism measured by Biolog Ecoplate. Previous exposure to MCs showed to be an important factor in the differentiation of the physiology of bacterioplankton communities (Fig. 3B) and it is most probably one of the drivers of the observed separation between physiologies of the water bodies under study. Some authors reported direct effect of MCs on freshwater microbial autotrophs physiology (Singh et al., 2001; Hu et al., 2004, 2005), on the other hand it has to be considered that changes in communities' structure caused by MCs (see Section 4.2) would then alter the observed physiological profile.

5. Conclusions

A complex pattern of relations and feedback appear to rule the production and degradation of MCs as well as the bacterioplankton communities structure and physiology in freshwater. In order to increase confidence in our results, here we used both DNA and physiological fingerprinting approaches to evaluate if exposure to MCs was able to affect bacterial communities. Not only exposure to MCs was able to affect both bacterioplankton communities' physiology and structure, but also communities' structure was related to half-life of MC-LR. Moreover DOC, nitrogen compounds and temperature had a significant effect in shaping bacterial communities' structure. This is the first report to our knowledge that is able to shed light at the relation between previous exposure to MCs and improved ability of the microbial communities to degrade such toxins. It appears that exposure to MCs is able to affect bacterioplankton physiology and structure and the later is then linked with degradation rate of MC-LR. Taking in account water chemical and physical parameters we suggest that nitrogen compounds, DOC and temperature drive bacterial communities structure both directly (i.e. nutrients availability and growth temperature) and indirectly (i.e. influencing the occurrence of cyanobacterial bloom and toxin production which then affect bacterial population). The identification of OTUs solely associated with water bodies regularly exposed to MCs requires further studies as, if confirmed, such OTUs could be used as bioindicator of water quality.

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