Freshwater bacterial diversity, functions and stability

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ABSTRACT

Biodiversity is declining worldwide with detrimental effects on ecosystems functions and services that it sustains. The relationship between biodiversity and freshwater purification remains unclear. Freshwater purification is of paramount importance for humankind as eighty percent of the world's population is exposed to high levels of threat in terms of water security. Bacteria are the most diverse and abundant organisms on Earth and they play, directly or indirectly, a key role in the majority of ecosystem services including water purification. The current work aimed, in freshwater systems, to unravel the relationships between microbial diversity and: (a) biodegradation of toxic compounds (i.e. specialised function); (b) respiration (i.e. broad function) and; (c) stability of broad functioning. Firstly, preliminary experiments were carried out to establish freshwater sample size to representatively evaluate bacterial communities' diversity and also suitable natural and man-made toxic compounds for freshwater incubation experiments. Then, the microbial communities' ability to degrade microcystin-LR was explored in the context of previous exposures and nutrient availability. Finally, we focused on the relationships between diversity and functioning. A decrease in microbial diversity caused a decrease in both broad and specialised ecosystem functions tested. Stability of broad functioning was also negatively affected by a decrease in microbial diversity. Both lakes (Scotland) and rivers (Australia) microcosms experiments resulted in comparable findings suggesting consistent relationships across different freshwater systems. These results highlight that, similarly to macro-organisms (plant and animals), declining diversity of the microbial communities has direct consequences for important ecosystem

functioning and services and therefore, microbial diversity should be explicitly considered in all biodiversity conservation debates.

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1. GENERAL INTRODUCTION

1.1 Biodiversity and bacteria

Biodiversity is the variability among living organisms from all sources, including terrestrial, marine, and other aquatic ecosystems and the ecological complexes of which they are part; this includes diversity within species, between species, and of ecosystems (Duraiappah et al., 2005). Biodiversity is declining worldwide (Barnosky et al., 2011) with detrimental consequences on ecosystem functions, stability and services provided to humanity (Cardinale et al., 2012, Hooper et al., 2012). The recognition of the importance of biodiversity in sustaining functional ecosystems as well as the evidence of its decline have stressed the need to strengthen scientific studies on this topic and includes evidence in national and international policies (Perrings et al., 2011). Of all living forms, bacteria are possibly the most ubiquitous, abundant and diverse (Whitman et al., 1998, Torsvik and Øvreås, 2002). The immense diversity of this group of organisms can possibly be explained by some characteristics: bacteria are small, can reproduce quickly and have been on earth longer than any other group of organisms. The size of bacteria allows them to colonise and differentiate in tiny niches and microenvironments without the need of wide areas as larger organisms do (Horner-Devine et al., 2004, Vos et al., 2013). The possibility to duplicate in times as short as a few hours, coupled with the fact that they have been on earth longer than any other organisms, enormously increase the possibility of DNA mutation which is at the base of evolution. Finally, bacteria are able to perform lateral gene transfer even between distantly related organisms, which "produces extremely dynamic genomes in which substantial amounts of DNA are introduced into

and deleted from the chromosome' (Ochman et al., 2000). These are probably some of the reasons why the great scientist Edwards Osborne Wilson considered the measurement of bacterial diversity 'beyond practical calculation' (Wilson, 1992). An increasing number of authors have been focusing on the topic of bacterial diversity and evidence of the exponential interest and amount of work in bacterial diversity is presented in Figure 1.1.

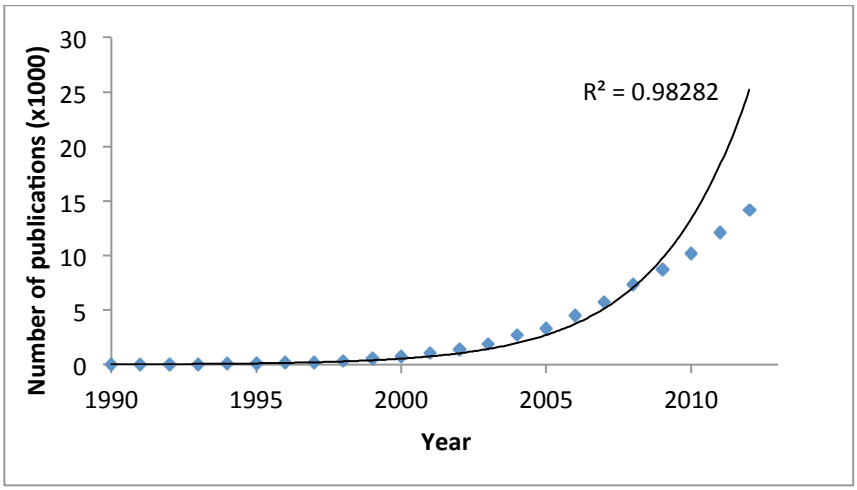

Figure 1.1 – Number of peer-reviewed publications with topic "bacterial diversity" from 1990 to 2012 - Data from ISI Web Of Knowledge (Analysis done on 20-03-2013).

Despite the large amount of research that is focused on bacterial diversity, this subject still has uncertainties about few important aspects. First of all, there is not a clear definition of bacterial species: the current criterion to distinguish bacterial species is not based on any theoretical justification. The criterion used is a cut-off level (70%) of pair wise genomic DNA-DNA hybridisation level which was chosen 20 years ago to match pre-existing species classification (Achtman and Wagner, 2008). Diversity has two components: richness and abundance. Currently there is limited understanding of the extent of microbial richness, even less knowledge about proportional abundance (Curtis, 2006). The issue arises from the fact the microbial world is

so vast and diverse that no techniques have been able to characterise it comprehensively. Most communities are dominated by a small number of species whereas the vast majority of populations are quite uncommon. Most of the current methods based on PCR are unable to detect the many uncommon members of these communities. While common organisms carry out most of the ecosystem functions in a specific time/space coordinate, uncommon organisms serve as a reservoir of functional and genetic diversity, often play key roles in ecosystems and become numerically important when environmental conditions changes (Bent and Forney, 2008, Sloan et al., 2007). Their role of genetic reservoir is particularly important because bacteria are able to perform lateral gene transfer between different taxa, and the rate of this process was shown to increase during stress conditions (Coombs and Barkay, 2004). Moreover, due to the evidence that bacteria respect taxa-area distribution (Woodcock et al., 2006), assessment of bacterial diversity in complex environments is futile without extensive sampling (Venter et al., 2004). Bacterial diversity is higher in soils than in planktonic communities (Zinger et al., 2012), and this may be explained by the lack of structure and resources in aquatic environment (Rainey et al., 2000, Rainey and Travisano, 1998).

1.2 Ecosystem functioning and bacterial diversity

Bacteria are essential for life on earth. They play a key role in biogeochemical cycling in both terrestrial and aquatic ecosystems (Nannipieri et al., 2003, Cotner and Biddanda, 2002). Being the most abundant organisms in both soils and waters, they constitute a large source and sink of nutrients. In terrestrial ecosystems they play a central role, amongst others, for control of greenhouse gas (Singh et al., 2010), plant health (Kim et al., 2011), bio-control of pests (Babalola, 2010), soil structure (Young et al., 1998) and biodegradation of toxic compounds (Singh, 2009). In aquatic environments, apart from being the main players in energy flow and biogeochemical cycling, bacteria perform biodegradation of the majority of toxic compounds both of natural origin (Edwards and Lawton, 2009) and man-made (Roh et al., 2009).

Many authors have suggested a key positive role of bacterial diversity on communities' functioning and stability (Bell et al., 2005b, Eisenhauer et al., 2012). Although there is a consensus of the positive effect of bacterial diversity on functioning, the shape of the relationships can vary upon the function studied (e.g. broad vs specific), the environmental complexity and a number of temporal, spatial and ecological interactions (Langenheder et al., 2010, Prosser, 2012). Hypothetical positive relationships between biodiversity and ecosystem functioning are represented in Figure 1.2.

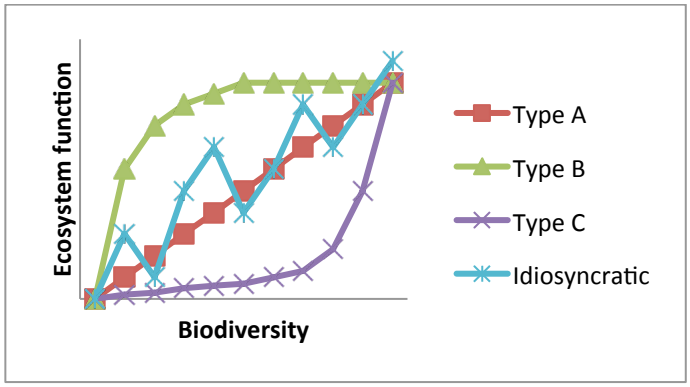

Figure 1.2 – Possible positive relationships between biodiversity and ecosystem functioning. Type A=all species have equal importance; Type B= vast redundancy; Type C= few species are responsible for most of the functioning; Idiosyncratic= the impact of diversity is dependent on communities structure

Figure 1.2 represents hypothetical positive relationships between biodiversity and ecosystem functioning that are enclosed between two "extreme" relationships: type B and type C. A type "A" curve would indicate that all species, even rare ones, are required to maintain healthy levels of ecosystem function (i.e. no redundancy). A type "B" relation, on the other hand, would suggest that ecosystems can lose much of their diversity without consequences to function (i.e. vast redundancy) (Yachi and Loreau, 1999). A type "C" relation would suggest that even a small decrease in diversity has a dramatic effect on the system functioning (i.e. no redundancy). Another possible relationship is idiosyncratic where not only bacterial diversity but also communities' composition plays an important role due to the ecological relationships (e.g. facilitation, competition and predation). The actual response of ecosystem functioning to a change in bacterial diversity may vary between types, and this relationship may be dependant, amongst others, upon the function measured. Two mechanisms were proposed to explain the relation between bacterial diversity and ecosystem functioning. The complementary

mechanism is based on the idea that different species use different resources, so communities with high species richness are more productive using more available resources. The selection mechanism is based on the concept that different species have a different effect on ecosystem functioning (e.g. different degradation rate of a compound between two species); due to this mechanism species rich communities are more productive because they are more likely to contain species with a significant effect (Bell et al., 2005b). Biodiversity affects the way in which ecosystems function, but the form of the relationship between bacterial diversity and functioning remain poorly understood. Studies of how bacterial diversity affects ecosystem function have been carried out with three main approaches: construction of communities from culturable microbes, dilution to extinction of natural communities and observation of natural bacterial communities. Unfortunately the outcome of the different approaches have given contrasting results (Bell et al., 2009). Each approach has its advantages and drawbacks.

The construction of artificial communities from culturable bacteria allows us to study not only the effect of diversity per se, but also to test the impact of each species on the function studies and also to get an insight on interactions between species. Unfortunately this approach is based on culturable bacteria which represent less than 1% of entire natural populations. Also, in the case that a scientist is aiming to test all possible combinations of a set of species to explore all interactions and species contribution to a determinate function, the number of initial species that he will be able to include in the study will be very limited: for example, all the combinations of 10 species will require more than one thousand microcosms. To our knowledge the study that employed the highest number of bacteria species was carried out by Bell et al. (2005b)

and included 72 species, but only a small percentage of the total species combination (i.e. > 4.7 X 10^{21}) was tested.

Removal approaches commonly rely on dilution-to-extinction reduction of bacteria diversity. This diversity manipulation method allows the study of the functioning of natural and highly diverse microbial communities, and how depletion in diversity affects it. Generally bacterial species relative abundance is heterogeneous in natural environments, hence the dilution approach has the effect of removing preferentially rare species. Possibly the main drawback of the dilution approach is that both a gradient in diversity and in biomass are generated. Thanks to the short duplication time in microbes, biomass can be recovered within few days in aquatic environments (Szabó et al., 2007) but it can be very lengthy in soils (Griffiths et al., 2001). During the biomass recovery step microbial communities' structure is likely to change and this can affects data interpretation if communities structure rather than diversity has a strong effect on functioning. Also, with this methodology much less information is available regarding the contribution to a certain function by each of the species present and the evaluation of ecological interactions between specific species becomes unfeasible.

The observational approach, where it is attempted to link natural occurring differences in microbial diversity to functioning has the main advantage to have as subject natural and possibly highly diverse systems. The finding of this approach might have high relevancy for the scientific community as they reflect dynamics in natural ecosystems. The first and great challenge with this approach is to find environments differing solely on microbial diversity. Natural systems are very complex and many drivers, both biotic and abiotic,

have effects on a certain ecosystem function. The complexity of natural systems does not imply a little influence of biodiversity on ecosystem functioning, but makes difficult the evaluation of the role of different factors. Because of our limited knowledge about the microbial diversity, natural system complexity and interactions, it is rather challenging to discriminate the importance of the different factors.

1.3 Freshwater quality

Water supports all life on earth. Freshwater is the most precious natural resource on terrestrial ecosystems and its biodiversity is a conservation priority. The United Nations General Assembly proclaimed 2005-2015 the International Decade for Action – "Water for life" (December 2003, resolution 58/217). eighty percent of the world population is exposed to high levels of threat in terms water security (Vörösmarty et al., 2010). Freshwater biodiversity has been highlighted as a key factor for freshwater preservation due to the ecosystem functions and services that it sustains (Dudgeon et al., 2005, Cardinale, 2011, Hooper et al., 2012). However the factors that have been pointed out that mainly affect water security are pollution and water resource development (Vörösmarty et al., 2010). Both factors are determined by human activities but water pollution has the potential to be remediated by biotic and abiotic factors.

1.3.1 Eutrophication, cyanobacteria and cyanotoxins

Natural waters have very low concentrations of nitrogen and phosphorous. Runoffs from farmlands along with wastewater deriving from urban and industrial activities increase nutrient levels. Increased loadings of phosphorus and nitrogen, two of the main pollution drivers listed in the work of Vörösmarty et al. (2010), determines the eutrophic status of a water body that often results in harmful cyanobacterial blooms (Conley et al., 2009). Currently eutrophication is one of the primary issues in most surface waters (Smith and Schindler, 2009) and the incidence of cyanobacterial blooms is likely to increase due to climate change (O'Neil et al., 2012, Paerl and Huisman, 2009).

Cyanobacteria are an ancient and diverse group of photosynthetic bacteria (Rasmussen et al., 2008, Tomitani et al., 2006). Cyanobacteria have been playing a fundamental role in shaping atmospheric composition (Kasting and Siefert, 2002) and in nitrogen cycling (Zehr, 2011). During their long evolution, this group of organisms have successfully colonised most environments present on earth and acquired the ability to produce a wide range of secondary metabolites: toxins, iron chelators, indole alkaloids and protein inhibitors (Gademann and Portmann, 2008). A number of scientific studies have focused on different aspects of cyanobacterial toxins as they represent a hazard to human and animal health (Chorus et al., 2000). Cyanotoxins can be categorised into: microcystins and nodularins (hepatotoxins and possibly carcinogens), saxitoxins and anotoxins (neurotoxins), and cylindrospermopsin (protein synthesis inhibitor) (Sivonen and Jones, 1999, Edwards and Lawton, 2009). Microcystins are the most widely occurring amongst cyanotoxins and are produced by several genera of cyanobacteria since ancient times (Rantala et al., 2004).

The long exposure to microcystins has possibly been selected for the ability in many bacterial species to be able to degrade them. One of the first occurrences of biodegradation of microcystins comes from a work of Jones and Orr (1994) with microcystin-LR: the most commonly occurring microcystin. The high toxicity of microcystin-LR (MacKintosh et al., 1990, Grosse et al., 2006) has led to the World Health Organisation to set a guideline value of 1 μg L^{-1} in drinking water (WHO, 1998). Microcystin-LR also occurs widely and can be very persistent in aquatic environments (Lahti et al., 1997). Early studies on biodegradation were followed by a number of works over the last two decades that have revealed various species able to degrade bacteria. The

majority of the isolated species responsible for microcystin-LR biodegradation belong to the *Proteobacteria* phylum (Edwards and Lawton, 2009), some exceptions are in the *Actinobacteria* (Manage et al., 2009b) and *Firmicutes* (Nybom et al., 2012). Other aspects of microcystin biodegradation that have been investigated include, but are not limited to, the effect of previous exposure on biodegradation ability/rate (Rapala et al., 1994, Christoffersen et al., 2002), the effectiveness of sand filters (Grützmacher et al., 2002, Ho et al., 2006), the biodegradation potential of biofilms (Babica et al., 2005), the characterisation of the genes involved in bacteria degradation (Bourne et al., 2001) and molecular methods to quantify these genes (Hoefel et al., 2009).

1.3.2 Pesticide and freshwater pollution

Pesticides were introduced to improve crop yields and face an increasing food demand, the latter due to the increasing global number of inhabitants along with a positive relationship of food requests per capita and country development. Industrial agriculture has its foundations on monoculture and heavy use of synthetic pesticides and fertilisers, hence their widespread environmental incidence (Gilliom, 2007). Once in the environment, the fate of a pesticide is determined by three processes: adsorption, transfer and degradation (Fishel, 1997). In the adsorption process the pesticides bind to soil particles influencing whether other processes can affect the pesticide. The transfer process is the route for widespread contamination of the surrounding ecosystem including surface water, groundwater and non-target species. Non-target plants are mainly affected by volatilisation and runoff of the pesticide. Runoff affects surface water quality and aquatic organisms; it then has an effect on all the living organisms, including humans, using the contaminated water. Runoff and leaching can lead to groundwater contamination (Fishel,

1997). Pesticides reach aquatic ecosystems mainly through agricultural runoff (Moore et al., 2008), but manufacturing plants and other human activities can contribute to the process.

Historically, the main two classes of pesticide are represented by organoclorine and organophosphorus. Organochlorines (e.g. dichlorodiphenyltrichloroethane - DDT) were initially widely used as broad-spectrum insecticides but a better understanding of their high stability, low degradation rates, high toxicity and tendency to bio-accumulate (Simonich and Hites, 1995) led to them to be taken out of use. Organochlorines were substituted by organophosphorus compounds that are still highly toxic but are more prone to biodegradation (Singh, 2009). Chlorpyrifos is one of the most widely used organophosphorus insecticides: in the United States alone approximately four thousand tonnes of chlorpyrifos were applied annually between 2000 and 2006 (EPA, 2009). Chlorpyrifos is employed for controlling a wide range of insect pests, mosquitoes, flies, termites and various soil and household pests (Singh et al., 2006b).

Evidence of rapid degradation of the OP pesticides chlorpyrifos and fenamiphos carried out by bacterial isolates, in both water and soil, was given by Singh et al. (2003, 2004, 2006b). Chlorpyrifos degrading bacteria were also isolated from activated sludge (Karpouzas and Singh, 2006). The identification of chlorpyrifos degrading fungi by Al-Mihanna et al. (1998) showed that the biodegradation of this pesticide is not carried out exclusively by bacteria. However in the natural environment half-life of chlorpyrifos ranges from days to years. While most of the studies have focused on persistence in soils and the mechanisms affecting it (Gebremariam et al., 2012), a report suggested a

long half-life (i.e. >100 days) in artificial wetlands (Budd et al., 2011). Chlorpyrifos can be found in various freshwater ecosystems posing a serious health risk to exposed organisms including humans (Gilliom, 2007). Different authors have provided evidence of chlorpyrifos toxicity to mammals including genotoxicity (Ojha et al., 2011), mutagenicity (Amer and Aly, 1992), increased cancer risk (Lee et al., 2007), endocrine disruption (Hodgson and Rose, 2008), reproductive toxicity (Farag et al., 2003) and developmental neurotoxicity (Flaskos, 2012).

1.3.3 Triclosan: incidence, biodegradation and toxicity

Triclosan is a chlorinated aromatic compound widely used as broad-spectrum antimicrobial agent. It was first registered as a pesticide in 1969 with the United States EPA and it has been mainly used as an antibacterial agent in a number of products including personal care, household and industrial products (Singer et al., 2002, Bester, 2003). Triclosan targets lipid synthesis in bacteria, fungi and algae by mimicking the natural substrate of the enzyme enoyl-acyl carrier protein reductase (McMurry et al., 1998, Levy et al., 1999). It has a bacterial inhibiting effect from a concentration of 10 μg L^{-1} (Bhargava and Leonard, 1996) while it can affect algae at concentrations as low as 0.4 μg L^{-1} (Yang et al., 2008). Annually hundreds of tonnes of triclosan are produced in Europe (Bester, 2005) and Unites States (Halden and Paull, 2005) followed by continuous release in the environment. The release in the environment is accentuated by the fact that personal care products are designed for external use and consequently they do not undergo metabolic changes resulting in high levels of triclosan being transferred to wastewater (von der Ohe et al., 2012).

Biodegradation of triclosan has been observed in activated sludge both in wastewater treatment plants (Sabaliunas et al., 2003, McAvoy et al., 2002) and in laboratory conditions (Federle et al., 2002). Biodegradation of triclosan can occur in both aerobic and anaerobic conditions (Gangadharan Puthiya Veetil et al., 2012) and can be catalysed by various bacteria belonging to different groups of bacteria (Lee et al., 2012, Roh et al., 2009, Meade et al., 2001). Some studies have reported dissipation rate in freshwater systems (Sabaliunas et al., 2003, Morrall et al., 2004) but were unable to estimate the extent of biotic and abiotic degradation. Although most of the triclosan is either biodegraded or sorbed to sludge in wastewater treatment plants (Bester, 2003, Singer et al., 2002, Kookana et al., 2011), a portion gets into the aquatic environment determining the contamination of most freshwater systems connected to human activities (Kookana et al., 2011, Dann and Hontela, 2011). The widespread use of triclosan has induced many bacterial strains to develop resistance to the compound which is often dangerously associated with antibiotics' cross resistance (Levy, 2001). Also, partial degradation of triclosan can result in formation to toxic dioxins (Buth et al., 2010, Mezcua et al., 2004). Triclosan has been found in human serum (Allmyr et al., 2006), breast milk (Adolfsson-Erici et al., 2002) liver and adipose tissue (Geens et al., 2012). Detection of triclosan in different human tissues is of concern as it has been shown that the antibacterial has endocrine disruption ability (Schuur et al., 1998) and ability to impair mitochondrial activity in animal cells (Newton et al., 2005).

1.4 Aims of the study

In the current age of extinction, increasing importance has been given to biodiversity and to its relationship with ecosystem functioning. Bacteria are the most abundant and diverse organisms on earth and play key roles in the majority of ecosystem functions and services including freshwater purification. Freshwater is the most precious resource in terrestrial ecosystems but eighty percent of the world population is exposed to high levels of threat in terms of water security. Water pollutants are one of the main factors affecting water security and their microbial biodegradation is the main dissipation mechanism. This study aims to examine how a decrease in microbial diversity can influence ecosystem functioning and stability in aquatic systems.

In this thesis, the following hypotheses were tested in freshwater microcosms:

I. *A decrease in microbial diversity will not affect the rate of a broad ecosystem function (i.e. respiration) according to the "insurance hypothesis" (Yachi and Loreau, 1999);*
II. *A decrease in microbial diversity will not affect the stability of a broad ecosystem function;*
III. *A decrease in microbial diversity will affect the rate of specific function (i.e. biodegradation of toxic compounds)*

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2. PRELIMINARY WORK

2.1 Introduction

Assessing the entire bacterial species richness and evenness of a complex environment remains an open ecological challenge unsolved due to practical and technological limitations (Hughes et al., 2001, Zinger et al., 2012). To determine the entire bacterial richness of an aquatic ecosystem and explore rare species extensive sampling effort is required (Venter et al., 2004). However, for diversity comparison across sets of samples, sampling and analysis effort can be much reduced without hindering the strength of the findings (Bell et al., 2005a).

Freshwater is the most precious resource of Earth and, globally, the most relevant water quality problem is eutrophication. In freshwater systems one of the most relevant class of natural toxic compounds is represented by cyanotoxins that are produced by cyanobacteria during eutrophication-triggered blooms (Paerl et al., 2001). While the ecological role of cyanotoxins has not been fully elucidated (Kaebernick and Neilan, 2001), their classification, structure, detection, toxicology and biodegradation have been described (Lawton et al., 1994, Chorus and Bartram, 1999, Edwards and Lawton, 2009) . The most common class of cyanobacterial toxins is represented by microcystins with microcystin-LR being its most common member. Microcystyin-LR is a potent inhibitor of protein phosphatases 1 and 2A (MacKintosh et al., 1990), hence hepatotoxic and possibly carcinogenic (Nishiwaki-Matsushima et al., 1992, Fujiki and Suganuma, 2009).

Pesticide runoff from agricultural land is a key issue in freshwater preservation where modern agricultural practices with intense use of pesticides have been implemented. Organophosphorus compounds are the most widely used insecticide (Singh and Walker, 2006) contaminating many ecosystems, biota and resources (Andreu and Picó, 2012, Bonansea et al., 2013, Wang et al., 2013). Biodegradation of organophosphorus insecticide has been widely investigated to elucidate ecology, organisms involved and mechanisms of degradation (Singh, 2009). Globally, widely used organophosphorus insectices include malathion, fenamiphos, parathion and chlorpyrifos. Chlorpyrifos is one of the most widely used organophosphorus insecticides (EPA, 2009) resulting in its widespread presence in the environment (Gilliom, 2007). That poses a serious health risk as chlorpyrifos was reported to disrupt endocrine functioning (Hodgson and Rose, 2008), being genotoxic (Ojha et al., 2011) and has a strong correlation with incidences of rectal cancer (Lee et al., 2007).

Triclosan is a chlorinated aromatic compound first registered as a pesticide in 1969 with the US EPA. Due to its antimicrobial activity (Regös et al., 1979) it has been widely used in a number of products including, but not limited to: toothpaste, mouthwash, hand soap, deodorants, facial tissues, shampoo, fabric softeners, fabric deodorant, antiseptics, medical devices, detergent, plastic additives, cutting boards, textiles, sport equipment, shoes, furniture and industrial machinery. A report from Bester (2005) suggests that about 1500 t of triclosan were produced annually worldwide and, due to its increasing popularity and widespread use, its production is likely to have increased in recent years and will rise further in coming years. The widespread usage of triclosan determines its continuous release in wastewaters that in most developed countries are processed in treatment plants. Most of the

triclosan is either biodegraded or sorbed to sludge in waste water treatment plants (Bester, 2003). However, the dissipation is often not complete and a portion gets into the aquatic environment determining the contamination of most freshwater systems connected to human activities (Kookana et al., 2011). Also, the application of the sludge on agricultural land determines contaminations of soils (Ying and Kookana, 2007). From a toxicological point of view, a review of Dann and Hontela (2011) reports that triclosan has bioaccumulation potential along with toxicity to aquatic organisms, endocrine disrupting effects, and the potential to trigger antibiotic resistance in bacteria.

For microcystin-LR many freshwater incubation studies have been successfully carried out (Edwards et al., 2008, Ho et al., 2012) and sturdy methodological approaches to detect the toxin at environmental concentration have been developed (Lawton et al., 1994, McElhiney and Lawton, 2005), but the same does not apply to chlorpyrifos and triclosan. The great majority of incubation studies for chlorpyrifos have been carried out in soils and the biodegradation of triclosan has been mainly tested in sludge either in wastewater treatment plant or in incubation experiments. Also, analytical methods to detect triclosan do not have the resolution to detect the antibacterial agent at concentrations commonly found in freshwater systems (i.e. $<10 \mu g\ L^{-1}$) given a small sample size.

In this chapter, results are reported and discussed from three experiments. The were carried out to gain essential data required to appropriately design experiments investigating diversity-function relationship in freshwater bacterial communities. The aims of these experiments were to:

- Investigate, within a practical range, how freshwater volume used for DNA extraction affects: a) DNA yield and; b) diversity of microbial communities assessed by DNA fingerprinting technique;
- Test methodologies and experimental design to successfully set up freshwater incubation experiments with: a) chlorpyrifos and: b) triclosan;
- Investigate the ability of natural freshwater microbial communities to degrade: a) microcystin-LR; b) chlorpyrifos and; c) triclosan.

2.2 Materials and methods

2.2.1 Sampling and experimental design of trial experiments

Experiments were carried out in Scotland and Australia in lakes and rivers, respectively.

In Scotland, sub-surface (i.e. within 10 cm of surface) freshwater samples collected from Loch Rescobie (R) (56.657029,-2.805339) on the 14th of July 2010 were employed to test if: a) freshwater volume filtered for DNA extraction affects DNA yield as well as composition and diversity of microbial communities and; b) natural microbial communities from this site were able to degrade natural (i.e. microcystin-LR) and man-made (chlorpyrifos) toxic compounds.

In Australia, sub-surface (i.e. within 10 cm of surface) freshwater samples were collected from four sites in New South Wales on the 19th of October 2012. The sites were: Grose River (G) (-33.614047, 150.62994), Hawkesbury River (H) (-33.573581, 150.738859), Farmers Creek at two sites (FP: -33.469397, 150.125899 and FC: -33.466712, 150.185895). Water was employed to test if natural microbial communities from these sites were able to degrade triclosan. Water was collected in sterile glass bottle wrapped in aluminium foil to ensure minimal exposure to light.

2.2.2 DNA extraction, DNA yield and microbial Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis from a range of freshwater volumes

For the first sampling (i.e. 14th July 2010) water was collected in triplicate into 10 L containers, previously acid washed and autoclaved to ensure sterility. Water temperature and pH were measured on site. Following sampling, the

water samples were processed within a few hours by filtration with a sterile metal sieve (pore size 150 µm) in sterile conditions to remove large particles and vegetation. On the same day, aliquots of 25, 50, 75, 100, 250 and 500 mL were filtered via individual 0.22 µm pore size membrane filter units (Stericup filter units, Millipore) by vacuum. The filters were removed in sterile conditions from the disposable filter units and stored in sterile Petri dishes at -20 ºC until DNA extraction. DNA extraction was carried out using PowerWater® DNA Isolation Kit (Mobio, Carlsbad, USA) following the manufacturers centrifuge protocol, except that the DNA was eluted in 50 µL and not in 100 µL as suggested by the manufacturer. After DNA isolation, the presence of DNA in the extract was confirmed by agarose gel electrophoresis. DNA was then stored at -20 ºC until further analyses.

Amplicons for terminal restriction fragment analysis were produced using three sets of primers that are listed in Table 2.1 with relative label, sequence, target region and specificity.

Table 2.1 - Primers used for amplification of bacterial, cyanobacterial and algal ribosomal DNA (rDNA).

Primer ID	Fluorescent Label	Sequence (5' to3')	Target region and specificity	Specificity
63f	VIC	CAGGCCTAACACATGCAAGTC	16S rRNA gene	Eubacteria
1087r	NONE	CTCGTTGCGGGACTTACCCC		
Cyan	NED	GGGGAATYTTCCGCAATGGG	16S rRNA gene	Cyanobacteria
Cyan	NONE	TCCCCTAGCTTTCGTCCC		
Algae	FAM	AATCAGTTATAGTTTATTTGRTGGTACC	18S rRNA gene	Microalgae
Algae	NONE	TCTCAGGCTCCCTCTCCGGA		

Within each set, one of the primers was labelled on the 5'-end with a fluorescent marker to allow 5'-terminal fragment detection and analysis by the T-RFLP method. To avoid any contamination, the PCR tubes/plates and master

mix tubes were UV-irradiated, before the PCR reaction, using a UV-crosslinker (CL-1000 model, UVP) for 2 minutes at 2.02 mJ cm^{-2}.

For PCR amplification of the bacteria 16S rRNA gene, the reaction mix (50 µL) consisted of: 1 x 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 were 63F-VIC and 1087R 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 minutes extension period at 72°C.

For algal 18S rRNA gene amplification, PCR reagents and conditions were identical to the ones used for bacteria except for the primers: Algae18sf p73 and Algae18sr p47. For algae, the cycle consisted of 5 min at 94°C, followed by 37 cycles of denaturing at 92°C for 50 s, annealing at 57 °C for 50 s, elongation at 72°C for 50 sec, and a last cycle of 10 minutes extension at 72°C.

For cyanobacterial 16S rRNA gene amplification, PCR reagent and conditions were identical to ones used for bacteria except primers which were used at 400 nM. For cyanobacteria, the cycle consisted of 5 min at 95°C, followed by 35 cycles of denaturing at 94°C for 30 s, annealing at 58 °C for 30s, elongation at 72 °C for 1 min, and a last cycle of 10 minutes extension at 72°C.

Successful PCR amplification was confirmed by agarose gel electrophoresis of PCR products. The relative concentration of DNA amplicons for the bacterial, algal and cyanobacterial rDNA were then visually evaluated from the band intensity on the gel electrophoresis. For each sample, aliquots of each of the three amplicons (i.e. bacteria, algae, cyanobacteria) were then mixed at the PCR purification stage, based on relative abundance data obtained from gel electrophoresis, in order to obtain comparable concentrations of the three amplicons in the mix. A higher concentration of PCR products for bacterial communities was added to the mix taking into account the higher number of peaks expected. PCR amplicons were purified using the Wizard® SV Gel and PCR clean up system (Promega) following the manufacturers instructions. Once the samples were purified using the commercial kit, the concentration and purity of DNA were measured using a NanoDrop™ 1000 spectrophotometer (Thermo Scientific). DNA concentration estimates were then used to normalise the amount of DNA at the restriction digestion step. The pools of bacterial, algal and cyanobacterial amplicons were digested at 37°C for 3 hours with the restriction enzymes *HhaI* (Promega) following manufacturer guidelines in a 10 µL reaction. DNA fragment analysis was carried out on an ABI PRISM® 3130*xl* Genetic Analyser (Applied Biosystems). After ensuring that the quality of the capillary electrophoresis run was satisfactory, relative abundance tables were obtained for statistical analysis that was carried out with GenStat (version 11.1, VSN). Before statistical analysis, only terminal fragments in the length range 30-500 base pairs were selected to comply with the range of the T-RFLP standard. Baseline was set up based on overall fluorescence noise of each run to exclude peaks resulting from technical artifacts. Also, peaks with relative abundance below 5% were

removed from analysis and remaining peaks were combined when differing for less than one base pair. In most cases 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 the treatment on the microbial communities. Data were also explored using canonical variate analysis (CVA) on the first 5 PC to visualise probable significant separations between groups.

2.2.3 Microcystin-LR and chlorpyrifos biodegradation assay in freshwater

Freshwater samples from Loch Rescobie were inoculated with filter sterilised microcystin-LR in 100% methanol or sterile chlorpyrifos in 100% methanol at a final concentration of 1 mg L^{-1} and 10 mg L^{-1}, respectively. The two treatments were incubated along with three controls. One control contained only freshwater without any toxic compound: to gain data about how microbial communities change over time due to the length and setting of the incubation. The other two controls were sterile freshwater inoculated with microcystin (MC) and chlorpyrifos (CPY): to evaluate abiotic degradation of the toxic compounds under study. For the sterile controls, freshwater was filter sterilised (0.2 µm filter, Stericup filter unit, Millipore). Treatment and all the controls were examined in triplicate in 250 mL conical glass flask containing 120 mL of freshwater. Flasks were kept sterile throughout the incubation assay. Incubation of samples containing MC was carried out for 28 days. Incubation of samples containing CPY was carried out for 64 days. The incubation was carried out in the dark at room temperature (25±3 ºC) and with continuous shaking at 70 rpm to ensure oxygenation. For HPLC analysis, aliquots of 0.5 mL were taken from each flask at regular intervals (*i.e.* 4 days

for MC and 7 days for CPY) under sterile conditions and immediately frozen. The samples were then freeze-dried and reconstituted in 125 µL of 50% aqueous methanol for HPLC analysis.

HPLC eluents were milli-Q water-0.05% trifluoroacetic acid (TFA) (Fisher Scientific, Leicestershire, UK) and acetonitrile (Rathburn, Walkerburn, UK) 0.05% TFA, the latter being used as ion pairing agent. The instrument used was a Waters 2695 Separation Module with a Waters 2996 Photodiode Array Detector (Waters, Elstree, UK) at a flow rate of 0.3 ml min^{-1}. The detector resolution was set at 1.2 µm and data were acquired in the wavelength range 200-400 µm. Separation was obtained with a Sunfire C18 column (2.1 mm i.d. x 150 mm long x 5 µm particle size) supplied by Waters (UK) kept at a temperature of 40 ºC. In order to detect microcystin-LR a solvent gradient (Lawton et al., 1994) was used (Table 2.2).

Table 2.2 – HPLC solvent gradients used in the detection of microcystin-LR and chlorpyrifos. Both solvents had 0.05%TFA as ion pairing agent.

	Chlorpyrifos Gradient		Microcystin Gradient	
Time (min)	MilliQ-water (%)	Acetonitrile (%)	MilliQ-water (%)	Acetonitrile (%)
0	65.0	35.0	85.0	15.0
25	5.0	95.0	35.0	65.0
27-29	0.0	100.0	0.0	100.0
34-40	65.0	35.0	85.0	15.0

Although some authors have previously been successful in detecting chlorpyrifos by HPLC with isocratic elution (Singh et al., 2002) and gas chromatography (Pablo et al., 2008), here HPLC with gradient elution was chosen as being a reliable method when compared to isocratic (Phillips et al., 2007) as well as offering the ability to detect the compound of interest without the need of internal standard required in gas chromatography. The solvent gradient used in the detection of microcystin was initially tested for the

detection of chlorpyrifos, which gave poor results. The gradient was then optimised and the final gradient used is reported in Table 2.2. This gradient allowed detection of CPY after 19 minutes of injection and of TCP after 7 minutes. At the end of the incubation 50 ml of water from each flask was filtered (0.2 µm, Stericup filter unit, Millipore) and the filters were stored in sealed sterile Petri dishes at -20ºC until DNA extraction for T-RFLP analysis. DNA extraction and all the following steps of T-RFLP analysis were carried out as described in previous section. Half-life (DT50) of microcystin-LR was measured assuming first order kinetics using the tool developed by the FOCUS Degradation Kinetics Workgroup and freely available online (http://focus.jrc.ec.europa.eu/dk/, last access 26-03-2013)

2.2.4 Triclosan biodegradation assay in freshwater

Freshwater samples (40 mL) from the sites G, H, FP and FC (see section 2.2.1 for details) were inoculated with triclosan to a final concentration of 10 μg L^{-1} along with a water control (i.e. no triclosan) and a sterile control (autoclaved water), all the treatments were performed in triplicate. Biodegradation assay for treatment and water control was started on the same day of sampling. For the sterile control, water was first autoclaved, cooled and then pH adjusted with 1M hydrocloridic acid to original value. All samples were kept in glass serum bottles (capacity 125 mL, Sigma Aldrich) sealed with butyl rubber stopper and sealed with aluminium crimp to ensure sterility. Bottles were wrapped with aluminium foil to ensure minimal exposure to light. The experiment was carried out for 12 days at 20 ±1°C shaking at 70 rpm in dark conditions. Serum bottles were opened in sterile conditions every two days to allow oxygenation and to take aliquots (i.e. 0.5 mL) for detection of triclosan. Aliquots were aseptically collected every 4 days, kept in brown glass vials (2 mL) with Teflon cap and immediately frozen. Due to low incubation concentration and experimental design with the inability to have large volume to process, the detection of triclosan with analytical methods (e.g. gas chromatography) was not feasible. Quantification of triclosan was achieved using a commercial kit (Abraxis kits, PA, USA) that applies the principles of enzyme linked immunosorbent assay (ELISA) with quantitation range from 0.05 to 2.5 ppb. Prior to analysis, samples were diluted with the diluent provided by the manufacturer to meet the assay specifications. Concentrations of triclosan obtained from the assay were then multiplied to the dilution factor. Half life (DT50) of triclosan was measured assuming first order kinetics using

the tool developed by the FOCUS Degradation Kinetics Workgroup (see section 2.2.3).

2.3 Results

2.3.1 DNA extraction, DNA yield and microbial T-RFLP analysis from a range of freshwater volumes

On the day of sampling, pH of the water was 8.66 with a temperature of 14°C and light rain. DNA extracted from the range of freshwater volumes (*i.e.* 25-50-75-100-250-500 mL) under study was visualised by gel electrophoresis (Figure 2.1).

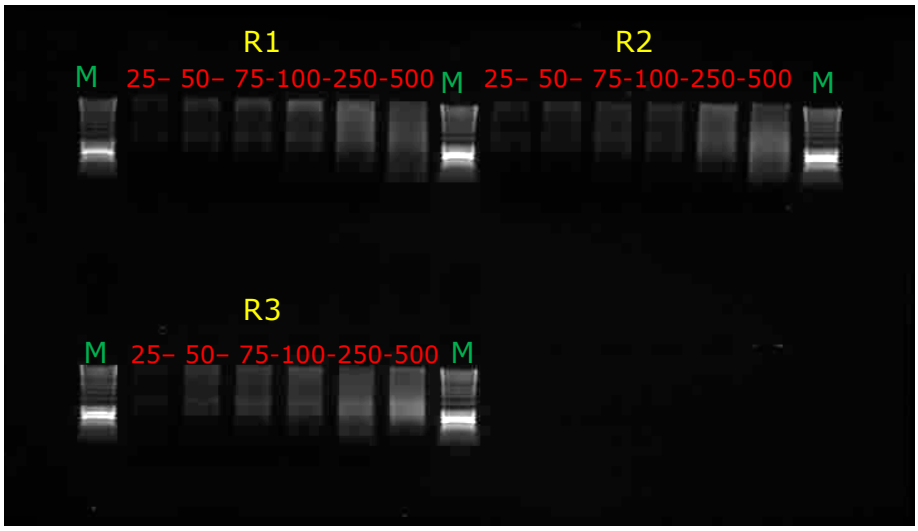

Figure 2.1 - Gel electrophoresis of total DNA extraction from a range of freshwater volumes (in triplicate: R1, R2, R3). Each well contained 4 µl of total DNA mixed with 4 µl of loading buffer, order of loading is outlined in the gel picture. M= 5 µl of Hyperladder I (Promega). Agarose 1% in 1x TBE buffer, voltage applied: 80V, run for 25 min.

The DNA yield was then quantified using a NanoDrop™ 1000 spectrophotometer (Thermo Scientific) and the DNA concentration obtained was plotted against freshwater volumes used for the extraction (Figure 2.2). Relation between water used in DNA extraction and DNA yield was linear up to 100 mL, but became exponential when higher volumes (i.e. 250 and 500 mL) were included in analysis (Figure 2.2).

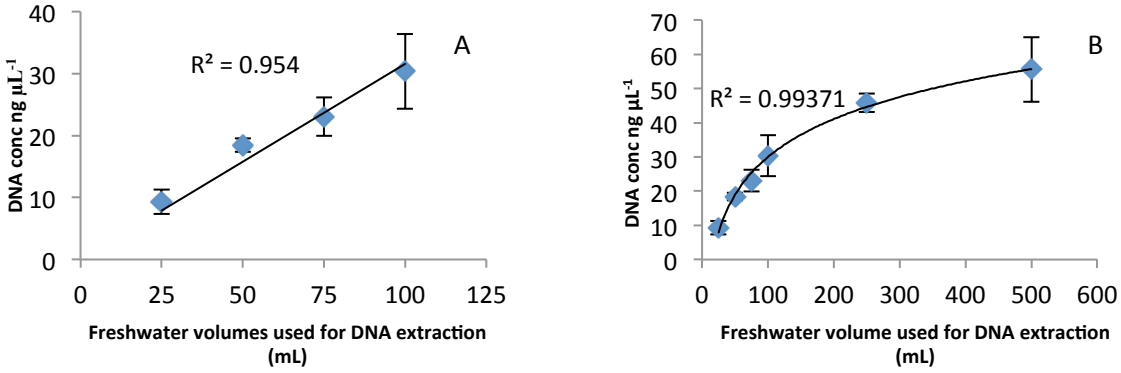

Figure 2.2 – Relationship between filtration volume used in DNA extraction and DNA recovery: A) range 25-100 ml; B) range 25-500 ml. Intercept set at 0 due to the initial negligible amount of DNA in the sterile filter. Freshwater filtered with Millipore Stericup. DNA extracted from Millipore filter with Power Water DNA isolation Kit (Mobio). DNA quantified using NanoDrop™ 1000 spectrophotometer (Thermo Scientific). Bars= standard deviation; n=3.

T-RFLP data for each sample were statistically analysed using analysis of variance (ANOVA) to determine if there was a significant difference amongst the communities deriving from the range of volumes tested. If a significant difference was found, data were further explored with a multiple comparison method (*i.e.* Tukey) to look for specific differences between pairs of groups. ANOVA and MANOVA analysis output for bacterioplankton communities are summarised in Table 2.3.

Table 2.3 – ANOVA analysis on 5 principal components of the terminal restriction fragments relative fluorescence data deriving from freshwater bacterioplankton T-RFLP analysis against the DNA extraction volume (treatment). Significant p values (< 0.05) are in bold. For Tukey multicomparison, letters indicates statistical difference (p<0.05) between treatments (i.e. volume of water).

		Tukey multicomparison					
Principal component (% variation)	**P value**	**25**	**50**	**75**	**100**	**250**	**500**
PC1 (59.4)	0.285						
PC2 (20.59)	**0.045**	*a*	*a*	*a*	*a*	*a*	*a*
PC3 (10.31)	0.191						
PC4 (3.33)	0.304						
PC5 (1.87)	0.191						
MANOVA (5PCs)	0.408						

Only ANOVA on the second principal component scores, accounting for 20.59% of the system variance, gave a significant P value of 0.045. However, when Tukey multiple comparison test was carried out, no significant difference ($P<0.05$) amongst DNA extraction volumes was found.

An identical statistical analysis was then carried out on the algae terminal restriction fragments relative fluorescence data. ANOVA and MANOVA analysis output for algal communities are summarised in Table 2.4.

Table 2.4 - ANOVA analysis on 5 principal components of the terminal restriction fragments relative fluorescence data deriving from freshwater algae T-RFLP analysis against the DNA extraction volume (treatment). Significant p values (< 0.05) are in bold. For Tukey multicomparison, letters indicate statistical difference ($p<0.05$) between treatments (i.e. volume of water).

		Tukey multicomparison					
Principal component (% variation)	**P value**	**25**	**50**	**75**	**100**	**250**	**500**
PC1 (76.75)	**0.024**	*ab*	*a*	*ab*	*ab*	*ab*	*b*
PC2 (11.54)	0.935						
PC3 (6.18)	**0.043**	*a*	*ab*	*ab*	*ab*	*b*	*ab*
PC4 (2.2)	0.579						
PC5 (1.41)	0.258						
MANOVA (5PCs)	**0.009**						

ANOVA on the first and third principal components, accounting together for 82.88% of the system variance, gave a significant P value ($P<0.05$). Tukey multiple comparison test was carried out on the first and third principal component scores. According to the Tukey multiple comparison test carried out on the first principal component scores, there was a significant difference between the algal communities extracted from 50 mL of freshwater (*a*) and the communities extracted from 500 mL (*b*). The communities deriving from the other freshwater volumes analysed were similar to each other (i.e. *ab*) and they represent a "trend" between *a* and *b* (not significantly different either from *a* or *b*). When the Tukey test was carried out on the third principal component scores of the algal terminal restriction fragments relative

fluorescence data, there was a significant difference between the algal communities extracted from 25 mL of freshwater (*a*) and the communities extracted from 250 mL (*b*). As above, the communities deriving from the other freshwater volumes analysed were similar to each other (i.e. *ab*) and they represent a "trend" between *a* and *b* (not significantly different either from *a* nor *b*).

Identical statistical analysis was then carried out on the cyanobacteria terminal restriction fragments relative fluorescence data. Neither ANOVA nor MANOVA on the main 5 principal components scores showed any significant effect between freshwater volume from which DNA was extracted and the shape of cyanobacterial communities.

Moreover, relative diversity for the different microbial communities was evaluated (Shannon diversity index) and a comparison of the diversity of the various aquatic microorganisms along the gradient of volumes used to extract DNA is presented in Figure 2.3. ANOVA followed by Tukey multicomparison was carried out to establish if significant differences were present and none were found. There was no impact of volume from which DNA was extracted on diversity measurements.

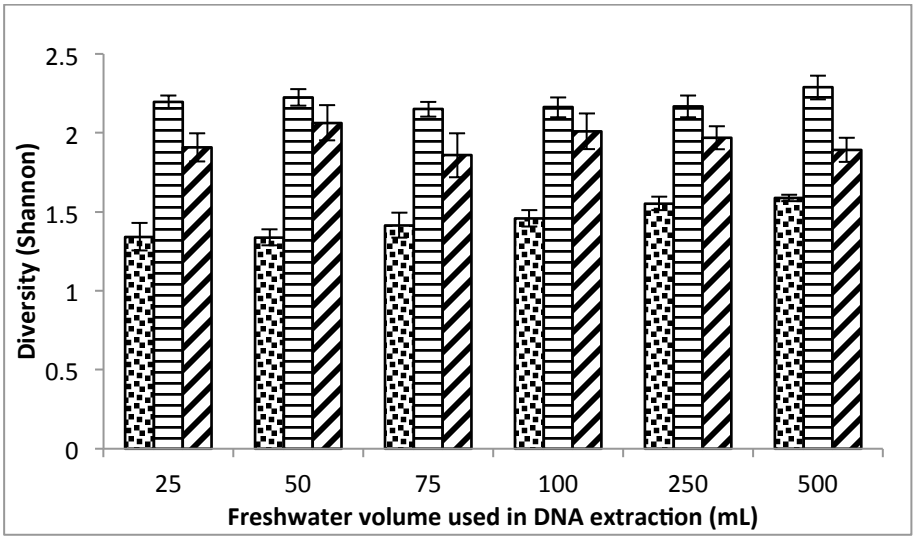

Figure 2.3 - Microbial relative diversity (T-RFLP data) along a range of freshwater volume used for DNA extraction. Algae ▩; Bacteria ▤; Cyanobacteria ▨ . Bars= standard error

2.3.2 Microcystin-LR and chlorpyrifos biodegradation assay in freshwater

In the study of the degradation of MC in freshwater under examination, first a calibration curve was prepared using a range of samples, in triplicate, with concentrations between 100 and 0.1 µg mL^{-1} (Figure 2.4).

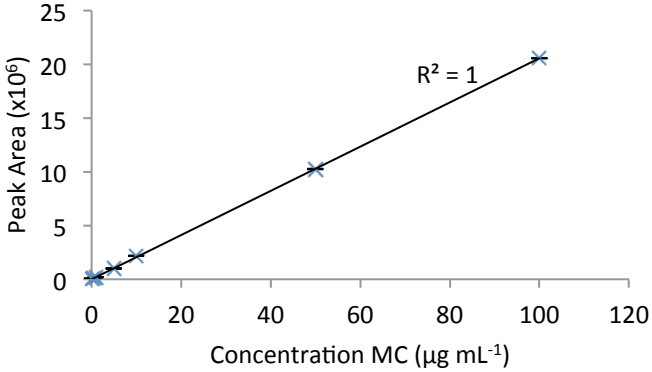

Figure 2.4 - Calibration curve for MC-LR. Analysis was carried out with a Waters 2695 Separation Module coupled with a Waters 2996 Photodiode Array Detector. Separation occurred in a Sunfire C-18 column over a gradient of milliQ-water and acetonitrile. Detection wavelength set at 232 nm. Injection volume: 25 µL. n=3

The preparation of the calibration curve allowed testing of the accuracy and repeatability of the HPLC system and of the user, along with enabling the user to calculate concentrations of unknown samples in the degradation assay. Then, 500 µL of samples were taken at regular intervals (*i.e.* 4 days) in sterile conditions from the degradation assay and were analysed using the same HPLC system in order to obtain degradation curves for both treatments (i.e. freshwater incubated with MC) and control (i.e. sterile freshwater incubated with MC) over a period of 28 days (Figure 2.5).

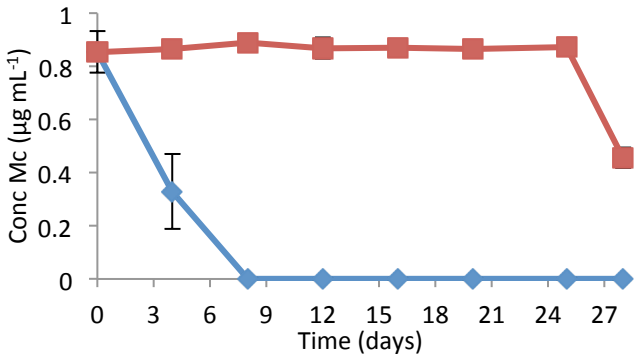

Figure 2.5 – Biodegradation of MC over time in Loch Rescobie freshwater and in sterile control. Freeze dried samples, aliquoted at regular intervals during the degradation assay, were re-suspended in 125 µL of 50% aqueous methanol, centrifuged, and 100µL of supernatant was immediately analysed with a Waters 2695 Separation Module coupled with a Waters 2996 Photodiode Array Detector. Separation occurred in a Sunfire C-18 column over a gradient of milliQ-water and acetonitrile. Detection wavelength set at 232 nm. Injection volume: 25 µL. Concentration MC in freshwater ◆; concentration MC in sterile freshwater ■. Bars = standard deviation. n=3.

HPLC analysis confirmed the ability of natural freshwater microbial communities to degrade MC, which was undetectable on day 8 with a DT50 of 2.4 days. Concentration of MC in the sterile control was constant for all the duration of the experiment except for the last measurement. Possible reasons for the decrease of concentration of MC in the sterile control will be explored in the discussion section 2.4.3.

The detection of CPY was initially investigated using an identical HPLC system, settings and solvent gradient used in the detection of MC. That led to the detection of a peak very late in the chromatography run (retention time ~30 minutes) causing it to overlap with the 100% acetonitrile wash at the end of each run. The gradient was then modified increasing the initial concentration of acetonitrile first to 25% and then to a final concentration of 95% over a 20 minutes gradient: however, this modified gradient did not significantly improved the quality of the chromatography. A new gradient starting at 35% acetonitrile to 95% in 20 minutes was tested giving satisfactory results with a retention time for CPY at ~19 minutes. The main degradation product of CPY in alkaline conditions is 3,5,6-trichloro-2-pyridinol (TCP) and that was detected too with the chosen solvent gradient of milliQ-water and acetonitrile with a retention time of ~7 minutes. The detection wavelength was modified from 238 nm used for the detection of MC to 295.3 nm in the detection of CPY. Despite that λ max for CPY was at 238 nm, the detection wavelength was adjusted to 295.3 nm due to a significant background noise that was present in the chromatograph at 238 nm, probably due to the high concentration of acetonitrile and steep gradient used in the detection of CPY. The background noise was removed when the detection wavelength was changed to 295.3 nm and a reliable detection of CPY and TCP was possible. Once the appropriate solvent gradient to detect both CPY and TCP in the HPLC system was developed, calibration curves were prepared for both compounds (Figure 2.6).

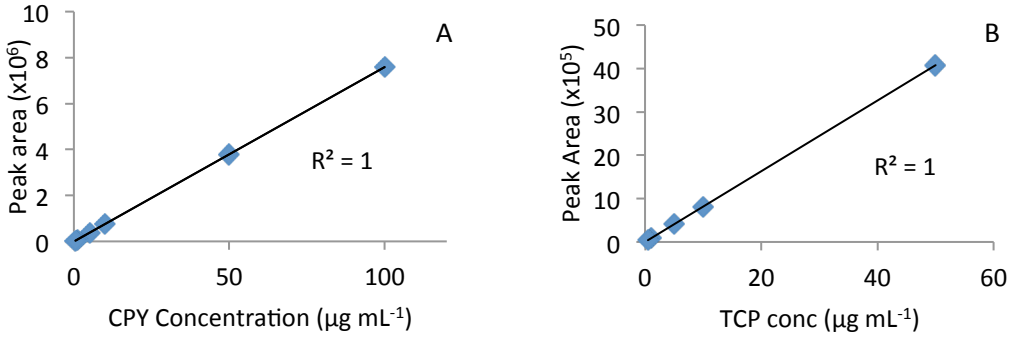

Figure 2.6 - Calibration curve CPY (A) and TCP (B). Samples were prepared from a stock solution of 10 mg mL^{-1} in 100% methanol, freshly diluted in 50% aqueous methanol and analysed with a Waters 2695 Separation Module coupled with a Waters 2996 Photodiode Array Detector. Separation occurred in a Sunfire C-18 column over a gradient of milliQ-water and acetonitrile. Detection wavelength set at 295.3 nm. Injection volume: 25 µL.

Freshwater samples from the degradation assay were analysed at regular intervals using the same HPLC system in order to obtain information about degradation curves of CPY with hypothetical formation of TCP for both treatment (i.e. freshwater incubated with MC) and control (i.e. sterile freshwater incubated with MC) over a period of 64 days (Figure 2.7).

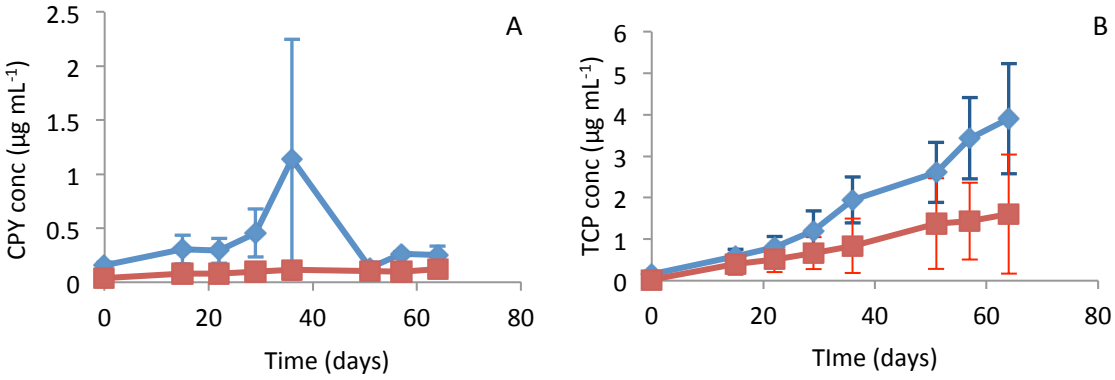

Figure 2.7 – Concentration of CPY (A) and TCP (B) over time in Loch Rescobie freshwater and in sterile control. Freeze dried samples, aliquoted at regular intervals during the degradation assay, were re-suspended in 125 µL of 50% methanol, centrifuged, and 100 µL of supernatant was immediately analysed with a Waters 2695 Separation Module coupled with a Waters 2996 Photodiode Array Detector. Separation occurred in a Sunfire C-18 column over a gradient of milliQ-water and acetonitrile. Detection wavelength set at 295.3 nm. Injection volumes: 25 µL (CPY). Concentration CPY (A) or TCP (B) in freshwater —◆—; concentration CPY (A) or TCP (B) in sterile freshwater —■—. Bars=one standard deviation. n=3

The results obtained by HPLC analysis for CPY were not those that were anticipated. At time zero, when concentration of CPY was known to be around 10 μg mL^{-1}, a very low concentration was observed followed by an unexplainable series of concentrations over the sampling period. Simultaneously, an increasing concentration of TCP was observed both in the treatment "freshwater + CPY" and in the control "sterile freshwater + CPY", with variations amongst replicates increasing along with time. Further investigations into chlorpyrifos solubility, storage and its behaviour into different solvents led to the conclusion that measuring its biodegradation in freshwater systems was not feasible in our system.

Bacterial T-RFLP data were produced at the end of the two biodegradation experiments (i.e. MC-LR and CPY) and combined with data from the beginning of the experiments. The statistical analysis of the complete dataset demonstrated that the first five PCs accounted for 84.55% of the variance. ANOVA of the PC scores, followed by Tukey multicomparison for significantly affected PCs, revealed that most of the difference in the communities was driven by the sampling time (i.e. begin vs end biodegradation assay) rather than the treatment (i.e. MC-LR and chlorpyrifos) (Table 2.5).

Table 2.5 - ANOVA analysis on 5 principal components of the terminal restriction fragments relative fluorescence data derived from freshwater bacteria against the time (begin vs end biodegradation assay) and treatment i.e. MC-LR and chlorpyrifos). Significant p values (< 0.05) are in bold. For Tukey multicomparison, letters indicate statistical difference ($p<0.05$) between treatments.

		Tukey multicomparison				
PC (%)	ANOVA	Begin assay	Water control (1 month)	Water control (2 months)	MC-LR (1 month)	CPY (2 months)
1 (29.54)	**<.001**	a	b	b	b	b
2 (22.14)	0.177					
3 (16.55)	**0.004**	a	b	b	b	b
4 (11.44)	0.155					
5 (4.97)	**0.038**	a	ab	ab	ab	b

Results were confirmed by canonical variate analysis (CVA) on the principal components (Figure 2.8).

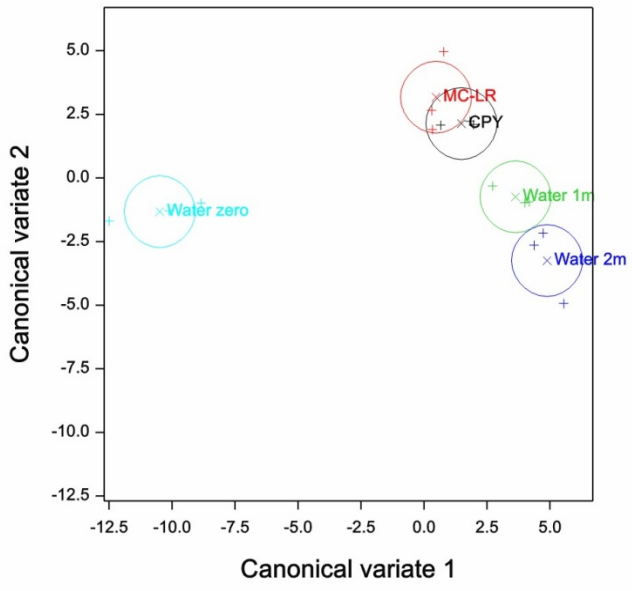

Figure 2.8 – Canonical variate analysis biplot showing the effect of sampling time and treatments on bacterioplankton (T-RFLP data). CVA on 5 PC scores, circles represent 95% confidence (CV1=77.48% - CV2=14.24%); Water zero=begin biodegradation assay; MC-LR= treatment with MC-LR (1 month incubation); CPY= treatment chlorpyrifos (2 months incubation); water 1m= water control one month incubation; water 2m= water control two months incubation.

Once established that the variation between start/end experiment was more important than the variation caused by the treatments (i.e. MC-LR and chlorpyrifos), statistical analysis was carried out focusing on the effect of the treatments at the end of the assay. ANOVA on the PC scores for samples terminated after one month did not show any significant effect of the microcystin-LR on the bacterial communities' structure. On the other hand ANOVA on the PC scores for samples terminated after two months showed a significant effect (P=0.025) of chlorpyrifos on the first principal component account for 66.26% of the variation of the system.

2.3.3 Triclosan biodegradation assay in freshwater

Along with the samples collected during the 12 days of the biodegradation assay a 6-points calibration curve in duplicate was incorporated into the ELISA analysis (Figure 2.9).

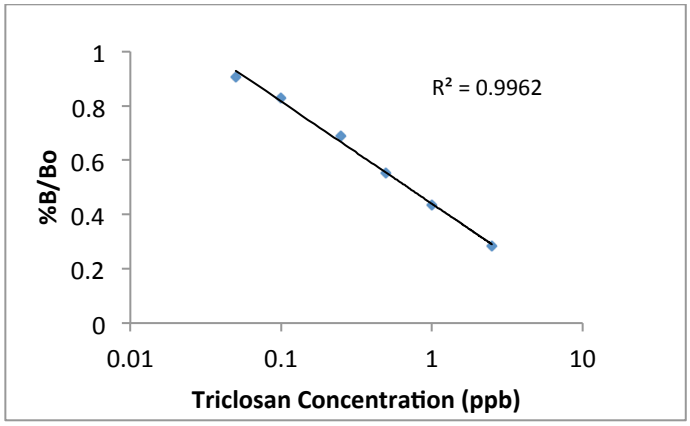

Figure 2.9 – Calibration curve for triclosan prepared following manufacturers protocol. Standards (i.e. 0.05, 0.1, 0.25, 0.5, 1.0, 2.5 ppb) were provided from the manufacturer. Dots represent average of absorbance readings (B) at 450 nm normalised by the absorbance of a zero standard (B_0). n=2

The calibration curve equation was then used to estimate concentrations of triclosan over time in the biodegradation assay. Results showed that triclosan was totally biodegraded from communities from the site H (DT50= 4.5 days) and partially from the site FP (DT50= 14.8 days). The sites G and FC did not show a clear degradation trend.

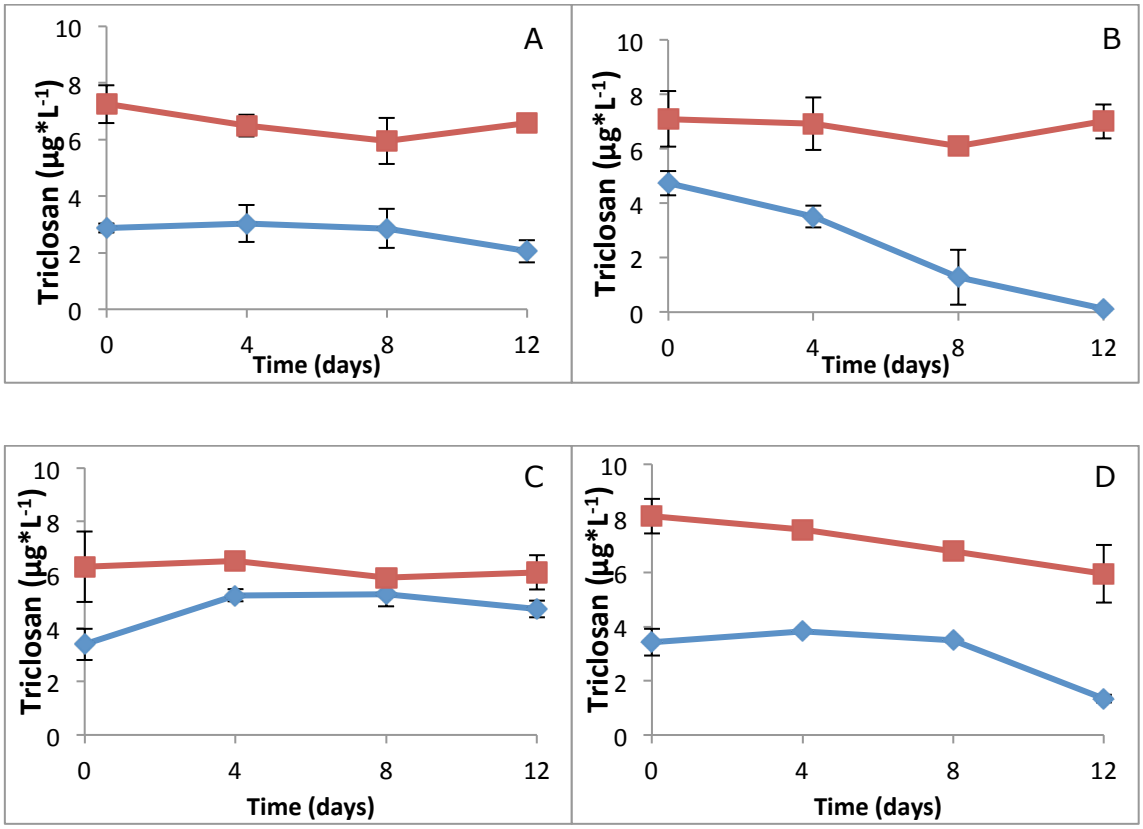

Figure 2.10 – Concentration of triclosan over time in site G (A), site H (B), site FC (C) and site FP (D) measured via triclosan ELISA kit (Abraxis) following manufacturers protocol.Treatment incubated with triclosan ; sterile control . Error bars represents standards error. n=3

The assay gave satisfactory analysis with low variability and consistent results. However, for all sites, a consistent discrepancy between initial concentration of triclosan in treatment and sterile control was observed.

2.4 Discussion

2.4.1 DNA extraction, detection and quantification from a range of freshwater volumes

DNA extraction from a range of freshwater volumes showed that the yield of DNA increased along with the water volume used in the extraction (Figure 2.1), with DNA concentrations ranging from 10 to 60 ng μL^{-1} (Figure 2.2B) when DNA was eluted in 50 µL. Manufacturer (*i.e.* MOBIO) suggests that the standard yield of DNA when extracted from 100 mL of lake freshwater ranges between 15 and 25 ng μL^{-1} (elution in 100 µL). In this work, when DNA was extracted from 100 mL of lake freshwater a yield of about 30 ng μL^{-1} was obtained. In this work, DNA was eluted in 50 µL and not 100 µL as suggested in the manufacturers protocol, in order to gain a higher and easier to detect concentration of DNA. The lower elution volume used explains that a higher DNA concentration was obtained when DNA was extracted from 100 mL. On the other hand, it was expected that a linear relation between DNA yield and water volume used in the extraction considering that the manufacturer stated a capacity of the filter to hold up to 20 µg of DNA: a concentration of 400 ng μL^{-1} when eluted in 50 µL. In this work, the relation between the DNA yield and the volume of freshwater used in the extraction was linear until the volumes 250 mL and 500 mL (Figure 2.2A). When highest volumes were included in the analysis the relation was logarithmic (Figure 2.2B). Some authors suggest that sample volume has a detrimental effect of DNA extraction efficiency (Boström et al., 2004). Here freshwater was filtered with Stericup filter units (Millipore) which have a filtration area of 40 cm^2 and then DNA was extracted with MOBIO Power water DNA isolation kit; however, the latter is designed for extraction of DNA from filters with an area of 13.46 cm^2.

Hence, even if the filters from the two manufacturers are both made out of the same material (*i.e.* polyethersulfon), it is possible that the bigger size of the Millipore filter affects the DNA extraction ability of the MOBIO DNA extraction kit when a high yield of DNA is trapped in the filter. The larger size of the Millipore filter may facilitate overlapping of areas of the filter in the MOBIO extraction tube designed for a smaller device, and that may cause the reduced DNA extraction capacity. Another possibility is the saturation of the kit filter used for DNA elution.

2.4.2 Bacterial, algal and cyanobacterial T-RFLP: communities' structure and relative diversity analysis from a range of freshwater volumes

The comparison between microbial T-RFLP data deriving from DNA extracted from a range of volumes showed that bacterial and cyanobacterial communities did not significantly differ across the range of volumes considered: neither in the community composition (Table 2.3) nor in relative diversity (Figure 2.3). A previous study on marine bacterial communities analysed by another common DNA fingerprinting technique (*i.e.* DGGE) reported little differences in communities extracted from 1 µL to 1 L (Long and Azam, 2001). Kirchman et al. (2001) showed little differences between bacterial communities *r*DNAs amplified with filter PCR with <10 mL samples and gels of amplicons from the standard approach with DNA isolated from about 2 L of coastal seawater. To our knowledge no other author before has attempted to gain an understanding of the role that freshwater volume used in DNA extraction has on bacterial and cyanobacterial communities. In freshwater environments some authors have used a range of water volumes for DNA extraction and then carried out molecular biological studies without considering the probable effect on their results (Pace et al., 1990). Bacterial

relative diversity measurement (T-RFLP data) for the different water volumes considered did not show significant differences. It is necessary to mention that the fingerprinting technique used in this work does not give a measure of absolute diversity but rather to relative diversity. Relative diversity measures are bound to the low resolution of the technique and its inability to detect rare species. For future studies other methodological approaches with higher resolution, such as pyrosequencing, may contribute to a better understanding of how the bacterial communities' structure and diversity change along with a different DNA extraction volume. On the other hand algal communities significantly differ across the range of freshwater volumes studied in community structure (Table 2.4): that was due to a significant difference between algal communities extracted from the smallest volumes (*i.e.* 25-50 mL) when compared to the highest volumes (*i.e.* 250-500 mL) (Table 2.4) but no significant difference was highlighted when diversity indexes along the volume gradient were compared. Although micro-algae diversity is considered to be huge, previous assessment of eukaryotic microalgae diversity in freshwater via T-RFLP found a limited number of OTUs (Dorigo et al., 2002). To conclude, differences in community composition of microalgae across the range of volumes considered in this work may be due to low evenness of the micro-algae communities, even amongst the low number of OTUs detected. The degradation assay carried out with freshwater from Loch Rescobie showed that the bacterial communities' structure changed mainly due to the incubation per se rather than the presence of microcystin-LR or chlorpyrifos. Microbial communities living in a natural freshwater system are exposed to a number of drivers (e.g. sunlight) that have a profound impact in shaping microbial composition. The experiment presented here was carried out in dark

conditions with continuous shaking and constant temperature: all factors that can significantly affect microbial communities' structure (Lindström et al., 2005)

2.4.3 Microcystin-LR biodegradation assay

Microcystin biodegradation by natural communities present in Loch Rescobie was completed by 8 days after incubation (Figure 2.5) with a half-life of 2.4 days. Christoffersen et al. (2002) showed total degradation of environmental relevant concentration of MC-LR within 6 days of inoculum in lake water. Bourne et al. (2006) showed a complete degradation of MC-LR within 12 days after incubation with an initial acclimation phase of 4 days. A previous work, carried out with water sampled from the same Scottish loch incubated with same concentration of MC-LR (Edwards et al., 2008), showed a complete biodegradation within 15 days, with most of the removal happening within the first 7 days after inoculum. Here no acclimation phase was observed, in accordance with the work of Edwards et al. (2008), and that is explained by a history of cyanobacterial bloom and microcystin occurrence in this water body. The sudden decrease of MC-LR in the sterile control observed in the last day of incubation (Figure 2.5) was not expected. The stability of MC-LR in aqueous solution would suggest a sampling error at last sampling. Malfunctions of the HPLC system are excluded because all samples for the incubation of MC-LR were analysed in large batches over two days along with standards, and all other samples and standards gave consistent results.

2.4.5 Chlorpyrifos biodegradation assay

Measurement of the concentration of chlorpyrifos (CPY) gave a range of results unexplained according to degradation of the compound over time

(Figure 2.7A); on the other hand the concentration of TCP increased over time, as expected when degradation of CPY takes place (Figure 2.7B). However, formation of TCP was also observed in the sterile control suggesting that the degradation of CPY was mainly driven by chemical-physical conditions rather than microorganisms. The formation of TCP as a result of abiotic degradation of CPY is probably due to alkaline hydrolysis which is pH dependent (Macalady and Wolfe, 1983): expected at the alkaline pH of the lake freshwater used in this experiment (pH 8.66). Moreover, degradation of CPY in water has been shown to be linked to temperature with a half life of 4.8 days at 21 ºC (Frank et al., 1991): in this experiment with an incubation temperature of 25±1 ºC the half life of CPY was then expected to be a few days. The low solubility of CPY in water (Bowman and Sans, 1983), coupled with its instability at alkaline pH and at room temperature determined the inability to evaluate the biodegradation activity of natural microbial communities in freshwater.

2.4.7 Triclosan biodegradation assay

A commercially available enzyme linked immunosorbent assay (ELISA) has been shown to be a reliable method for detecting triclosan at environmental concentrations with limited sampling material in wastewater samples (Brun et al., 2008, Kantiani et al., 2008). Here the assay was successfully applied to track biodegradation of triclosan in microcosm experiments of four rivers present in New South Wales, Australia. The Hawkesbury-Nepean river system covers about 22,000 square kilometres and provides 95% of drinking freshwater to millions of people in Sydney area along with supporting agriculture in the region. In this system, the four chosen sampling sites were differentiated by their water quality linked to pollution/nutrients exposure. Two

sampling sites (i.e. H and FP) represented polluted/high nutrients sites with wastewater treatment plants closely upstream and urban and/or agricultural runoff feeding into the system. The other two sampling sites (i.e. G and FC) represent pristine sites with no documented source of pollution within 30 km upstream of the sites. The triclosan biodegradation ability of the microbial communities differed amongst sites. Microbial communities that have been experiencing chronic pollution and very likely exposure to triclosan (i.e. H and FP) showed an ability to degrade triclosan (Figure 2.10B-D). On the other hand microbial communities which do not chronically experience pollution and exposure to triclosan (i.e.G and FC) did not show any biodegradation activity (Figure 2.10A-C). Triclosan has been reported to be present in many terrestrial and aquatic environments in Australia (Kookana et al., 2011) and worldwide (Dann and Hontela, 2011). The main gateway of entrance for triclosan to the environment is wastewater treatment plants in which triclosan is largely degraded mainly by biological activities (Kookana et al., 2011). In aquatic environments biodegradation, sorption and photocatalysis contribute to triclosan degradation with their contribution differing upon environmental factors (Sabaliunas et al., 2003). The results of the present experiment suggest that biodegradation is the main mechanism of triclosan dispersal in freshwater incubation experiments in dark conditions, confirming the work of Nakada et al. (2008). Results also show that triclosan biodegradation ability of microbial freshwater is not ubiquitous (at least not within the incubation length of this experiment), but rather it is linked to previous exposure of triclosan suggesting an exposure-driven enhanced biodegradation of triclosan in the environment.

2.5 Conclusions and future work

The results of the experiments presented in this chapter suggest that:

- Freshwater volume (i.e. from 25 to 500mL) employed in DNA extraction: a) does influence the DNA yield; b) does influence the shape of algal communities and; c) does not influence bacterial communities' diversity and structure in freshwater environments;
- Microcystin-LR was promptly degraded from bacterial communities present in a freshwater body with previous exposure/biodegradation activity;
- For experimental design and media of this study, chlorpyrifos is not suitable for biodegradation assay due to its low solubility;
- Experimental incubation time, rather than the presence the microcystin-LR or chlorpyrifos, has a strong effect in shaping bacterial communities in the system tested;
- Triclosan showed different patterns of biodegradability in different water bodies depending upon their previous exposure.

Future studies should therefore focus on bacterial freshwater communities with particular interest in:

- The effect that previous exposure and environmental factors have on the microcystin-LR biodegradation ability from freshwater bacterial communities;
- The relation between bacterial diversity and: a) broad ecosystem function (i.e. respiration); b) specific ecosystem function (i.e. biodegradation) and; c) ecosystem stability.

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3. BACTERIAL COMMUNITIES' RESPONSE TO MICROCYSTINS EXPOSURE AND NUTRIENT AVAILABILITY: LINKING DEGRADATION CAPACITY TO COMMUNITY STRUCTURE

3.1 Introduction

Freshwater is one of the most precious natural resources on the planet. Natural waters have very low concentrations of nitrates and phosphorous. Runoff from farm lands, along with wastewater derived 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 possibly carcinogens), saxitoxins and anatoxins (neurotoxins), 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 characterised and MC-LR has been the 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 requires adequate ways for screening toxicity and for evaluating water quality of exposed water bodies (Codd et al., 2005). A number of works has been published investigating biodegradation of MCs in freshwater (Jones and Orr, 1994, Cousins et al., 1996). 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 throughout history starting from observation with a magnifying glass by Antony van Leeuwenhoek back in the 17th century (van Leeuwenhoek, 1702) and arriving at newly-developed high-output DNA sequencing (Gobet et al., 2011). 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., 2006a). Here T-RFLP was used to evaluate bacterial communities' structure and Biolog EcoPlate to determine their physiology.

This work investigated the relationship 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.

3.2 Materials and methods

3.2.1 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 latter via a small stream (approximately 600 m long) with both water bodies 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, 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. Details about cyanobacterial bloom history, MC-LR exposure and half-life of MC-LR in these water bodies are outlined in Table 3.1.

Table 3.1 - 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 ± 0.0	7.4 ± 0.0	7.8 ± 0.0	8.5 ± 0.0	7.9 ± 0.0	7.8 ± 0.0
Temperature	11.1 ± 0.0	10.5±0.0	11.6 ± 0.0	9.5 ± 0.0	8.6 ± 0.0	8.2 ± 0.0
MC-LR half-life (days) (source Edwards et al., 2008)	4	9	4	5	13	14

[a] 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

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: Whatman, 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.

3.2.2 Biodegradation of MC-LR

The half-life data of MC-LR were derived from the work of Edwards et al. (2008) for the same water bodies 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 $\times$ *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.

3.2.3 T-RFLP analysis

DNA was extracted from each sample using half of the polyethersulfone filter obtained from the filter unit (Millipore Stericup) that were stored at –20°C. DNA extraction was carried out using the Power Soil DNA Extraction Kit (Mo Bio, Carlsbad, California) following the manufacturer's 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 30s, annealing at 55 °C for 30s, 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 hrs 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.

3.2.4 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, the other 3 micro wells do not have any source of carbon and are used as controls. All water samples were processed under sterile conditions within 24 hrs 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 $\pm$ 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 hrs 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).

3.2.5 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 (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).

3.3 Results

3.3.1 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 3.1.

3.3.2 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 (Figure 3.1A) 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 shifts 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 (Figure 3.1B) revealed significant shifts in the structure of the bacterial community due to natural exposure to MC-LR.

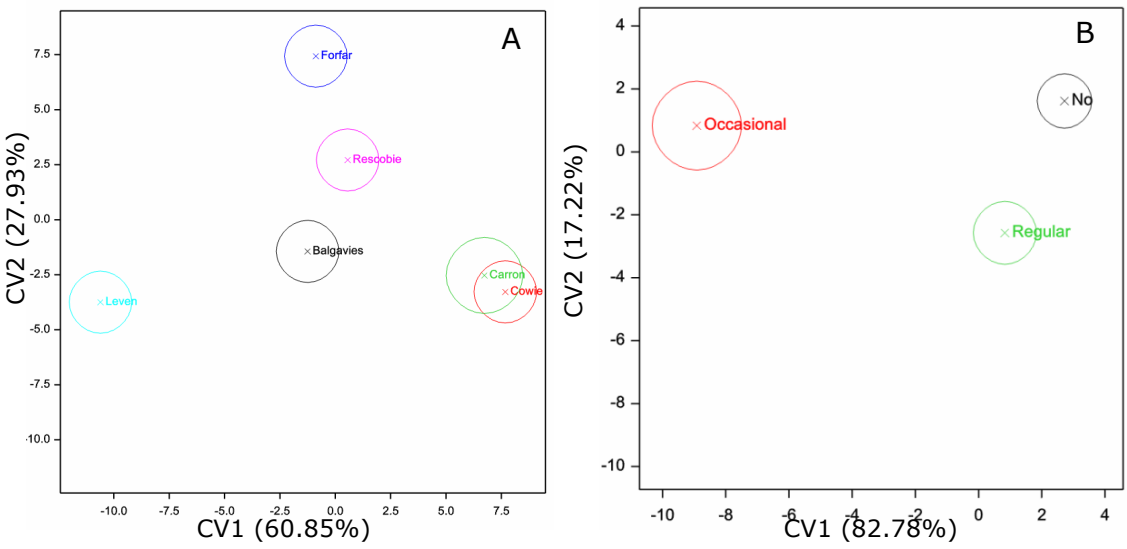

Figure 3.1 – Canonical variate analysis biplots. A) Effect of location on bacterioplankton communities' structure (T-RFLP data). CVA on 5 PC scores, circles represent 95% confidence (CV1=60.85% - CV2=27.93%); B) Effect of natural exposure to microcystins on freshwater bacterioplankton structure (T-RFLP 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.

Linear regression analysis was carried out to explore whether bacterial community structure had an impact on rate of MC-LR degradation. It showed a significant correlation between the bacterial communities' structure and the half-life of MC-LR (see Table 3.2), as well as a significant correlation ($P < 0.001$) between previous exposure to MC-LR and half-life of MC-LR.

Table 3.2 - 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

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 Figure 3.2). 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 Section 3.2.5 Statistical methods). 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 was shown to be positively correlated with the bacterioplankton of Loch Rescobie.

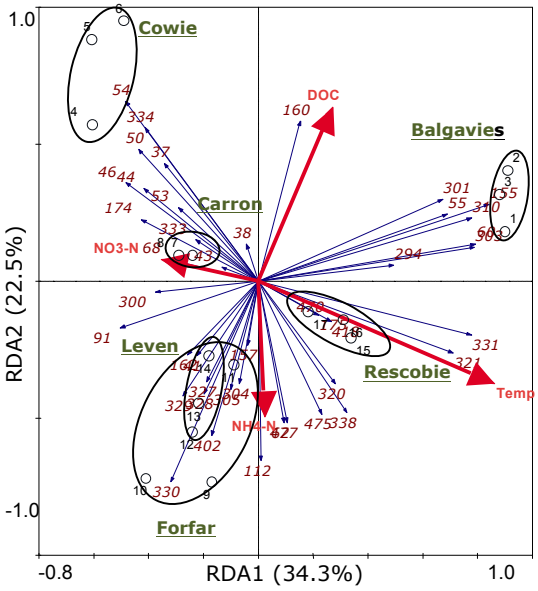

Figure 3.2 – Redundancy analysis triplot. Effect of water chemistry on bacterioplankton communities. T-RFs as species and chemistry as environmental variables. Only significant ($p<0.05$) environmental variables are showed. Variance explained by the variables selected: 56%

3.3.3 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. 3.3A). Biolog EcoPlate data after 48 h incubation were used in this analysis because they showed the greatest 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. 3.3B). No significant correlation was found between living community physiology profiles and MC-LR half-life.

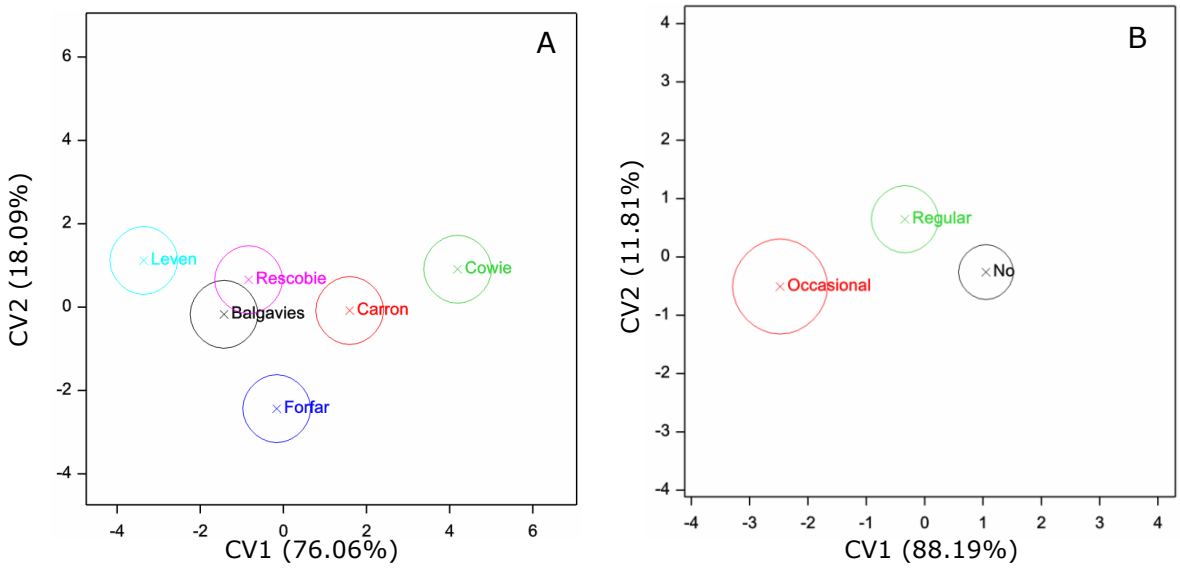

Figure 3.3 – Canonical variate analysis biplots. A) Effect of location on living community physiology (Biolog EcoPlate™ data after 48 hrs). CVA on 5 PC scores, circles represent 95% confidence. B) Effect of natural exposure to microcystins on living community physiology (Biolog EcoPlate™ data after 48hrs 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.

3.4 Discussion

3.4.1 Bacterioplankton communities structures

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 (Figure 3.1A). The magnitude in difference between the lakes investigated in this work observed in Figure 3.1 A 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 (Figure 3.1A), 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 Figure 3.1B. Chemical signalling, inhibitors/stimulators of growth activities along with toxicity to microorganisms are the most plausible direct effect of MCs on bacterial communities' structure (Christoffersen, 1996, Kearns and Hunter, 2000, Babica et al., 2006). On the other hand MCs can be toxic to macro-organisms (MacKintosh et al., 1990, Christoffersen, 1996), and their toxicity cause death or differential feeding patterns at different levels in the food web and that in turn affect the bacterial

communities' structure (Kaebernick and Neilan, 2001). 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 leads 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 3.2). 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 selectively appear in water bodies with regular exposure to MCs also in other systems as they could be used as bio-indicators for water quality. If such bio-indicators 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.

3.4.2 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 (Figure 3.2). 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-fixing and non-nitrogen-fixing cyanobacteria (Kaebernick and Neilan, 2001). Some of the water bodies studied in this work have a 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 was expected. Temperature is a key factor in regulating freshwater bacterial growth (White et al., 1991, Felip et al., 1996) and communities' structure (Yannarell and Triplett, 2004, Yannarell and Triplett, 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 appears to be a parameter closely related with bacterial communities structure (Figure 3.2).

3.4.3 Biolog EcoPlate

Bacterial carbon substrate utilisation profiles, determined with Biolog Ecoplate, were used to establish differences amongst bacterial communities' physiology. Biolog EcoPlates has been used in various investigations to study aquatic microbial communities (Comte and Del Giorgio, 2009) and their ability to distinguish between 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 (Figure 3.3A). 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 was shown to be an important factor in the differentiation of the physiology of bacterioplankton communities (Figure 3.3B) 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 effects of MCs on freshwater microbial autotrophs physiology (Singh et al., 2001, Hu et al., 2004), on the other hand it has to be considered that changes in communities' structure caused by MCs (see Section 3.5.2) would then alter the observed physiological profile.

3.5 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 was exposure to MCs 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. Although previous studies have shown a link between previous exposure to MCs and improved ability of the microbial communities to degrade such toxins, this is the first report to our knowledge to demonstrate that a change in bacterioplankton communities' structure is the link between previous exposure and biodegradation ability. It appears that exposure to MCs is able to affect bacterioplankton physiology and structure and the latter is then linked with degradation rate of MC-LR. Taking into 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 study as, if confirmed, such OTUs could be used as bio-indicators of water quality.

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4. BACTERIAL DIVERSITY, FUNCTION AND STABILITY: RELATIONS IN LAKE WATER

4.1 Introduction

In the current age of extinction (Barnosky et al., 2011), increasing attention and importance has been given to biological diversity as a key factor governing ecosystems and their functioning (Naeem et al., 2012). The most tangible effects of biodiversity on humankind are the "services" that it provides (Duraiappah et al., 2005), which are a result of essential functions that biodiversity sustains (Duffy, 2008). A review by Cardinale et al. (2012) highlighted the last 20 years of research that focused on how biodiversity influences ecosystem functions and services, pointing out consensus and emerging trends obtained from literature. In the review it was reported that freshwater purification is one of the few ecosystem services for which studies conflict with prediction and give not significant relationship. A large number of species for both micro and macro-organisms have important roles in freshwater purification, but bacteria cover a key position in biogeochemical cycling, biodegradation and biomass production (Newton et al., 2011). Bacterial diversity vitally contributes to improve water quality, but diverse bacteria are also able to produce a very wide range of secondary metabolites with many being of industrial importance (Singh, 2010) and some toxic (Harada, 2004). The most relevant class of natural toxic compounds in aquatic environments is represented by cyanotoxins that are generally produced by cyanobacteria during eutrophication-triggered blooms (Paerl et al., 2001). Bell et al. (2009) categorised the different approaches to study the relationship between diversity and functions in microbial communities in: a) microcosm

experiments of artificial communities constructed from culturable microbes; b) removal experiments (e.g. dilution-to-extinction approach) and; c) observational studies (i.e. attempt to link *in-situ* diversity to functions). Despite the increasing interest in biodiversity ecosystem function relation, only one study explored how loss in microbial diversity affects biodegradation of toxic compounds (Cook et al., 2006). To test this relationship in this study, microcosms were set up using batch cultures of sterile freshwater inoculated with serial freshwater dilution (Salonius, 1981). After regrowth, the impact of the removal experiment on freshwater bacterial diversity was evaluated by both DNA fingerprinting and high-throughput sequencing. Also measured was how the dilution to extinction experiment affected carbon mineralisation, stability of the system (Tilman et al., 2006) and biodegradation of microcystin-LR (i.e. the most relevant cyanotoxins). We then explored relations between the measured bacterial diversity and functions/properties mentioned above.

4.2 Materials and Methods

4.2.1 Freshwater Sampling

For the first experiment, sub-surface water (i.e. within 10 cm from surface) was sampled in triplicate on the 6th of June 2011 from two large deep Scottish lakes: Loch Rescobie (NO 525 515) and Loch Freuchie (NN 854 381). Loch Rescobie (area 0.59km^2) is a well studied lake and harbours microcystin producing cyanobacteria as well as microcystin degrading bacteria (see Chapter 2 and Chapter 3). At the time of sampling its water temperature was 14°C and pH 7.4. Loch Rescobie suffers from two sources of diffuse pollution (i.e. arable farming and sewage disposal) and its overall status was categorised as "poor" by the Scottish Environmental Protection Agency (SEPA) (SEPA, 2010a). Loch Freuchie (area 1.39km^2) is a water body highlighted by SEPA (personal communication) to be one of the most pristine lakes in Scotland. At the time of sampling its water temperature was 11°C and pH 6.4. Loch Freuchie does not suffer from any known environmental pressure and its overall status was categorised as "good" by SEPA (SEPA, 2010b). Water was sampled in sterile glass bottles and kept at constant temperature while transported to the laboratory facilities. On the same day of sampling, all water from both sites was filtered via glass fibre filters (1.2 µm pore size, GF-C, Whatman, Maidstone, United Kingdom) to remove large particles and bacterial grazers. In addition, a portion of the water collected from Loch Rescobie was filter sterilised via Stericup filter units (0.22 µm, Millipore, Bedford, MA, USA). Finally, water to be used as sterile controls in the MC-LR biodegradation experiment (Loch Freuchie) was autoclaved at 121°C for 20 minutes along with the water to be used as sterile control and for the diversity manipulation step in the diversity/function experiment with Loch Rescobie.

For the second experiment, subsurface water was sampled in triplicate on the 25th of August 2011 from Loch Rescobie and Loch Freuchie. At the moment of sampling Loch Rescobie water temperature was 16°C and pH 8.6, while Loch Freuchie water temperature was 12°C and pH 6.7. For this sampling time sampled water was not filtered via glass fibre filters, differently to what had been done in previous sampling time (see above), as this step was considered to be responsible for the absence of biodegradation activity. Otherwise samples were treated in the exact same way as performed in previous sampling time.

For ease of identification, from this section on, experiments done with freshwater sampled in June 2011 will be named Loch Rescobie (1) and Loch Freuchie (1); the one with freshwater sampled in August 2011 will be named Loch Rescobie (2) and Loch Freuchie (2).

4.2.2 Bacterial diversity manipulations and biomass recovery monitoring

To achieve a bacterial diversity gradient without need of culturing, a dilution-to-extinction approach was employed. In brief, water sampled from Loch Rescobie (1), previously filtered via 1.2 μm filters, was serially diluted with water sampled from Loch Rescobie (1) which had been filter sterilised and autoclaved to ensure sterility. Ten-fold serial dilutions were carried out in triplicate under sterile laminar flow and three dilutions were used for further studies: 1) $10exp^{-1}$; 2) $10exp^{-4}$; 3) $10exp^{-7}$ (Figure 4.1). The whole range of dilutions (i.e. from $10exp^{-1}$ down to $10exp^{-7}$) was not kept for logistic reasons, as it would have been unfeasible to carry out the whole range of analysis on all dilutions due to time constraints. On the other hand the dilutions kept (i.e. $10exp^{-1}$, $10exp^{-4}$, $10exp^{-7}$) gave a range of extremes and intermediate

diversity levels, allowing significant reduction in the number of samples to make the experiment more feasible.

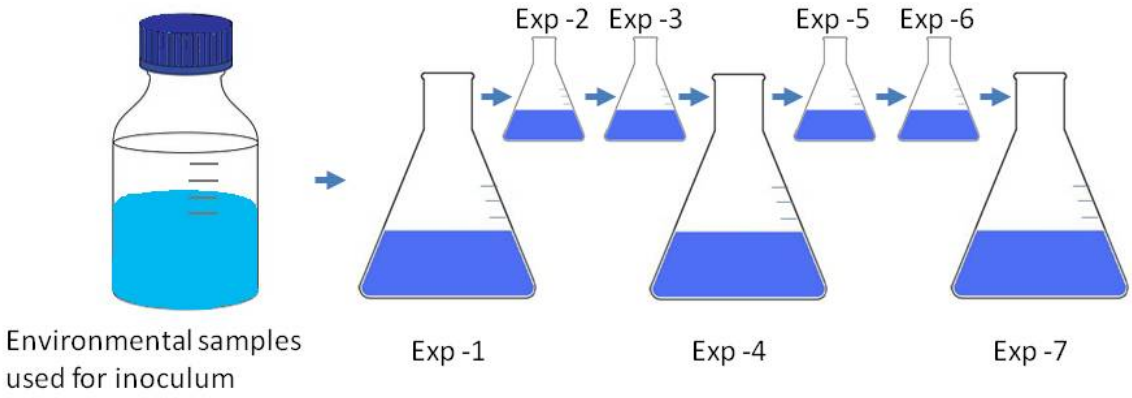

Figure 4.1 – Schematic representation of bacterial diversity manipulation via dilution-to-extinction approach. Arrows indicate 10-fold dilution. Large conical flasks indicate dilutions kept for further studies. Small conical flasks indicate dilutions discharged.

The diversity gradient for Loch Rescobie (2) and Loch Freuchie (2) was achieved following the same approaches described above for Loch Rescobie (1) with the only difference being that freshwater was not filtered via 1.2 µm filters as this step was considered to be responsible for lack of biodegradation activity in Loch Rescobie (1) experiment.

Once dilutions were achieved in a final volume of 180 mL, flasks were incubated for biomass recovery at 20°C shaking at 70 rpm in dark conditions. Biomass recovery was tested by means of bacterial 16S qPCR every three days starting from hour zero (i.e. the hour when dilutions were made). For 16S qPCR, initially 25 mL of water was filtered via 0.2 µm Stericup filter units, the filters were then cut from the units using a sterile scalpel and placed aseptically into 5 mL tubes ready for DNA extraction using the PowerWater DNA isolation kit (MoBio Laboratories Inc.). DNA extraction was carried out following manufacturers instructions. Once DNA was extracted, 16S qPCR was

carried out using a modified protocol of Fierer et al. (2005) for the detection of all bacteria. qPCR assays were carried out on a Rotor Gene-3000 (Corbett Research, Cambridge, United Kingdom) in polypropylene thin-walled tubes. Each 25 µL reaction contained: 12.5 µL of GoTaq® qPCR Master Mix (Promega), 1 µL of bovine serum albumin (20 mg mL^{-1}; Roche), 0.625 µL of primer EUB338 (20 µM, Seq: ACTCCTACGGGAGGCAGCAG) (Kolb et al., 2003), 0.625 µL of primer EUB518 (20 µM, Seq: ATTACCGCGGCTGCTGG) (Muyzer et al., 1993), 5.25 µL of nuclease-free water (Promega) and 5 µL of template. PCR conditions were: 5 min at 95°C, followed by 30 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 60 s and 83°C for 15 s. To produce an amplicon standard, a plasmid containing the target regions was constructed and used as template for PCR. Amplified products were run on 2% agarose gel to confirm specificity. Standard curves were generated in duplicate via 10-fold dilutions of the quantified PCR amplicon. At least five non-zero standard concentrations per assay were included, with standard concentration ranging from $10exp^{9}$ to $10exp^{2}$ copies $µL^{-1}$. Melting curve analysis was carried out following each assay during the optimisation stage of the assay to verify the specificity of the fluorescence signal, however, once the assay gave optimal results (i.e. $R^2 \geq 0.99$ and efficiency at 100±5%) melting curve was removed to shorten the assay run time. Target copy numbers for each reaction were calculated assuming a product size of 200 bp from the standard curves, which in all assays gave optimal correlation coefficient and efficiency. Once biomass recovery was achieved, samples were used in microcystin-LR biodegradation experiments.

4.2.3 Biodegradation assays of microcystin-LR

Three biodegradation assays were set up: a) biodegradation of MC-LR with water from Loch Freuchie (1) with its sterile control; b) biodegradation of MC-LR for each diversity level with water from Loch Rescobie (1) with its sterile and water controls and; c) biodegradation of MC-LR for each diversity level with water from Loch Rescobie(2) and Loch Freuchie (2) with respective sterile and water controls.

4.2.3.1 Biodegradation assay of MC-LR in Loch Freuchie (1)

Freshwater samples (40mL) from Loch Freuchie (1) were added with filter sterilised microcystin-LR (1 mg mL^{-1} in milli-Q water) to a final concentration of 1 mg L^{-1} for biodegradation study. A sterile control with same amount of microcystin-LR was maintained. Treatment and control were tested in glass serum bottles (capacity 125 mL, Sigma Aldrich) sealed with butyl rubber stoppers and sealed with aluminium crimp to ensure sterility. The experiment was carried out at 20 ±1°C shaking at 70 rpm in dark conditions. Experiment was maintained for 14 days. Serum bottles were opened in sterile conditions every three days to allow oxygenation and to take aliquots (i.e. 0.5 mL) for analytical analysis (i.e. HPLC to detect MC-LR) and frozen immediately. Samples were then freeze-dried and, at the time of HPLC analysis, reconstituted in 125 µL of 50% aqueous methanol. Quantification of MC-LR over time was evaluated with the method outlined in section 2.2.3.

4.2.3.2 Biodegradation assay of MC-LR at different diversity levels in Loch Rescobie (1), Loch Rescobie (2) and Loch Freuchie (2)

Samples were incubated for 14 days for Loch Rescobie (1) and 13 days for Rescobie (2) and Loch Freuchie (2) with MC-LR following the procedures

described in previous section. Additionally, respiration, as a proxy of total biological activity, was measured using infrared gas analysers (IRGA) (EGM-4, PP systems). Gaseous sample (10 mL) from the headspace of each serum bottle were taken using syringes just before opening the bottle for atmospheric re-equilibration at each sampling time. The gaseous samples were then injected to the IRGA saturating the infrared cell and giving immediate readings of CO_2 concentration.

Additionally, for Loch Rescobie (2) and Loch Freuchie (2), biodegradation experiments were set up with undiluted water to test if even one ten-fold dilution could be the cause of the lack of biodegradation activity. Experiments of undiluted samples were kept for 14 days with three measurements of concentration of MC-LR over time (i.e. day 0, day 7, day 14), otherwise experiments were set up and maintained as described in section above. Due to time constraints, no respiration measurements or molecular biological analyses were carried out on undiluted samples.

4.2.4 Relative bacterial diversity and richness along a diversity gradient in Loch Rescobie (1), Loch Rescobie (2) and Loch Freuchie (2) (T-RFLP data)

At the end of the incubation, 25 mL of water from each bottle were filtered (0.2 µm, Stericup filter unit, Millipore) and the filters were stored in sealed sterile Petri dishes at -20 ºC until DNA extraction for T-RFLP analysis. DNA extraction and all the following steps for T-RFLP analysis were carried out as described in section 2.2.2 for bacterial communities. The only difference was that both primers in the PCR were labelled with a fluorescent tag (i.e. 63f-VIC and 1087r-FAM). T-RFs data were then analysed to extrapolate measures of bacterial diversity (i.e. Shannon diversity index) and bacterial richness (i.e.

number of Operational Taxonomic Units-OTUs). Estimates for richness and diversity were obtained by averaging the results of forward and reverse terminal restriction fragments data.

4.2.5 Bacterial diversity and richness: pyrosequencing

Due to low concentration of DNA in individual samples, for the pyrosequencing template 16s rRNA gene amplicons were used as template. The amplicons were obtained using the same primers and conditions described in section 2.2.2 except that unlabelled primers were used. The amplicons were then cleaned up using Wizard® SV Gel and PCR Clean-Up System (Promega) following manufacturers protocol. Pyrosequencing of 16S rRNA gene was performed on a Roche Junior Titanium Series. A 466-bp fragment of 16S rRNA gene was amplified using the modified primers PRK341F (5'-CCTAYGGGRBGCASCAG-3') and PRK806R (5'-GGACTACNNGGGTATCTAAT-3') (Yu et al., 2005, Xu et al., 2012). Data analysis was performed using the 'Quantitative Insights Into Microbial Ecology' (QIIME v 1.6.0) software package (Caporaso et al., 2010). Barcode, linker primer and reverse primer sequences were removed from the raw sequence reads using the 'split_libraries.py' script while setting minimum sequence length of 200 and minimum quality score of 20. 'Acacia' tool was used with default options to remove pyrosequencing noise (Bragg et al., 2012). Potential chimeras were removed using the UCHIME chimera detection utility of the USEARCH v6.0.307 tool (Edgar et al., 2011). Similar sequences were binned into OTUs using 'UCLUST' method (minimum pairwise identity of 97%). Observed species (OTUs) metric and alpha-diversity with Shannon entropy were then measured.

4.3 Results

4.3.1 Bacterial biomass recovery and its trend over time

Before testing the hypothesis related to the diversity-function relation, biomass of the manipulated levels of biodiversity was monitored over time to determine its recovery. Biomass recovery along the dilution (diversity) range was achieved within 6 days for Loch Rescobie (1) and maintained a constant magnitude until the termination of the biodegradation assay (Figure 4.2A).

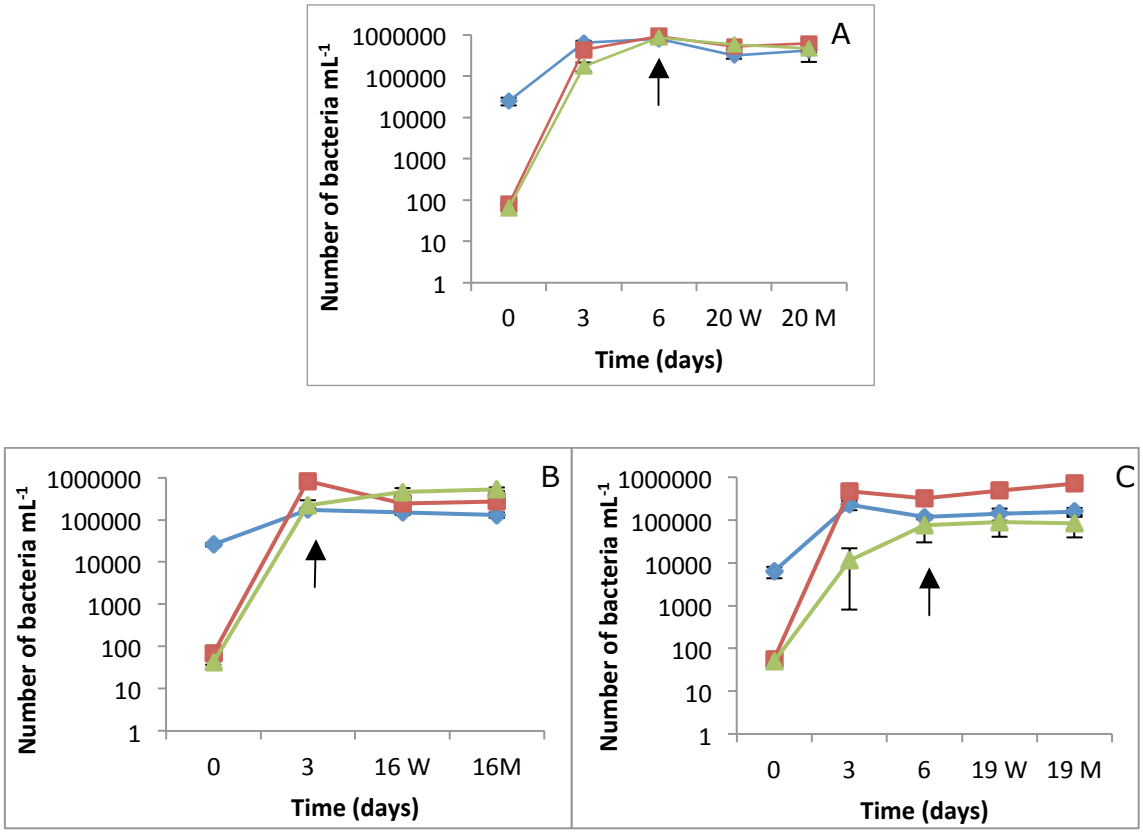

Figure 4.2 – Bacterial biomass over time at different dilution levels: data of gene copy number mL^{-1} of bacterial 16S rRNA gene number of bacteria in original sample from: A) Loch Rescobie (1), B)Loch Rescobie (2) and C) Loch Freuchie (2). =exp^{-1}; = exp^{-4}; = exp^{-7}. W=water control, M=incubated with MC-LR. Arrow indicates time point when biomass recovery was achieved. Error bars indicate standard error. n=3

Biomass recovery along the dilution (diversity) range was achieved within 3 days for Loch Rescobie (2) and 6 days for Loch Freuchie (2) and maintained a

constant magnitude until the termination of the biodegradation assay (Figure 4.2B-C).

4.3.2 Bacterial diversity and richness along the dilution gradient: DNA fingerprinting and pyrosequencing

Once biomass recovery was achieved, relative bacterial diversity was estimated via T-RFLP analysis. OTUs relative abundance data from both forward and reverse terminal restriction fragments matrices were obtained and used to estimate diversity and richness of the bacterial communities. Diversity was measured by the Shannon diversity index (Equation 4.1) which is the most widely used diversity index, originally introduced by C.E. Shannon (1948) as a entropy measure.

Equation 4.1 – Shannon Diversity Index (H'). S = total number of species; p_i = relative abundance on each species.

$$H' = \sum_{i=1}^{S} p_i \ln(p_i)$$

Richness was measured as total number of OTUs. The mean of Shannon diversity indexes deriving from forward and reverse terminal fragment restriction analysis was used as overall relative diversity estimate. Same procedure was followed for richness.

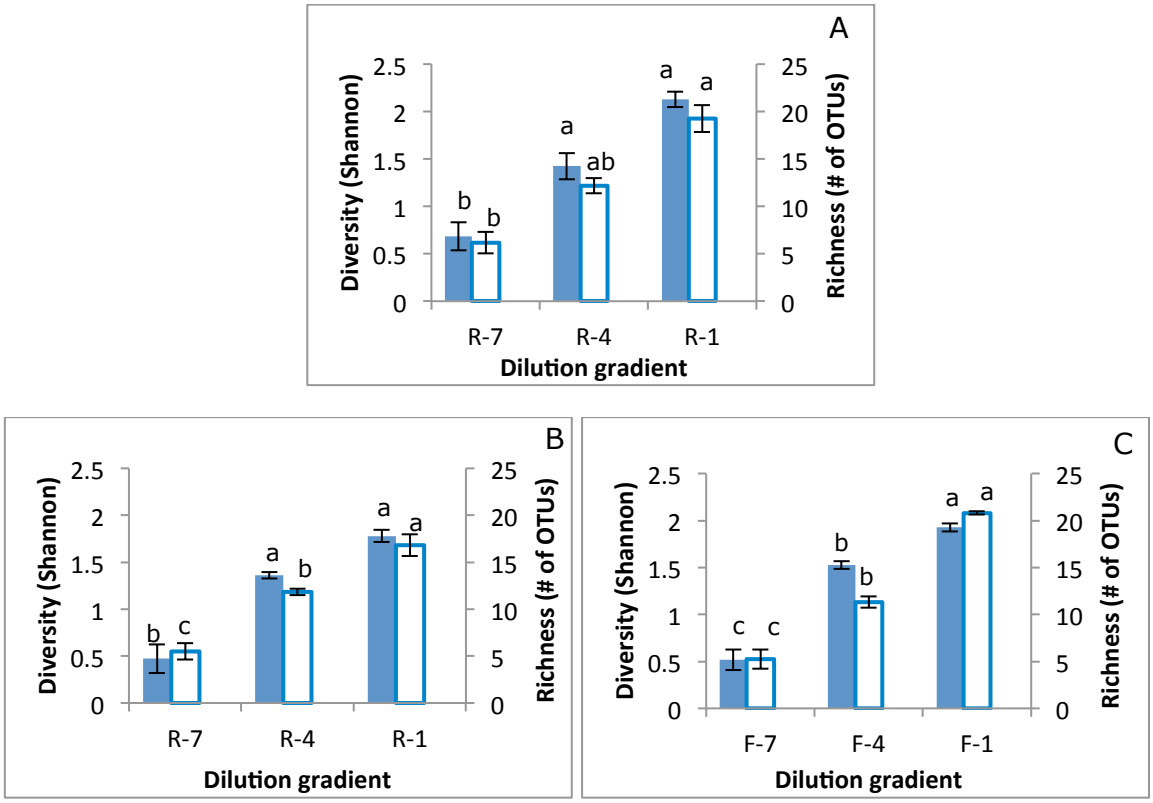

Figure 4.3 – Bar plots presenting relative diversity and richness (based on T-RFLP data) along the dilution gradient of: A) Loch Rescobie (1), B) Loch Rescobie (2) and C) Loch Freuchie (2). Solid columns=diversity, empty columns=richness. R=Loch Rescobie, F=Loch Freuchie. Values with different letters differ significantly ($P<0.05$). Error bars indicates standard error. n=3

The dilution to extinction approach was an effective method to manipulate diversity and richness of bacterial communities in freshwater.

For Loch Rescobie (1) (Figure 4.3A) the dilution significantly affected both diversity ($P=0.009$) and richness ($P=0.014$) with an overall 68% reduction (i.e. comparison R-1 to R-7) in both diversity and richness. For Loch Rescobie (2) (Figure 4.3B) the dilution significantly affected both diversity ($P<0.001$) and richness ($P<0.001$) with an overall reduction (i.e. comparison R-1 to R-7) in diversity and richness of 73% and 67%, respectively. For Loch Freuchie (2) (Figure 4.3C) the dilution treatment significantly affected both diversity

(P<0.001) and richness (P<0.001) with an overall reduction (i.e. comparison R-1 to R-7) in diversity and richness of 73% and 75%, respectively.

Along with using DNA fingerprinting to ascertain if a significant gradient of relative diversity was established via the dilution-to-extinction approach, pyrosequencing of bacterial 16S rRNA gene was employed to confirm and strengthen findings. Pyrosequencing was carried out on samples from Loch Rescobie (2) and Loch Freuchie (2).

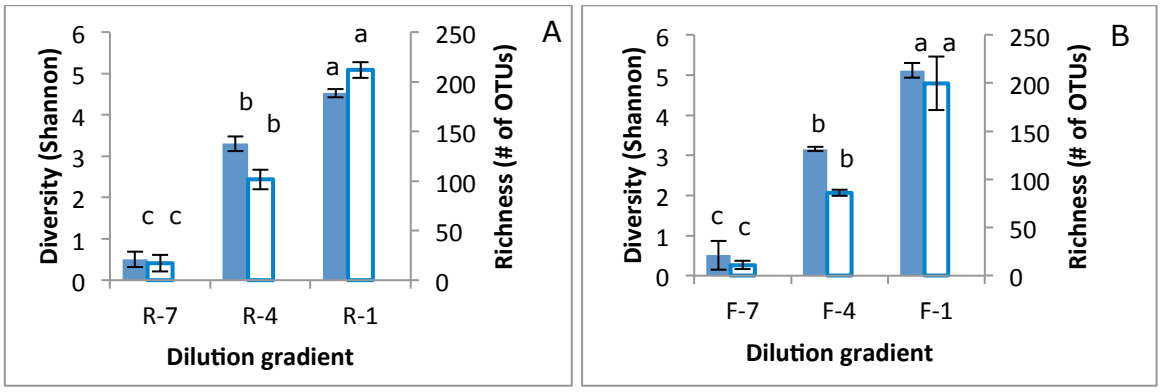

Figure 4.4 – Bar plots presenting bacterial diversity and richness (based on pyrosequencing data) along the dilution gradient of: A) Loch Rescobie (2) and B) Loch Freuchie (2). Solid columns=diversity, empty columns=richness. R=Loch Rescobie, F=Loch Freuchie. Values with different letters differ significantly (P<0.05). Error bars indicate standard error. n=3

Analysis carried out on processed pyrosequencing data at a depth of 4672 sequences per sample confirmed an effective reduction in communities' richness and diversity induced via the dilution-to-extinction approach.

A significant reduction (P<0.001) in bacterial richness was measured in Loch Rescobie (2) that on average harboured: 212 species at its most diverse level (i.e. exp^{-1}), 102 (52% reduction) at its intermediate diversity level (i.e. exp^{-4}) and 17 (92% reduction) at its least diverse level (i.e. exp^{-7}). The reduction in

richness in Loch Freuchie (2) was also significant (P=0.002) with 200 species at exp^{-1}, 86 (57% reduction) species at exp^{-4} and 11 (97% reduction) at exp^{-7}.

Similarly, a significant reduction (P<0.001) in bacterial diversity based on Shannon index was measured for Loch Rescobie (2) accounting for a reduction of 27% (i.e. exp^{-4}) and 89% (i.e. exp^{-7}) compared to the most diverse level of diversity (i.e. exp-1). Reduction in bacterial diversity induced via the dilution-to-extinction approach was also significant (P<0.001) for Loch Freuchie (2) with 38% (i.e. exp^{-4}) and 90% (i.e. exp^{-7}) comparing to the most diverse level of diversity (i.e. exp-1).

As diversity and richness were estimated by two techniques (i.e. T-RFLP and pyrosequencing), it was of interest to investigate if the results between the two techniques would correlate, if there was a discrepancy and if the discrepancy would differ along the diversity gradient.

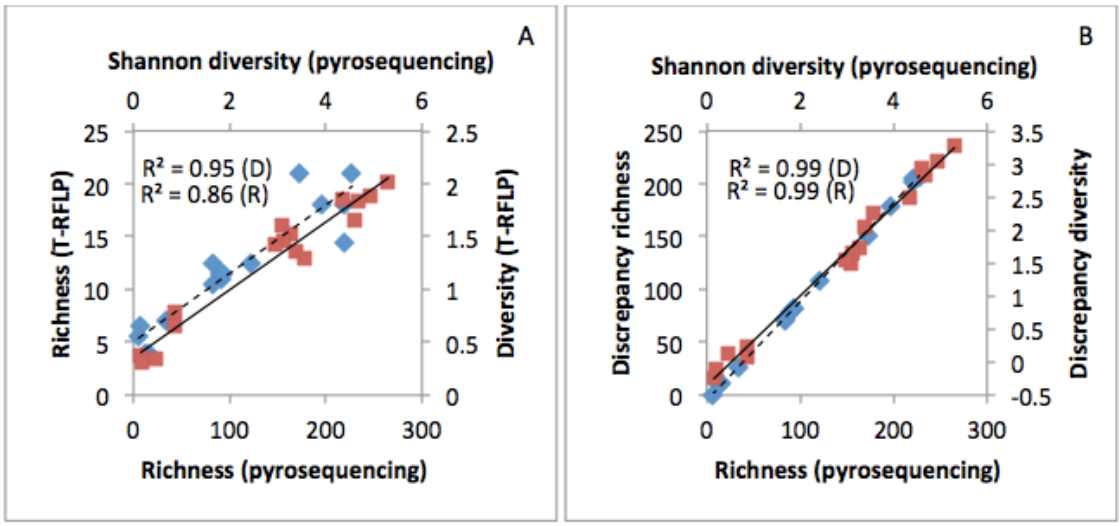

Figure 4.5 – Scatter plots presenting: A) Correlation between diversity and richness (number of OTUs) estimated via pyrosequencing against T-RFLP; B) correlation between diversity and richness (number of OTUs) estimated via pyrosequecing against discrepancy between diversity and richness between pyrosequencing and T-RFLP (i.e. discrepancy richness= richness pyrosequenicing – richness T-RFLP; discrepancy Shannon diversity= Shannon diversity pyrosequencing – Shannon diversity T-RFLP). Richness=■; diversity=◆. Continuous regression line= Shannon diversity, dashed regression line= richness.

As expected, richness estimated via pyrosequening was strongly correlated to richness estimated via T-RFLP ($P<0.001$) and similarly a strong correlation was found for Shannon index ($P<0.001$). The discrepancies between the two techniques significantly ($P<0.001$) positively related to the total diversity/richness of the communities. In other words, a strong linear relation describes that the higher the diversity/richness of the communities the less T-RFLP analysis achieves to measure them.

4.3.3 Activity and stability along a dilution gradient

Once biomass recovery was achieved, an experiment to test the relationship between diversity and function was initiated. Two functions were tested: total communities' activity and ability to degrade microcystin-LR. Respiration data provided overall information on the total communities' activity at the different levels of diversity. Additionally, based on respiration data, the stability of the systems at the different diversity levels was measured as mean-standard deviation ratio (Tilman et al., 2006) (Figure 4.6).

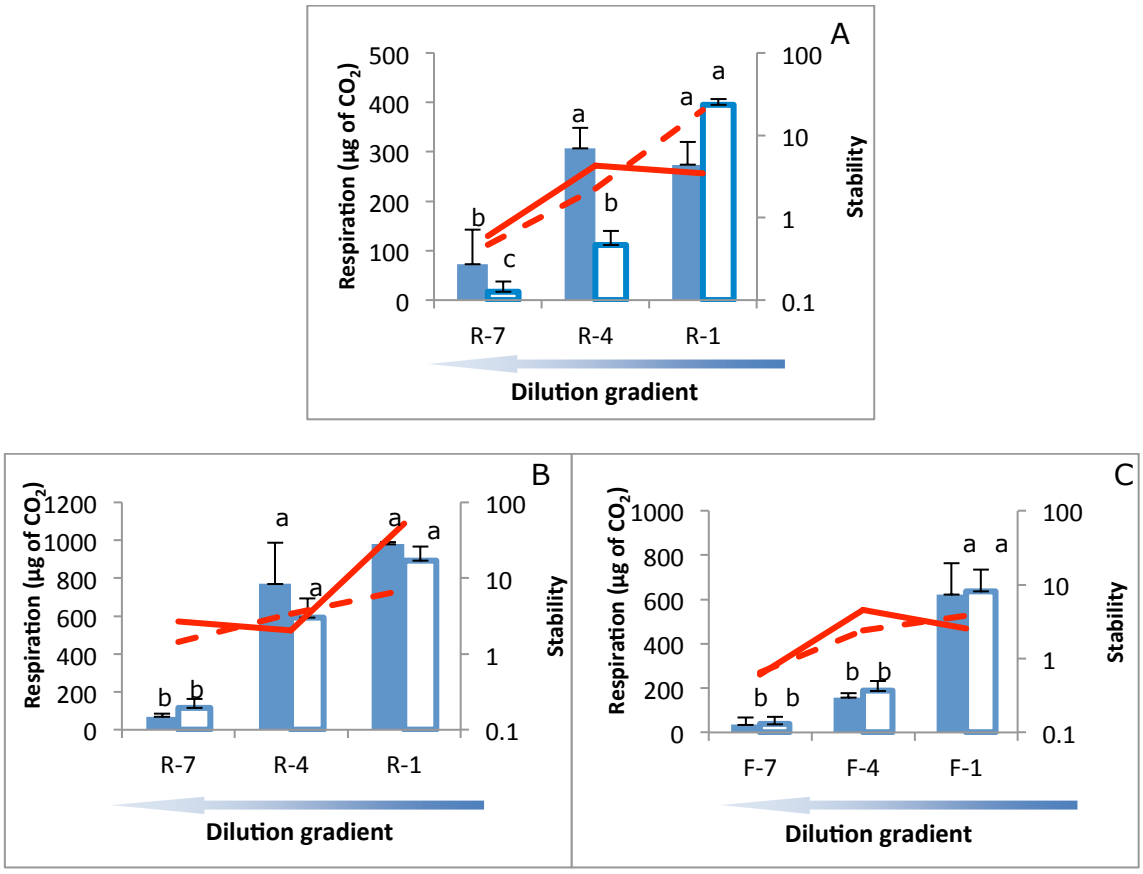

Figure 4.6 – Overall respiration and stability along the dilution gradient for: A) Loch Rescobie (1), B) Loch Rescobie (2) and C) Loch Freuchie (2). Columns indicate respiration (i.e. solid columns=water control, empty columns=incubated with MC-LR). Lines indicate variability (i.e. continuous line= water control, dashed line= incubated with MC-LR). Values with different letter differ significantly ($P<0.05$). Error bars represent standard error. n=3

The diversity gradient produced via the dilution to extinction approach in the experiment Loch Rescobie (1) significantly affected overall respiration of freshwater communities in both water control ($P=0.044$) and treatment incubated with microcystin-LR ($P<0.001$). Overall reduction in respiration (i.e. comparison R-1 to R-7) was 73% in the water control and 95% in the treatment incubated with microcystin-LR. On the other hand, there was an overall reduction in stability for water control and treatment incubated with microcystin-LR of 83% and 98%, respectively.

The diversity gradient produced via the dilution to extinction approach in the experiment Loch Rescobie (2) significantly affected overall respiration of freshwater communities in both water control ($P=0.005$) and treatment incubated with microcystin-LR ($P=0.001$). Overall reduction in respiration (i.e. comparison R-1 to R-7) was 93% in the water control and 87% in the treatment incubated with microcystin-LR. The overall reduction in stability (i.e. comparison R-1 to R-7) was quantified to be 95% for water control and 79% for treatment incubated with microcystin-LR.

The diversity gradient produced via the dilution to extinction approach in the experiment Loch Freuchie (2) significantly affected overall respiration of freshwater communities in both water control ($P=0.006$) and treatment incubated with microcystin-LR ($P=0.002$). Overall reduction in respiration (i.e. comparison R-1 to R-7) was 94% in both the water control the treatment incubated with microcystin-LR. The overall reduction in stability (i.e. comparison R-1 to R-7) was quantified to be 76% for water control and 83% for treatment incubated with microcystin-LR.

4.3.4 Bacterial diversity and richness: relations with functions and variability

The relationships between diversity and function/stability in Loch Rescobie (1)(2) and Loch Freuchie (2) were visualised by plotting the relative bacterial diversity and richness, estimated via T-RFLP analysis, against: a) cumulative carbon dioxide produced, as a proxy of communities function and; b) stability of the systems measured as mean-standard deviation ratio (Tilman et al., 2006) derived from respiration data (Figure 4.7,Figure 4.8).

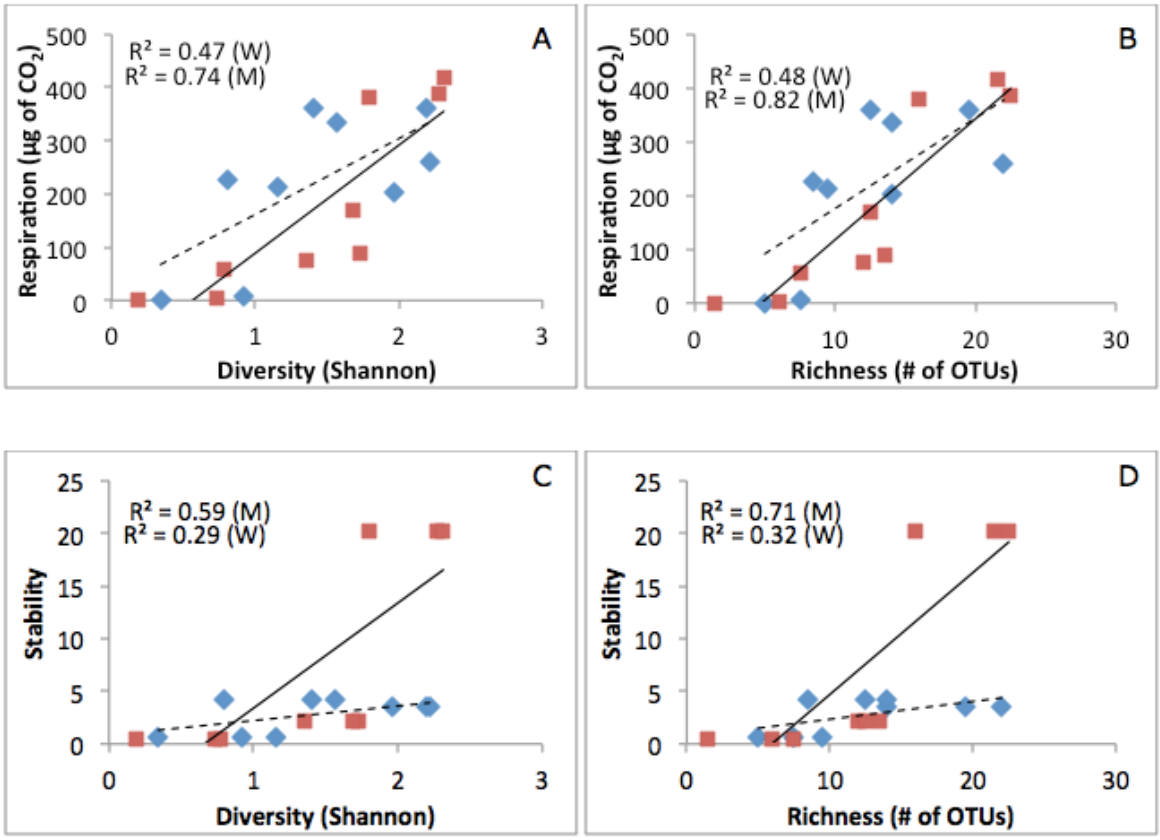

Figure 4.7 – Scatter plots showing dependence of communities' activity on bacterial diversity (A) and richness (B) for Loch Rescobie (1) experiment. Dependence of communities' stability on bacterial diversity (C) and richness (D) for Loch Rescobie (1) experiment. Bacterial diversity and richness estimated via T-RFLP. For data points: sample incubated with MC-LR=■; Water control=◆. For regression values: M= samples incubated with MC-LR; W= water control; continuous line= regression line MC-LR; dashed line= regression line water control.

For Loch Rescobie (1), a strong positive relationship between diversity and function was confirmed when analysed in a univariate regression analysis: both bacterial diversity and richness had a highly significant effect (P<0.001) on communities' activity in both treatments tested (i.e. water control and incubated with microcystin-LR). When analysed in a univariate regression analysis, bacterial richness had a high significant effect (P<0.001) on communities' stability in both treatments tested. Bacterial diversity also had a significant impact on communities' stability of both water control and treatment incubated with MC-LR with P<0.001 and P=0.002, respectively.

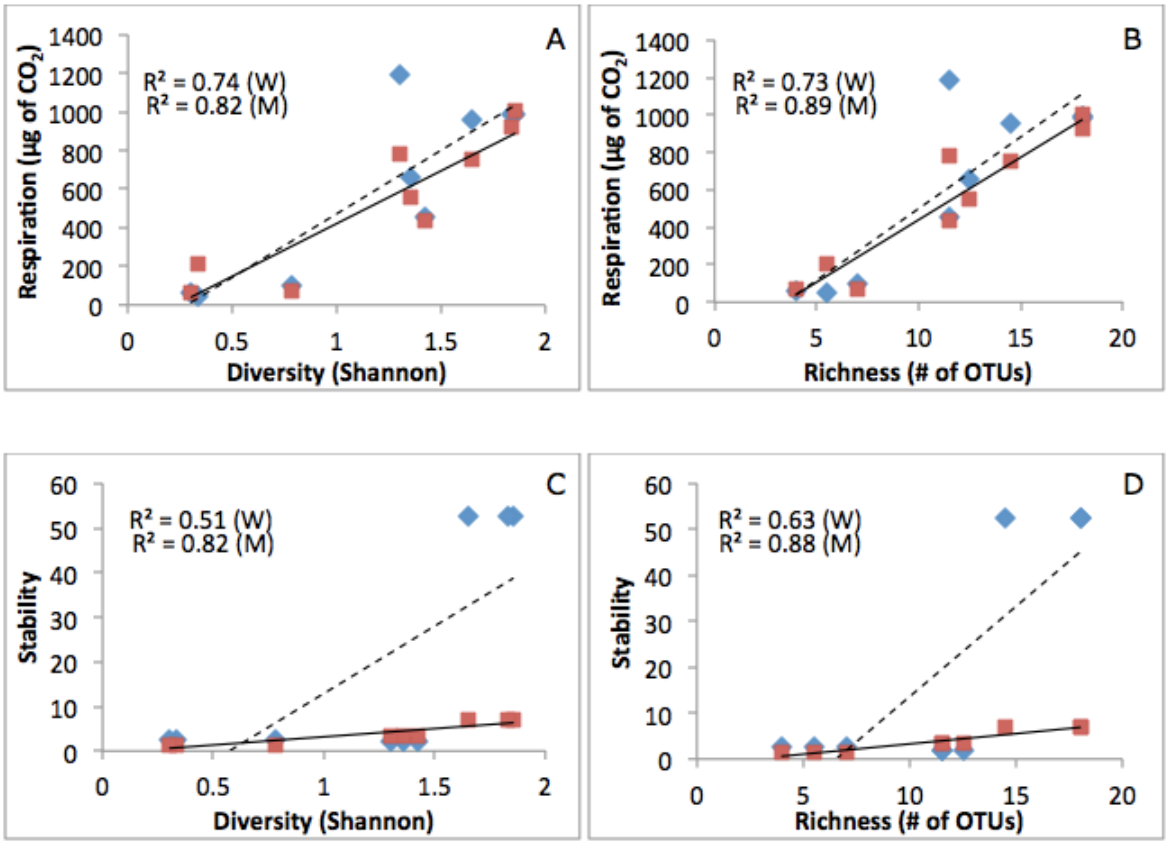

Figure 4.8 – Scatter plots showing dependence of communities' activity on bacterial diversity (A) and richness (B) for Loch Rescobie (2). Dependence of communities' stability on bacterial diversity (C) and richness (D) for Loch Rescobie (2). Bacterial diversity and richness estimated via T-RFLP. For data points: sample incubated with MC-LR=■; Water control=◆. For regression values: M= samples incubated with MC-LR; W= water control; continuous line= regression line MC-LR; dotted line= regression line water control.

For Loch Rescobie (2), a strong positive relationship between diversity and function was confirmed when analysed in a univariate regression analysis: both bacterial diversity and richness had a highly significant effect ($P<0.001$) on communities' activity in both treatments tested (i.e. water control and incubated with microcystin-LR).

On the other hand, bacterial richness had a highly significant effect on communities' stability in the water control ($P=0.004$) and treatment incubated with MC-LR ($P<0.001$). Bacterial diversity also had a significant impact on communities' stability in the water control ($P=0.005$) and treatment incubated with MC-LR ($P<0.001$).

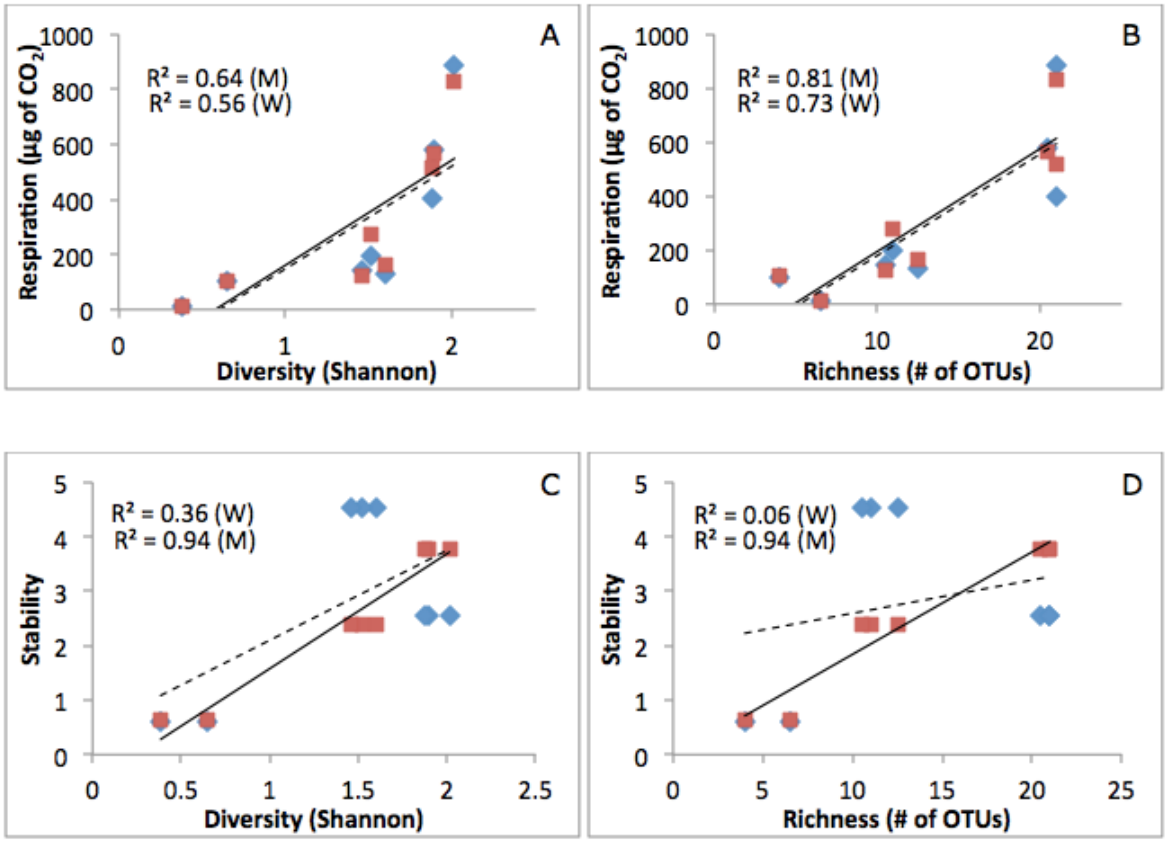

Figure 4.9 – Scatter plots showing the dependence of communities' activity on bacterial diversity (A) and richness (B) for Loch Freuchie (2). Dependence of communities' stability on bacterial diversity (C) and richness (D) for Loch Freuchie (2). Bacterial diversity and richness estimated via T-RFLP. For data points: sample incubated with MC-LR=■; Water control=◆. For regression values: M= samples incubated with MC-LR; W= water control; continuous line= regression line MC-LR; dotted line= regression line water control.

For Loch Freuchie (2), a strong positive relationship between diversity and function was confirmed when analysed in a univariate regression analysis: richness had a highly significant effect ($P<0.001$) on communities' activity in both treatments tested. Diversity had a significant impact on communities' activity in water control ($P=0.002$) and treatment incubated with MC-LR ($P<0.001$).

Bacterial diversity had a highly significant effect ($P<0.001$) on communities' stability in both treatments tested, likewise did bacterial richness on stability of water control ($P=0.004$) and treatment incubated with MC-LR ($P<0.001$).

Along with the informative T-RFLP data presented so far in this section, pyrosequencing data were available for Loch Rescobie (2) and Loch Freuchie (2) experiments allowing a deeper understanding of the total diversity/richness of the system (Figure 4.4) and a refined view of the relation between diversity/richness and ecosystems functioning/stability.

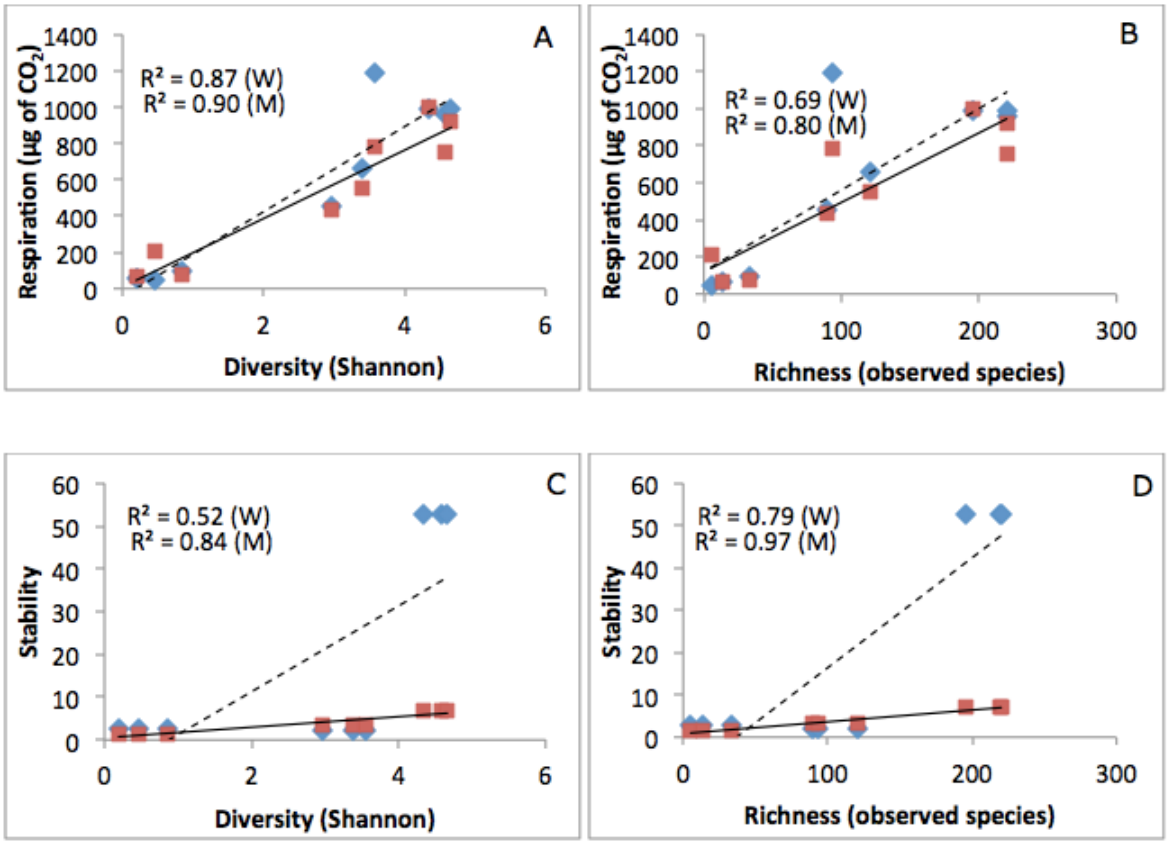

Figure 4.10 - Dependence of communities' activity on bacterial diversity (A) and richness (B) for Loch Rescobie (2). Dependence of communities' stability on bacterial diversity (C) and richness (D) for Loch Rescobie (2). Bacterial diversity and richness estimated via pyrosequencing. For data points: sample incubated with MC-LR=■; Water control=◆. For regression values: M= samples incubated with MC-LR; W= water control; continuous line= regression line MC-LR; dotted line= regression line water control.

For Loch Rescobie (2), a strong positive relationship between diversity and function was confirmed when analysed in a univariate regression analysis: both bacterial diversity and richness had a highly significant effect ($P<0.001$)

on communities' activity in both treatments tested (i.e. water control and incubated with microcystin-LR).

On the other hand, bacterial richness had a high significant effect (P<0.001) on communities' stability in both treatments tested. Bacterial diversity also had a significant impact on communities' stability in the water control (P=0.003) and treatment incubated with MC-LR (P<0.001).

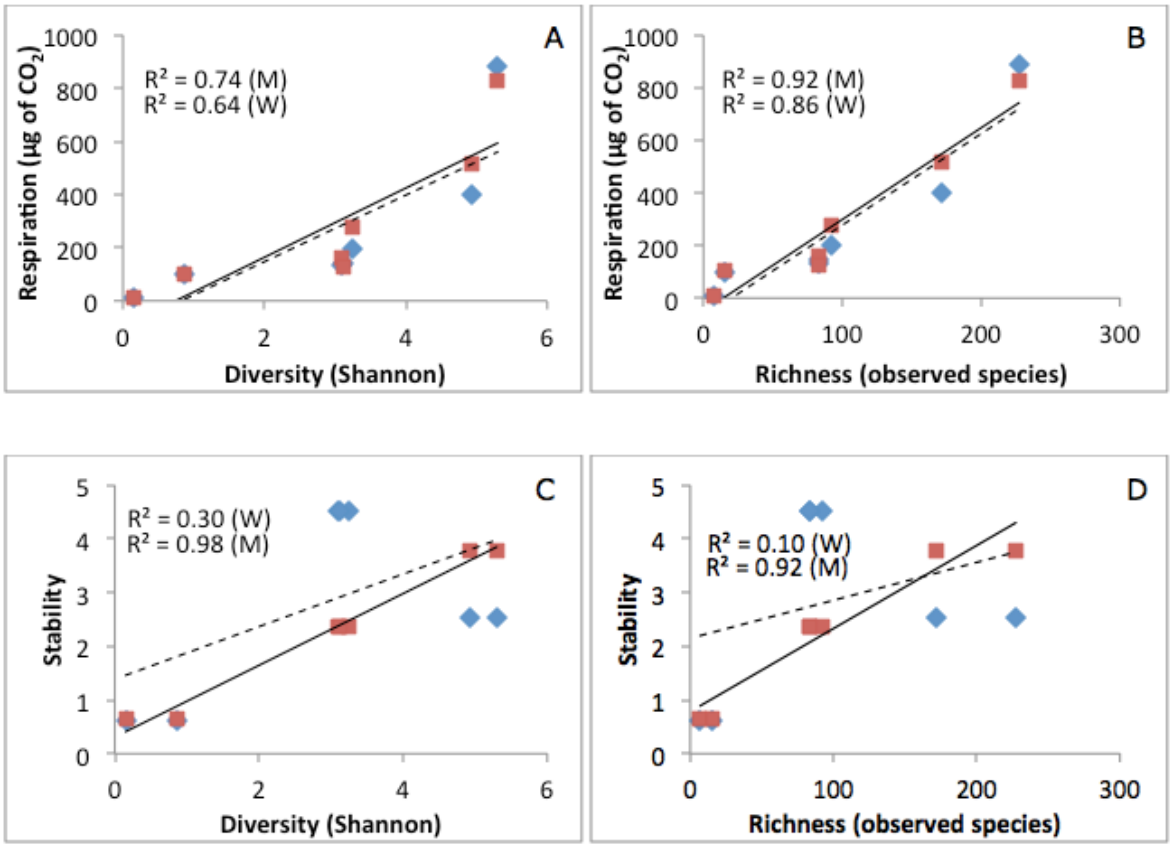

Figure 4.11 – Scatter plots showing dependence of communities' activity on bacterial diversity (A) and richness (B) for Loch Freuchie (2). Dependence of communities' stability on bacterial diversity (C) and richness (D) for Loch Freuchie (2). Bacterial diversity and richness estimated via pyrosequencing. For data points: sample incubated with MC-LR=■; Water control=◆. For regression values: M= samples incubated with MC-LR; W= water control; continuous line= regression line MC-LR; dotted line= regression line water control.

For Loch Freuchie (2), a strong positive relationship between diversity and function was confirmed when analysed in a univariate regression analysis:

richness had a highly significant effect ($P<0.001$) on communities' activity in both treatments tested. Diversity had a significant impact on communities' activity in water control ($P=0.003$) and treatment incubated with MC-LR ($P<0.001$).

Bacterial diversity had a highly significant effect ($P<0.001$) on communities' stability of treatment incubated with microcystin-LR a significant impact ($P=0.004$) in the water control, likewise did bacterial richness on stability of water control ($P=0.002$) and treatment incubated with MC-LR ($P<0.001$).

4.3.5 Biodegradation of MC-LR along a diversity gradient

Biodegradation of MC-LR was monitored from Loch Freuchie (1), Loch Rescobie (1), Loch Rescobie (2) and Loch Freuchie (2) by HPLC (Figure 4.12).

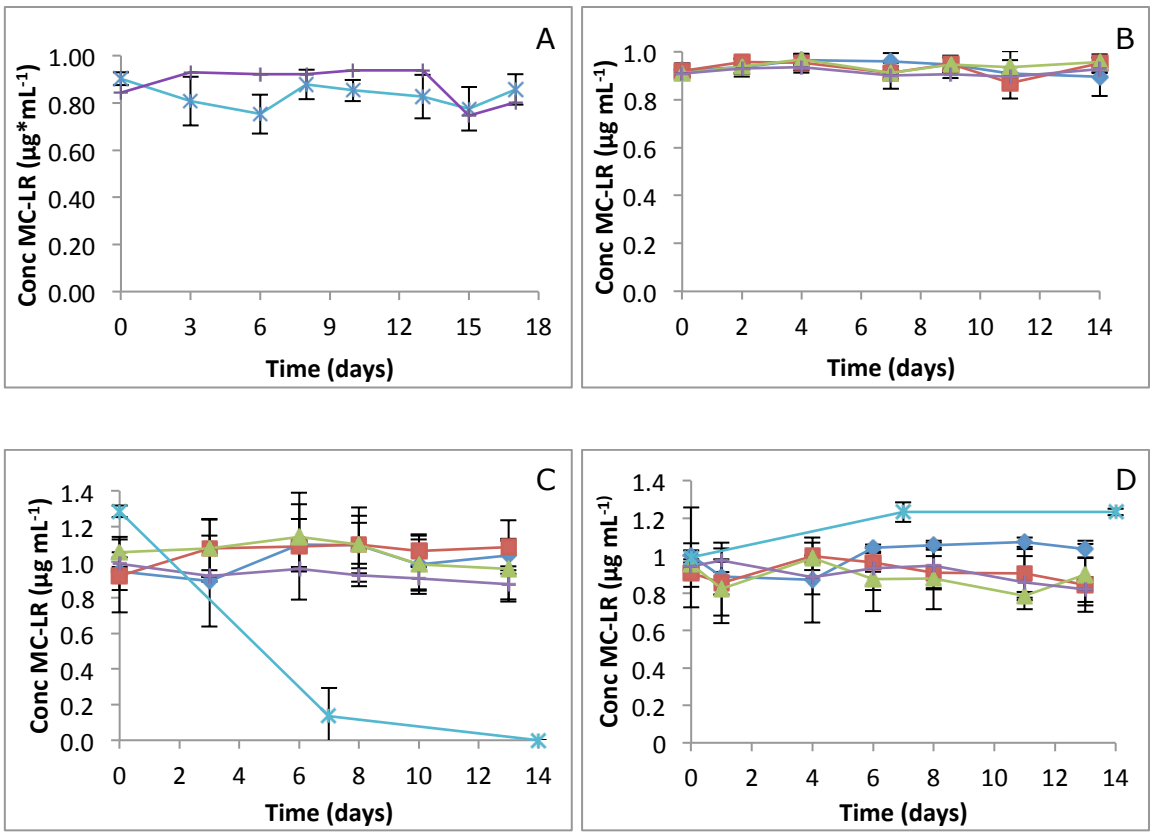

Figure 4.12 – Trend of concentration of MC-LR over time in: A) Loch Freuchie (1), B) Loch Rescobie (1),C) Loch Rescobie (2) and D) Loch Freuchie (2). No dilutions MC-LR= ; Exp^{-1} MC-LR= ; Exp^{-4} MC-LR= ; Exp^{-7} MC-LR= ; Sterile control MC-LR= . Error bars indicates standard deviation.

HPLC analysis of samples showed no biodegradation of microcystin-LR for Loch Freuchie (1) and Loch Rescobie (1). As microbial communities from Loch Rescobie have been reported to consistently be able to degrade microcystin-LR (see Chapter 3), it was hypothesised that the lack of biodegradation ability of microbial communities from this water body was linked to filtration via 1.2 µm filter (see section 4.2.1). In experiment Loch Rescobie (2) water was not filtered, however the biodegradation capability was absent in all dilutions

levels studied (i.e. exp^{-1}, exp^{-4}, exp^{-7}). However, for Loch Rescobie (2) and Loch Freuchie (2), undiluted treatment was set up to test if even a ten-fold dilution could be the cause of the lack of biodegradation ability. Microbial communities from undiluted samples for Loch Rescobie (2) degrade 90% of microcystin-LR by day 7 with a half-life of 2.1 days. Microbial communities from undiluted samples from Loch Freuchie (2) did not show microcystin-LR biodegradation ability even in undiluted samples.

4.4 Discussion

4.4.1 Dilution-to-extinction, biomass recovery and diversity measures

The aim of this work was to evaluate the impact of a reduction in freshwater microbial diversity on the functioning and stability of freshwater ecosystems. A microbial diversity gradient was obtained via a dilution-to-extinction approach that preferentially removes rare species (Salonius, 1981). Since the dilution approach also creates a gradient in biomass, the microcosms were pre-incubated to ensure biomass recovery that was, in all cases, achieved within 6 days (Figure 4.2). The rapid bacterial biomass recovery is consistent with other works in aquatic environments (Szabó et al., 2007) and opposed to the lengthy bacterial biomass recovery required in soils (Griffiths et al., 2001). To assess how bacterial communities' diversity and richness were affected by the dilution manipulation, 16S rRNA gene amplicon T-RFLP analysis and pyrosequencing were performed at the end of the biomass recovery period. The dilution-to extinction approach was, as shown from previous authors (Wertz et al., 2006), effective in reducing diversity of bacterial communities (Figure 4.3). However, some reports (Franklin et al., 2001, Franklin and Mills, 2006) showed contrasting results, where dilution treatment did not follow a significant reduction in diversity/richness when measured via DNA fingerprinting techniques. In this work, considering DNA fingerprinting data for all sampling sites/times presented, a three orders of magnitude dilution difference (i.e. comparison exp-1 to exp-4) produced a 28%±6 reduction in diversity and a 33%±6 reduction in richness. A six orders of magnitude dilution difference (i.e. comparison exp-1 to exp-7) produced a very consistent reduction in diversity and richness of 71% ±2 and 70%±4, respectively. When the diversity and richness of bacterial communities were analysed via

pyrosequencing, a three orders of magnitude dilution difference (i.e. comparison exp-1 to exp-4) produced a 33%±6 reduction in diversity and a 54%±2 reduction in richness. A six orders of magnitude dilution difference (i.e. comparison exp-1 to exp-7) produced a very consistent reduction in diversity and richness of 93% ±1 and 89%±1, respectively. The reproducibility of pyrosequencing compared to T-RFLP in the evaluation of aquatic microbial diversity was reported by Pilloni et al.(2012). In this work it was shown that the two techniques are able to effectively measure differences in bacterial diversity along a steep dilution gradient. However, the comparison with pyrosequencing showed that the proportion of species that T-RFLP analysis measured was negatively and strongly related with the diversity of the system. These findings confirms the "tragedy of the uncommon" (Bent and Forney, 2008) when evaluating the diversity of microbial communities via DNA fingerprinting approaches, and add that the diversity of the system dictates the scale of the "tragedy".

4.4.2 Communities functioning and stability along the dilution gradient

Of the three approaches available to study the relation between microbial diversity and function (i.e. microcosm, removal and observational studies) (Bell et al., 2009), when using the removal approach authors have reported mixed results for the relation between diversity and functions. A detrimental effect of dilutions treatments on aquatic microbial functioning was reported by Peter et al. (2011) where the authors showed a decline in chitin and cellulose degradation rates along the dilution gradient. Franklin et al. (2001) reported that dilution treatments reduces the number of carbon sources that raw sewage water microbial communities are able to metabolise different carbon sources, while Franklin and Mills (2006) did not find a significant effect of a

dilution gradient on metabolism of glucose, acetate, citrate and an amino acid mixture. Wertz et al. (2006) reported that dilutions spanning over seven order of magnitude did not significantly affect carbon mineralisation or denitrification activity. Contrasting results in literature depends, amongst others, upon the function measured, the sample origin, experimental approach, environmental factors and the combination of these factors. In this work, it was found that a consistent significant effect of the dilution treatment on overall microbial activity and stability of the system tested (Section 4.4.2.2) without a constant effect of the addition of microcystin-LR (Figure 4.6). Microcystin-LR is highly toxic to animals and humans due to its hepatotoxicity (Edwards and Lawton, 2009) and potential carcinogen activity (Grosse et al., 2006). However, studies of its effect on bacterial communities have given contrasting results (Martins et al., 2011) and the ecological role of cyanotoxins has not been fully understood (Kaebernick and Neilan, 2001). In this perspective these results are not surprising showing an inconsistent effect of microcystin-LR on function and stability of microbial communities.

4.4.3 Diversity, richness, overall communities functioning and stability

Although many investigations have attempted to study the relation between microbial diversity and functioning with a dilution-to-extinction approach, only few have then observed a positive relation between diversity estimates and measured functions. Of the studies that did not show such relationships, either they did not measure a diversity gradient along the dilution gradient (Franklin et al., 2001, Franklin and Mills, 2006) or did not report function/stability differences along the diversity gradient (Griffiths et al., 2001, Wertz et al., 2007). A study based on observational data has reported a positive relation between increased diversity and increased stability after perturbation (Girvan et al., 2005). Moreover, to date, the study of artificial communities including the highest number of species (i.e.72) in various combinations to study the relation between bacterial diversity and function in microcosm experiment showed the species composition and richness are both significant in influencing functions, with the latter having the higher impact (Bell et al., 2005b). A recent work based on dilution-to-extinction manipulation of microbial diversity by Philippot et al. (2013) showed a significant reduction in potential denitrification activity in soils following a dilution treatment where a reduction of 75% in OTUs followed a reduction in activity of 48 to 88%. This work, independent of the technique used to assess bacterial diversity and richness, showed that a significant ($P<0.05$) reduction in bacterial diversity that was achieved via a dilution-to-extinction approach followed a significant reduction in carbon mineralisation ($P<0.05$). It was also shown that, in all experiments sites/time considered, both carbon mineralisation and stability on the microbial communities were significantly ($P<0.05$) affected by both bacterial diversity and richness (Section 4.3.5). Based on the more informative pyrosequencing

data, considering the extreme level of diversity (i.e. exp-1 and exp-7), a reduction in diversity of 89-90% (92-97% richness) followed a reduction in carbon mineralisation from 55 to 95% and in stability from 76 to 97%. In the current "age of extinction" (Naeem et al., 2012) these findings stress the importance of biodiversity in maintaining ecosystem functioning and stability.

4.4.4 Biodegradation of microcystin-LR along the dilution gradient

Because of the expected degradation of microcystin-LR by microbial communities in Loch Rescobie (Edwards et al., 2008), lack of biodegradation observed in Loch Rescobie (1) experiment was unexpected. Additionally, the absence of biodegradation activity even in the most bio-diverse microcosms did not allow investigating the relation between biodiversity and a specific function such as biodegradation of microcystin-LR. The cause of the absence of biodegradation activity was initially attributed to the filtering via 1.2 µm filter that was carried out in the attempt to minimise the effect of bacterial grazers (Szabó et al., 2007, Bell et al., 2010). Due to the extreme variable size of prokaryotes (Koch, 1996) the filtration did not only remove most of the bacterial grazers, but also a number of bacteria. Also, it has been shown that microbes attached to particles are the most active in aquatic environment (Kirchman and Mitchell, 1982) and the filtration would remove them. As no previous studies have been done in investigating the role of depletion of microbial diversity on biodegradation activity of microcystins, it was not considered that the lack of biodegradation could be caused by a single ten-fold dilution of environmental sample. Upon this assumption, the lack of degradation observed in the Loch Freuchie (1) experiment was also attributed to the filtration. However, when a second set of experiments were carried out again, this time without filtration, with freshwater from both lakes (i.e. Loch

Rescobie (2) and Loch Freuchie (2)) the results suggested that the cause of the lack of degradation of microbial communities from Loch Rescobie was the ten-fold dilution, while even undiluted microbial communities from Loch Freuchie were not able to degrade microcystin-LR within the duration of the experiment. More studies need to be done to confirm findings, however, the data from the Loch Rescobie (2) experiment suggests that even a little reduction in diversity is able to determine the loss of microcystin-LR biodegradation ability.

4.5 Conclusions and future work

For microbial communities, previous biodiversity-ecosystem functioning experiments have shown contrasting results, and it is commonly thought that microbial communities are highly redundant (Wertz et al., 2006). In this work, a significant reduction in microbial diversity achieved by a dilution-to-extinction approach always followed a significant reduction in carbon mineralisation. Bacterial diversity and carbon mineralisation were linked by a significant linear relationship. Also significant was the impact of the removal experiment on stability of the system, and again stability was significantly correlated to diversity and richness of the system. Microbial ability to degrade microcystin-LR was lost with a single ten-fold dilution suggesting that even a small reduction in diversity can have a dramatic effect on ecosystem functioning.

While results for carbon mineralisation are very consistent amongst sampling time/sites, stability of the system and biodegradation of microcystin-LR needs further exploration to confirm findings. For future work, it will be important to include undiluted treatments in experiments, as this will give information on diversity and functioning of the system at its natural biodiversity level. Also, it will strengthen the findings if the hypothesis will be tested in different freshwater systems and/or geographical locations. It will also be beneficial to test the relationship between microbial diversity and biodegradation of relevant toxic man-made compounds, as these are continuously introduced in the environment by human activities.

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5. BACTERIAL DIVERSITY, FUNCTIONS AND STABILITY: RELATIONS IN RIVER WATER

5.1 Introduction

Results from Chapter 4 suggest that microbial diversity plays a key role on ecosystem functioning and services in the Scottish lakes tested. Due to the lack of literature and contrasting evidence on the relationship between biodiversity and water purification (Cardinale et al., 2012), findings of Chapter 4 are of extreme interest but need to be confirmed in other systems. To confirm results presented in Chapter 4, it was decided to test whether diversity-ecosystem functioning relationships found are consistent in river water that harbour different microbial communities (Pernthaler, 2013). One of the main man-made pollutants of river systems is triclosan. Triclosan is a widely used antimicrobial agent (Bester, 2005) that continuously enters freshwater systems due to incomplete dissipation in wastewater treatment plants (Kookana et al., 2011). Triclosan targets lipid synthesis in bacteria (McMurry et al., 1998) and is effective from a concentration of 10 μg L^{-1} (Bhargava and Leonard, 1996). Numerous evidence suggests that triclosan has the ability to promote bacterial cross-resistance with antibiotics (Levy, 2001). In higher organisms triclosan has adverse effects on the endocrine system (Schuur et al., 1998) and ability to impair mitochondrial activity (Newton et al., 2005). That is of particular concern as the antibacterial agent has been detected in various human tissues including serum (Allmyr et al., 2006) and adipose tissue (Geens et al., 2012).

To further investigate the role that microbial species diversity play on ecosystem functions and stability in freshwater systems three hypotheses were tested in river systems: (1) a decrease in diversity will affect broad ecosystem function in opposition to "insurance hypothesis" (Yachi and Loreau, 1999) but in accordance with the findings of Chapter 4; (2) a decrease in diversity will have a negative impact on system stability and ; (3) a decrease in diversity will have a dramatic impact on specialised ecosystem functions (i.e. microcystin-LR and triclosan biodegradation).

5.2 Materials and Methods

5.2.1 Freshwater Sampling

Two sampling events separated by three days were carried out.

For the first sampling, sub-surface water (i.e. within 10 cm from surface) was sampled on the 4th of February 2013 from the Hawkesbury River (H) and Farmers Creek (F) (downstream of Lithgow). The two sites represent polluted/high nutrient rivers belonging to the Hawkesbury-Napean river system in New South Wales, Australia. For details about geographic coordinates and characteristics of sampling sites refer to section 2.2.1 and 2.4.6. Water was sampled in 1 L glass bottles wrapped in aluminium foil to minimise the input of light, water temperature was 14.8°C for F site and 23.5°C for H site. Once in the laboratory, water pH was measured (H=6.85, F=7.21), then water was filter sterilised via Stericup filter units (0.22 µm, Millipore) and autoclaved. After cooling overnight, pH was measured again and was found to be higher than original sample as reported from similar studies (Peter et al., 2011). pH was adjusted to 7.04 for H and 7.27 for F with 1M hydrocloridic acid. Water was then stored at 4°C ready to be used for next steps.

For the second sampling, sub-surface water (i.e. within 10 cm from surface) was sampled in triplicate on the 7th of February 2013 from the same sites mentioned above. Water was sampled in 500 mL glass wrapped in aluminium foil to minimise the input of light, water temperature was 16.0°C for F and 28.0°C for H. Once in the laboratory water pH was measured (H=7.33±0.03, F=7.32±0.01). An AQ2 discrete analyser (Seal Analytical Inc., Maquon WI, USA) was used to determine water concentration of ortho-phosphate-P

(method: EPA-118-A Rev.5), nitrate (method: EPA-127-A Rev.7) and ammonia (method: EPA-103-A Rev.6). Total nitrogen and total dissolved carbon were measured using a TOC-L analyser (Shimadzu, Kyoto, Japan).

5.2.2 Bacterial diversity manipulations and biomass recovery monitoring

To achieve a bacterial diversity gradient without need of culturing a "dilution to extinction" approach was used as described in Chapter 4. In brief, water sampled from H and F on the second sampling time was serially diluted with water sampled from H and F on the first sampling, which had been filter sterilised and autoclaved to ensure sterility. Ten-fold serial dilutions were carried out in triplicate under laminar flow and four treatments were kept for further studies: 1) undiluted; 2) 10 exp^{-1}; 3) 10 exp^{-2}; 4) 10 exp^{-4}. Once dilutions were achieved in a final volume of 225 mL contained in 500 mL glass conical flasks, they were incubated for biomass recovery at 20°C shaking at 70 rpm in dark conditions. Biomass recovery was tested as described in section 4.2.2.

5.2.3 Biodegradation assays: microcystin-LR and triclosan

Once biomass recovery was achieved, a biodegradation assay was set up with all dilution levels obtained from both sites in triplicate. Microcystin-LR (0.5 mg L^{-1}) and triclosan (10 µg L^{-1}) biodegradation ability of the microbial communities were tested along with water control for all dilution levels. Also sterile controls were included. Microcystin-LR treatment was set up and samples analysed as described in section 4.2.3.2. Triclosan treatment was set up and samples analysed as described in section 2.2.4. HPLC (microcystin-LR) and ELISA (triclosan) analysis were carried out on aliquots from both

treatments that were taken every 4 days and immediately frozen as described in sections mentioned above.

5.2.4 Estimation of richness and diversity: T-RFLP and pyrosequencing

T-RFLP and pyrosequencing analysis were carried out as described in sections 4.2.4 and 4.2.5.

5.3 Results

5.3.1 Freshwater chemical analysis

Chemical analysis was carried out on freshwater to determine concentrations of some important water chemistry parameters (Table 5.1).

Table 5.1 – Concentration of water chemistry parameters for Hawkesbury River and Farmers Creek. Mean values expressed in mg L^{-1}± standard deviation.

	Total dissolved carbon	Total nitrogen	Ortophosphate	Ammonia	Nitrate
Hawkesbury River	10.85±0.1	1.24±0	0.01±0	0.01±0	0.63±0
Farmers Creek	5.19±0.2	4.19±0.2	0.01±0	0.06±0	3.41±0

Both total nitrogen and nitrate were well above the national values considered able to trigger negative impact on the ecosystem (Anzecc, 2000).

5.3.1 Bacterial biomass recovery and its trend over time

Before to test the hypothesis related to the diversity-function relation, biomass of the manipulated levels of biodiversity was monitored over time to check its recovery. Biomass recovery along the dilution (diversity) range was achieved within 3 days for both Hawkesbury River and Farmers Creek and maintained a constant magnitude until the termination of the biodegradation assay (Figure 5.1).

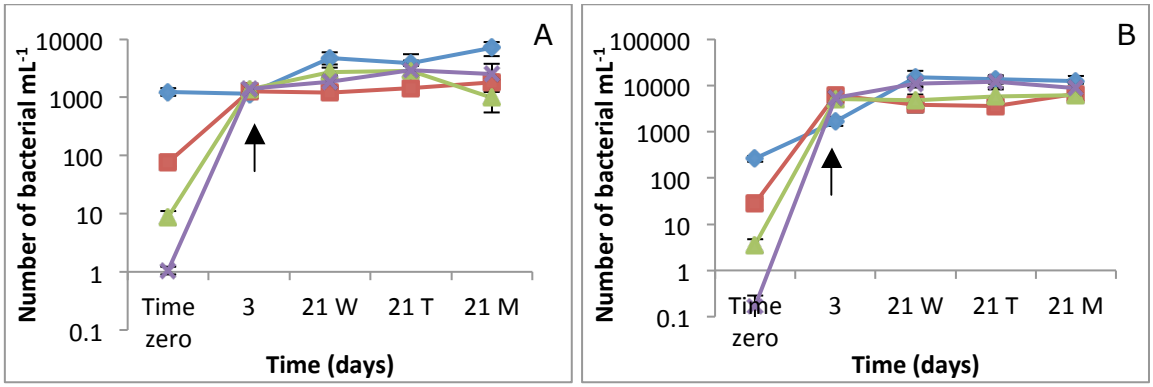

Figure 5.1 - Bacterial biomass over time at different dilution levels: data of gene copy number mL^{-1} of bacterial 16S rRNA gene number of bacteria in original sample from: A) Hawkesbury River and; B) Farmers Creek. =undiluted; = exp^{-1}; = exp^{-2}; = exp^{-4}. W=water control, T=incubated with triclosan. M=incubated with MC-LR. Arrows indicate time point when biomass recovery was achieved. Error bars indicate standard error. n=3

5.3.2 Bacterial diversity and richness along the dilution gradient: DNA fingerprinting and pyrosequencing

Once biomass recovery was achieved, relative bacterial diversity was estimated via T-RFLP analysis. Richness and diversity estimates were obtained as described in section 4.3.2.

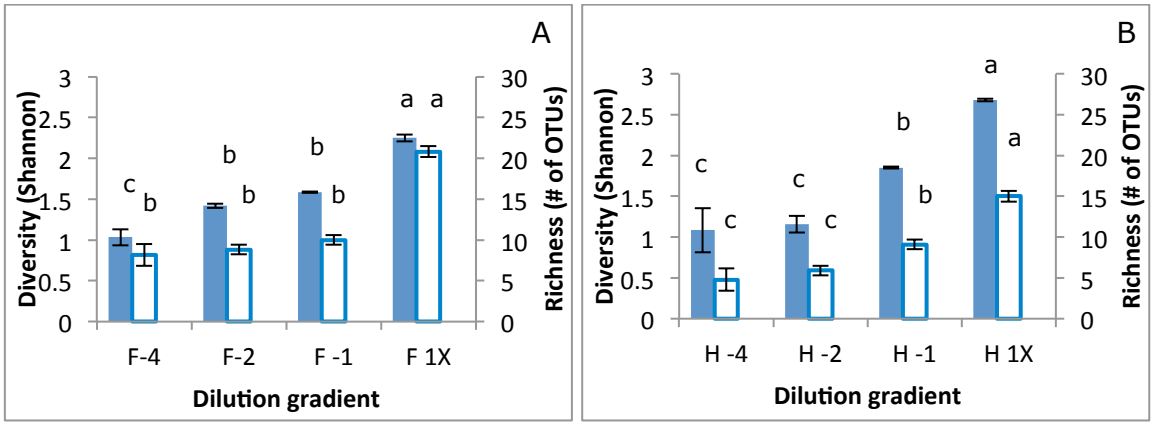

Figure 5.2 – Bar plots showing relative diversity and richness (based on T-RFLP data) along the dilution gradient of: A) Farmers Creek and; B) Hawkesbury River. Solid columns=diversity, empty columns=richness. F=Farmers Creek, H=Hawkesbury River. Values with different letters differ significantly ($P<0.05$). Error bars indicate standard error. n=3

Based on T-RFLP data, the dilution to extinction approach was an effective method to manipulate diversity and richness of bacterial communities in freshwater. For Farmers Creek (Figure 5.2A) the dilution significantly affected both diversity ($P<0.001$) and richness ($P<0.001$) with an overall reduction (i.e. comparison undiluted to exp^{-4}) of 54% in diversity and 61% in richness. For Hawkesbury River (Figure 5.2B) the dilution significantly affected both diversity ($P<0.001$) and richness ($P<0.001$) with an overall reduction in diversity and richness of 52% and 59%, respectively.

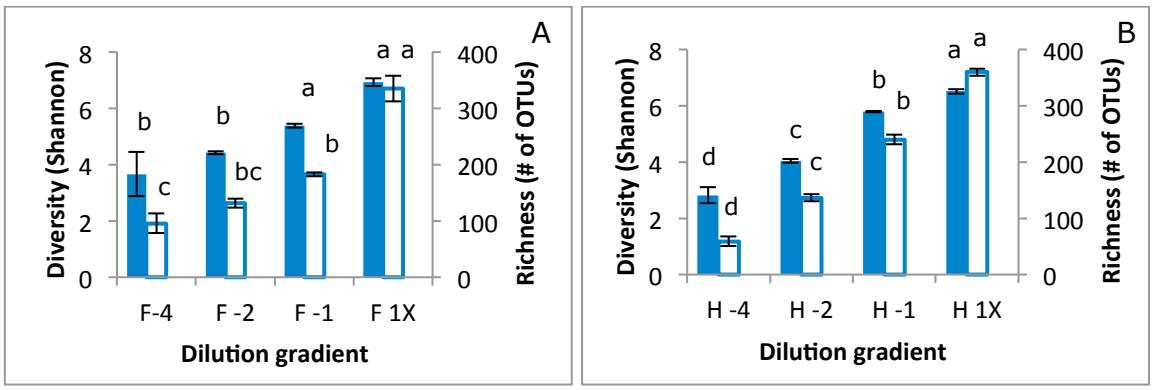

Figure 5.3 – Bar plots showing diversity and richness (based on pyrosequencing data) along the dilution gradient of: A) Farmers Creek and; B) Hawkesbury River. Solid columns=diversity, empty columns=richness. F=Farmers Creek, H=Hawkesbury River. Values with different letters differ significantly (P<0.05). Error bars indicates standard error. n=3

Based on pyrosequencing data, the dilution-to-extinction approach was an effective method to manipulate diversity and richness of bacterial communities in freshwater. For Farmers Creek (Figure 5.3A) the dilution significantly affected both diversity (P=0.002) and richness (P<0.001) with an overall reduction (i.e. comparison undiluted to exp^{-4}) of 47% in diversity and 71% in richness. For Hawkesbury River (Figure 5.3B) the dilution significantly affected both diversity (P<0.001) and richness (P<0.001) with an overall reduction in diversity and richness of 57% and 84%, respectively.

As diversity and richness were estimated by two techniques (i.e. T-RFLP and pyrosequencing), it was of interest to investigate whether the results between the two techniques would correlate, if there was a discrepancy and if the discrepancy would differ along the diversity gradient.

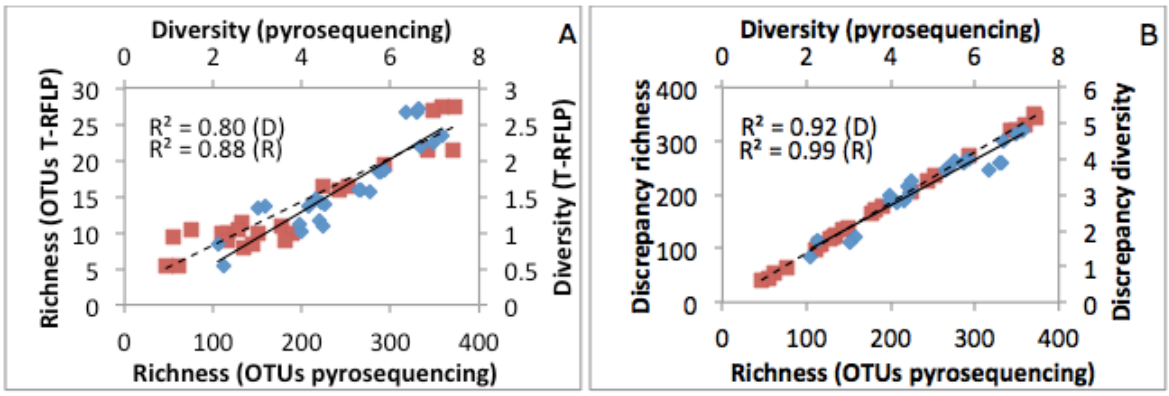

Figure 5.4 – Scatter plots showing: A) Correlation between diversity (D) and richness (R) estimated via pyrosequencing against T-RFLP; B) correlation between diversity and richness estimated via pyrosequencing against discrepancy between diversity and richness between pyrosequencing and T-RFLP (i.e. discrepancy richness= richness pyrosequencing – richness T-RFLP; discrepancy Shannon diversity= Shannon diversity pyrosequencing – Shannon diversity T-RFLP). Richness=■; diversity=◆. Continuous regression line= Shannon diversity, dashed regression line= richness.

As expected, richness estimated via pyrosequencing was strongly correlated to richness estimated via T-RFLP ($P<0.001$) and similarly a strong correlation was found for Shannon index ($P<0.001$). The discrepancies between the two techniques significantly ($P<0.001$) positively related to the total diversity/richness of the communities. In other terms, a strong linear relation describes that the higher the diversity/richness of the communities, the less T-RFLP analysis achieves to measure them.

5.3.3 Activity and stability along a dilution gradient

Once biomass recovery was achieved, an experiment to test the relation between diversity and function was initiated with focus on one general function (i.e. carbon mineralisation) and two specialised functions (i.e. ability to degrade microcystin-LR and ability to degrade triclosan). Additionally, based on respiration data, the stability of the systems at the different diversity levels was measured as mean-standard deviation ratio.

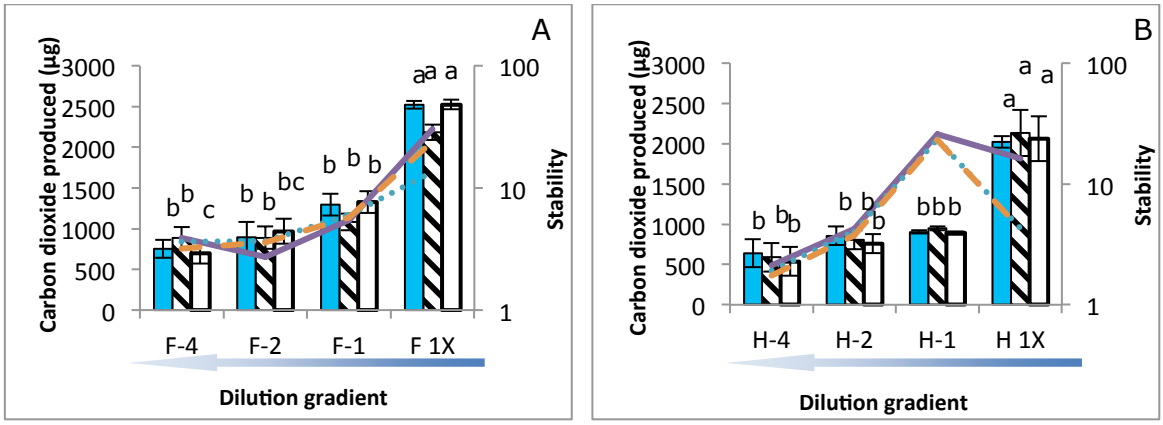

Figure 5.5 - Overall respiration and stability along the dilution gradient for: A) Farmers Creek and; B) Hawkesbury River. Columns indicate respiration (i.e. solid columns=water control; striped column= incubated with triclosan; empty columns=incubated with MC-LR). Lines indicate variability (i.e. continuous line= water control; dotted line=incubated with triclosan; dashed line= incubated with MC-LR). Values with different letters differ significantly ($P<0.05$). Error bars represent standard error. n=3

The diversity gradient produced via the dilution-to-extinction approach in the experiment with freshwater from Farmers Creek significantly affected ($P<0.001$) overall respiration of freshwater communities in all treatments considered (i.e. water control, incubated with MC-LR and incubated with triclosan). Overall reduction in respiration (i.e. comparison undiluted to exp^{-4}) was 70% in the water control, 72% in the treatment incubated with microcystin-LR and 46% in the treatment incubated with triclosan. On the

other hand, there was an overall reduction in stability of 87% for water control, 87% for treatment incubated with microcystin-LR and 73% for treatment incubated with triclosan.

The diversity gradient produced via the dilution-to-extinction approach in the experiment with freshwater from Hawkesbury River significantly affected ($P \leq 0.001$) overall respiration of freshwater communities in all treatments considered. Overall reduction in respiration was 68% in the water control, 74% in the treatment incubated with microcystin-LR and 72% in the treatment incubated with triclosan. The overall reduction in stability was quantified to be 87% for water control, 59% for treatment incubated with microcystin-LR and 55% for the treatment incubated with triclosan.

5.3.4 Bacterial diversity and richness: relations with functions and variability

5.3.4.1 Relations based on T-RFLP estimates of diversity and richness

The relationships between diversity and function/stability in Farmers Creek and Hawkesbury River were visualised by plotting the relative bacterial diversity and richness, estimated via T-RFLP analysis, against: a) cumulative carbon dioxide produced, as a proxy of communities' function and; b) stability of the systems measured as mean-standard deviation ratio (Tilman et al., 2006) derived from respiration data.

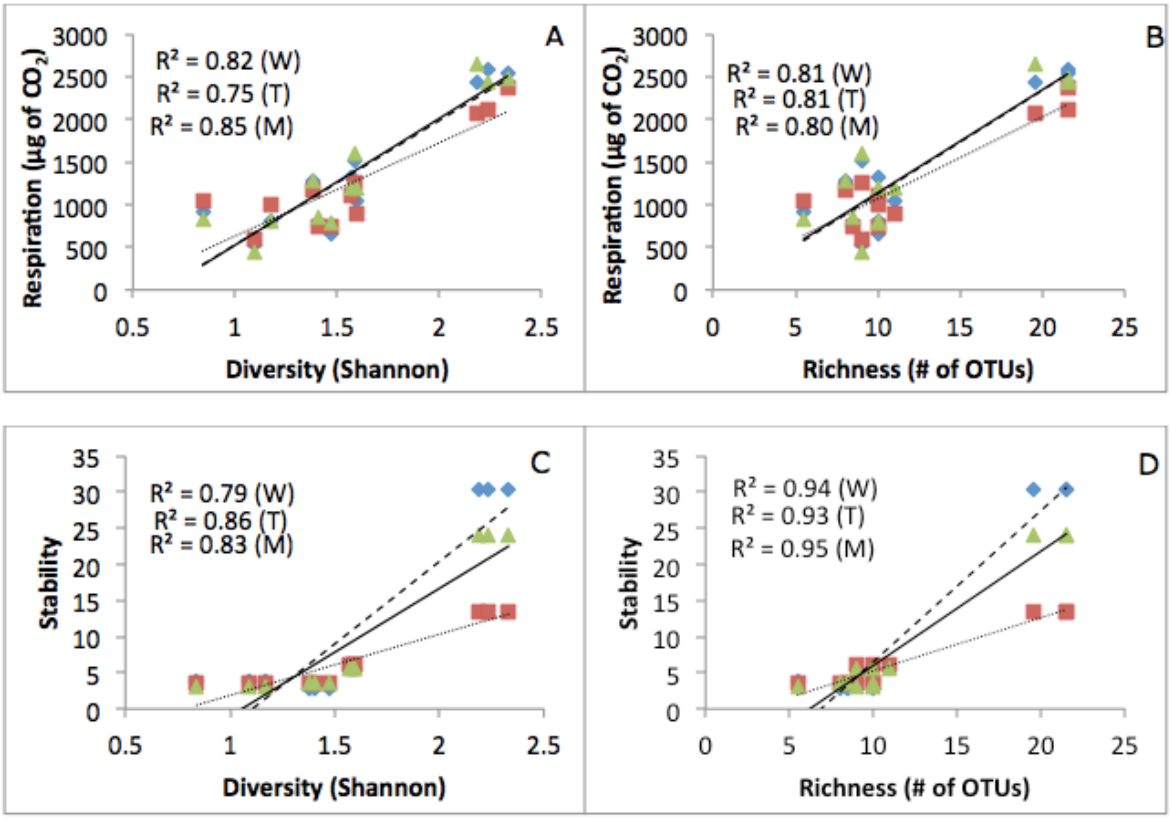

Figure 5.6 – Scatter plots showing the dependence of communities' activity on bacterial diversity (A) and richness (B) for Farmers Creek experiment. Dependence of communities' stability on bacterial diversity (C) and richness (D) for Farmers Creek experiment. Bacterial diversity and richness estimated via T-RFLP. For data points: Water control=◆; samples incubated with MC-LR= ▲; samples incubated with triclosan=■. For regression values: W= water control; M= samples incubated with MC-LR; T=samples incubated with triclosan. Dashed line= regression line water control; continuous line= regression line MC-LR; dotted line=regression line triclosan.

For freshwater microbial communities from Farmers Creek, a strong positive relationship between diversity and function was confirmed when analysed in a univariate regression analysis: both bacterial diversity and richness had a highly significant effect ($P<0.001$) on communities' activity in all treatments tested (i.e. water control, incubated with microcystin-LR and incubated with triclosan).

At the same time, both bacterial diversity and richness had a highly significant effect ($P<0.001$) on communities' stability in all treatments tested.

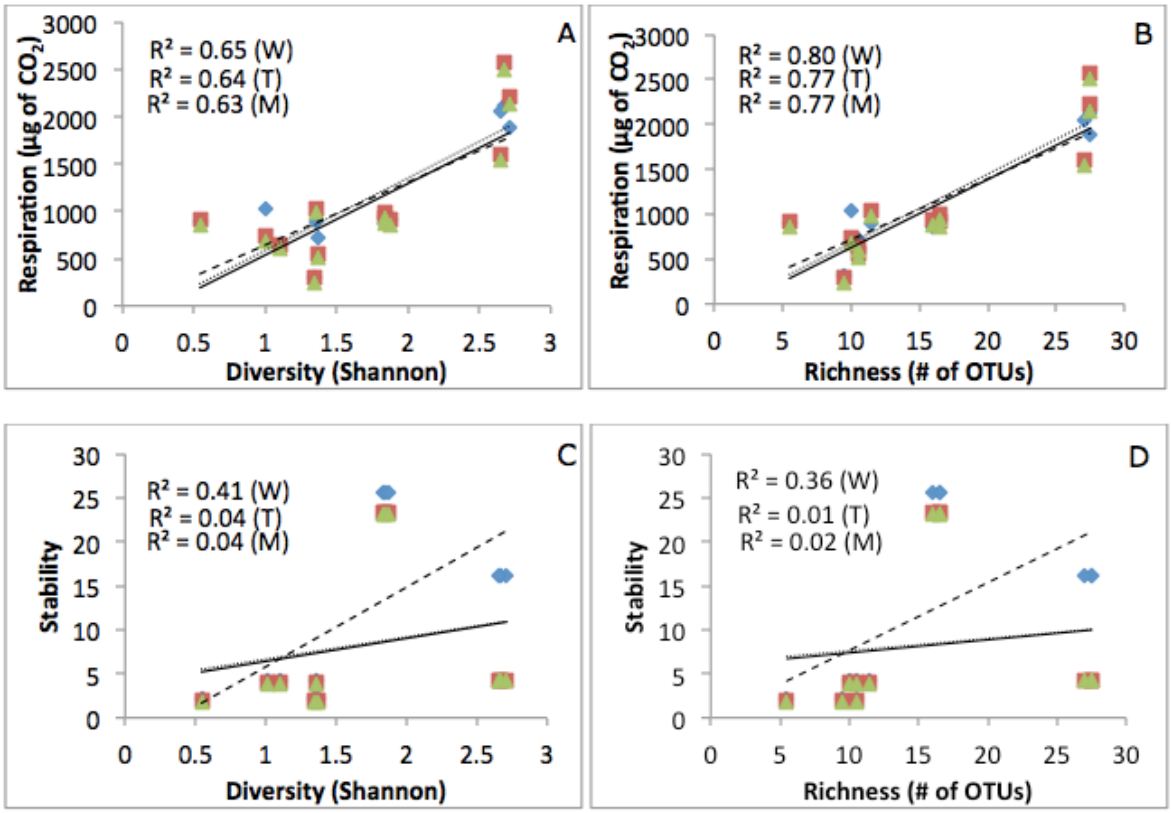

Figure 5.7 – Scatter plots showing the dependence of communities' activity on bacterial diversity (A) and richness (B) for Hawkesbury River experiment. Dependence of communities' stability on bacterial diversity (C) and richness (D) for Hawkesbury River experiment. Bacterial diversity and richness estimated via T-RFLP. For data points: Water control=◆; samples incubated with MC-LR=▲; samples incubated with triclosan=■. For regression values: W= water control; M= samples incubated with MC-LR; T=samples incubated with triclosan. Dashed line= regression line water control; continuous line= regression line MC-LR; dotted line=regression line triclosan.

For freshwater microbial communities from Hawkesbury River, a strong positive relationship between diversity and function was confirmed when analysed in a univariate regression analysis: both bacterial diversity and richness had a highly significant effect ($P<0.001$) on communities' activity in all treatments tested (i.e. water control, incubated with microcystin-LR and incubated with triclosan).

Regarding the relationship between bacterial diversity/richness with stability of the system that appeared to be weaker in scatter plot (Figure 5.7C-D), when analysed via linear regression analysis it always showed a significant effect of diversity/richness on stability ($P<0.05$). In detail, diversity was significantly correlated with stability of water control ($P<0.001$), treatment incubated with triclosan ($P=0.008$) and treatment incubated with microcystin-LR ($P=0.008$). Bacterial richness was significantly correlated with stability of water control ($P<0.001$), treatment incubated with triclosan ($P=0.013$) and incubated with microcystin-LR ($P=0.014$).

5.3.4.2 Relations based on pyrosequencing estimates of diversity and richness

The relationships between diversity and function/stability in Farmers Creek and Hawkesbury River were visualised by plotting bacterial diversity and richness estimated via pyrosequencing against: a) cumulative carbon dioxide produced, as a proxy of communities' function and; b) stability of the systems measured as mean-standard deviation ratio derived from respiration data.

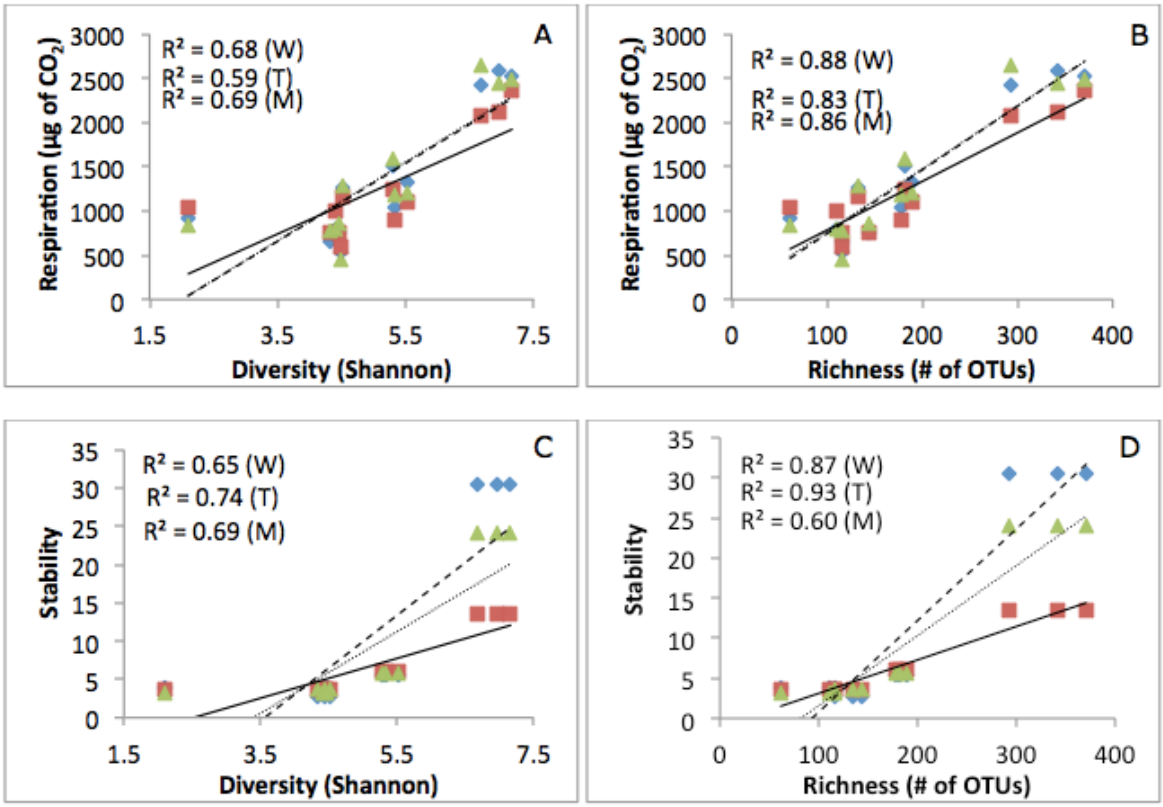

Figure 5.8 – Scatter plots showing the dependence of communities' activity on bacterial diversity (A) and richness (B) estimated via pyrosequencing for Farmers Creek experiment. Dependence of communities' stability on bacterial diversity (C) and richness (D) for Farmers Creek experiment. For data points: Water control=◆; samples incubated with MC-LR=▲; samples incubated with triclosan=■. For regression values: W= water control; M= samples incubated with MC-LR; T=samples incubated with triclosan. Dashed line= regression line water control; continuous line= regression line MC-LR; dotted line=regression line triclosan.

For freshwater microbial communities from Farmers Creek, a strong positive relationship between diversity and function was confirmed when analysed in a univariate regression analysis: both bacterial diversity and richness had a highly significant effect ($P<0.001$) on communities' activity in all treatments tested (i.e. water control, incubated with microcystin-LR and incubated with triclosan).

At the same time, both bacterial diversity and richness had a highly significant effect ($P<0.001$) on communities' stability in all treatments tested.

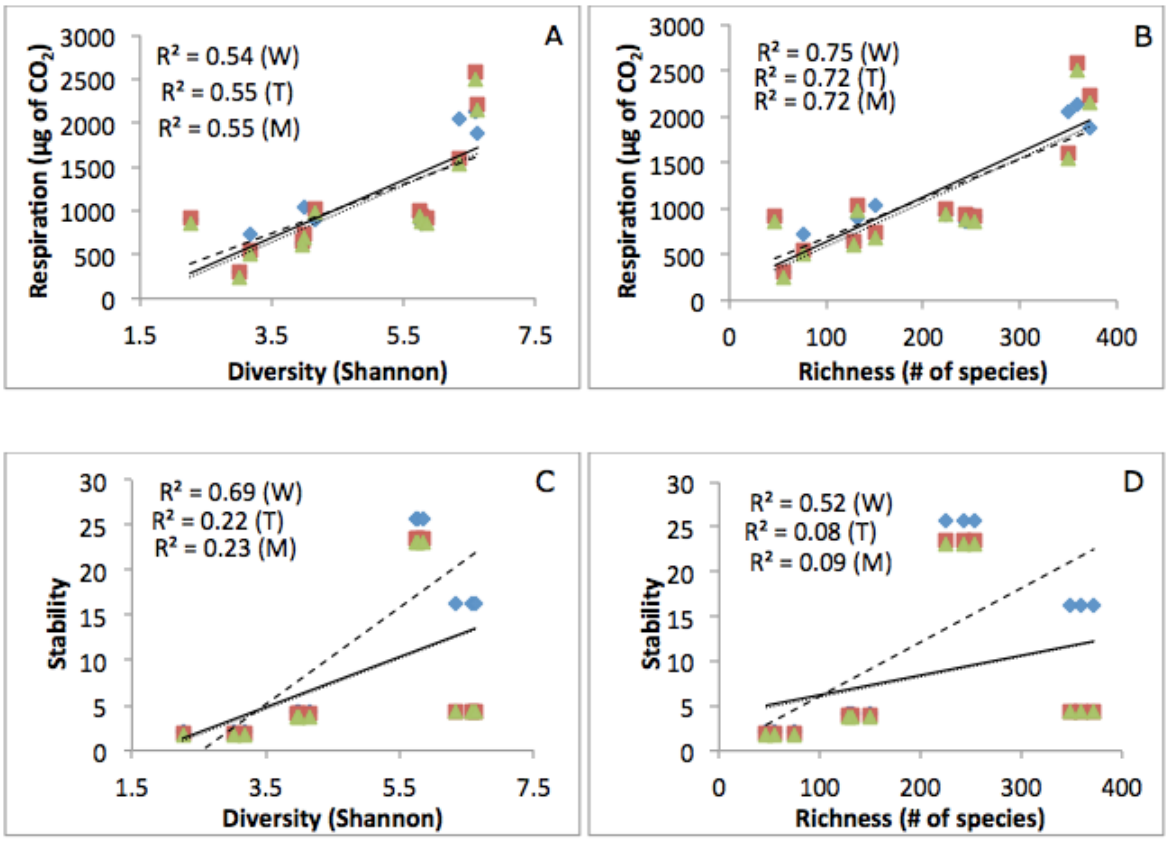

Figure 5.9 – Scatter plots showing the dependence of communities' activity on bacterial diversity (A) and richness (B) estimated via pyrosequencing for Hawkesbury River experiment. Dependence of communities' stability on bacterial diversity (C) and richness (D) for Hawkesbury River experiment. For data points: Water control=◆; samples incubated with MC-LR=▲; samples incubated with triclosan=■. For regression values: W= water control; M= samples incubated with MC-LR; T=samples incubated with triclosan. Dashed line= regression line water control; continuous line= regression line MC-LR; dotted line=regression line triclosan.

Based on pyrosequencing data, a strong positive relationship between diversity of freshwater microbial communities from Hawkesbury River and function was confirmed when analysed in a univariate regression analysis: both bacterial diversity and richness had a highly significant effect ($P<0.001$) on communities' activity in all treatments tested (i.e. water control, incubated with microcystin-LR and incubated with triclosan).

Regarding the relationship between bacterial diversity/richness with stability of the system that appeared to be weaker in scatter plot (Figure 5.9C-D), when

analysed via linear regression analysis it always showed a significant effect of diversity/richness on stability ($P<0.05$). In detail, diversity was significantly correlated with stability of water control ($P<0.001$), treatment incubated with triclosan ($P=0.002$) and treatment incubated with microcystin-LR ($P=0.003$). Bacterial richness was significantly correlated with stability of water control ($P<0.001$), treatment incubated with triclosan ($P=0.007$) and incubated with microcystin-LR ($P=0.007$).

5.3.5 Biodegradation of triclosan along the dilution gradient

Triclosan concentration was measured every four days in each of the dilution levels deriving from the dilution-to-extinction approach. Data for both Farmers Creek and Hawkesbury River showed different rates of degradation in the different dilution levels (Figure 5.10).

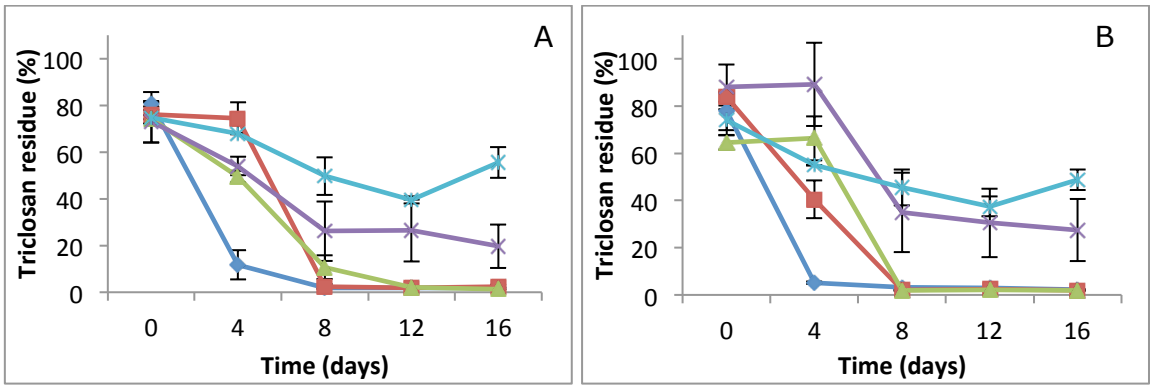

Figure 5.10 – Biodegradation of triclosan along the dilution gradient over time of: A) Farmers Creek and; B) Hawkesbury River. =undiluted; = exp^{-1}; = exp^{-2}; = exp^{-4}; = sterile control. For all dilution levels n=3; for sterile control n=2. Error bars indicate standard error.

Half-life (DT50) of triclosan was estimated as described in section 2.2.4 and results are graphically presented in Figure 5.11.

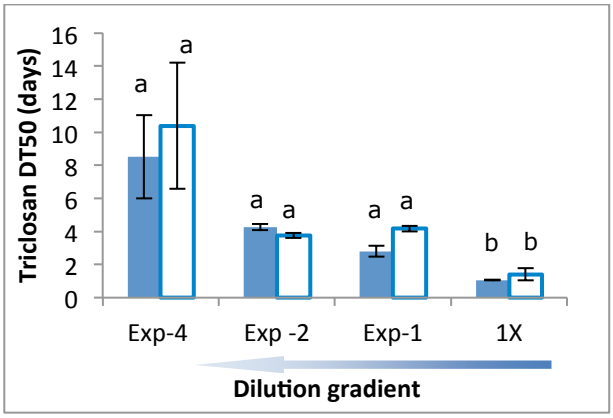

Figure 5.11 – Bar plot showing triclosan half-life (DT50) along the dilution gradient. Solid columns=Hawkesbury River; Empty columns=Farmers Creek. Values with different letters differ significantly ($P<0.05$). Error bars indicate standard error. n=3

There was a significant difference in triclosan DT50 along the dilution gradient for both Farmers Creek (P=0.05) and Hawkesbury River (P=0.02). In Farmers Creek triclosan DT50 values went, on average, from 1.4 days (undiluted) to 10.4 days (exp-4). In Hawkesbury River, triclosan DT50 values went from 1.1 to 8.5 days. Also, although a clear pattern in stability (i.e. standard deviation-mean ratio) was not observed along the three most diverse dilution levels (i.e. 1X, exp^{-1}, exp^{-2}; data not shown), a high variability was observed for the least diverse dilution level.

5.3.5 Biodegradation of microcystin-LR along the dilution gradient

Microcystin-LR concentration was measured every four days in each of the dilution levels deriving from the dilution-to-extinction approach. Data for both Farmers Creek and Hawkesbury River showed different rates of degradation in the different dilution levels (Figure 5.12).

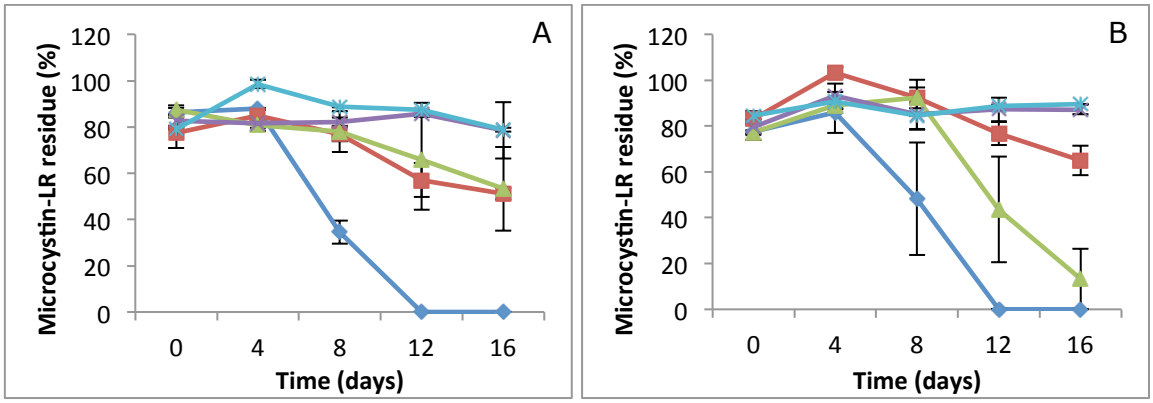

Figure 5.12 - Biodegradation of microcystin-LR along the dilution gradient of: A) Farmers Creek and; B) Hawkesbury River. =undiluted; = exp^{-1}; = exp^{-2}; = exp^{-4}; = sterile control. For all dilution levels n=3; for sterile control n=2. Error bars indicate standard error.

Half-life (DT50) of microcystin-LR was estimated as described in section 2.2.4 and results are graphically presented in Figure 5.13.

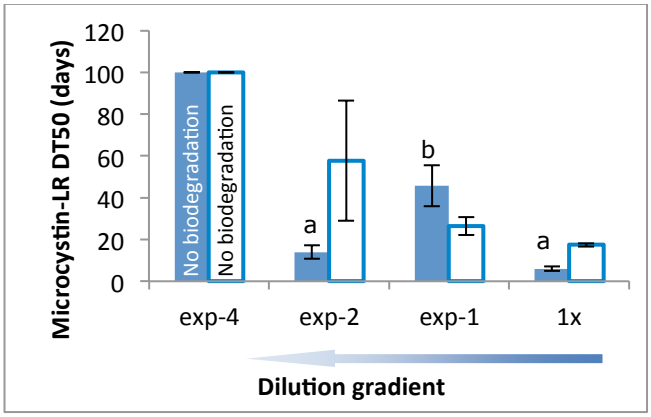

Figure 5.13 – Bar plot showing microcystin-LR half-life (DT50) along the dilution gradient. Solid columns=Hawkesbury River; Empty columns=Farmers Creek. Values with different letter differ significantly ($P<0.05$). Error bars indicate standard error. n=3

No significant differences were present between DT50 of microcystin-LR in the various dilution levels in Farmers Creek despite DT50 increased steadily on average from undiluted (17 days) to exp^{-1} dilution (26 days) and exp^{-2} dilution (58 days). Lack of significance was attributed to very high variability measured for exp^{-2} dilution where DT50 ranged from 8 to 108 days. The most diluted treatment (exp^{-4}) was not included in analysis as no biodegradation occurred.

Significant differences ($P=0.008$) were present between DT50 of microcystin-LR in the various dilution levels in Hawkesbury River. DT50 trend did not follow dilution gradient with, on average, a half-life of 6 days on the undiluted, 46 days in the exp^{-1} and 14 in the exp^{-2} dilution level. Again, the most diluted treatment (exp^{-4}) was not included in analysis as no biodegradation occurred.

5.3.6 Relationships between bacterial commmunities' diversity and specific functions

The relationships between bacterial communities' diversity and triclosan/microcystin-LR biodegradation ability in Farmers Creek and Hawkesbury River were visualised by plotting the bacterial diversity estimated via T-RFLP and pyrosequencing against the degradation rate constant (k) for each sample. Same analyses were carried out for bacterial communities' richness. Degradation rate constants were derived using the tool developed by the FOCUS Degradation Kinetics Workgroup and freely available online (http://focus.jrc.ec.europa.eu/dk/, last access 26-03-2013). Data were then explored via a linear regression analysis to assess the significance of the relationship.

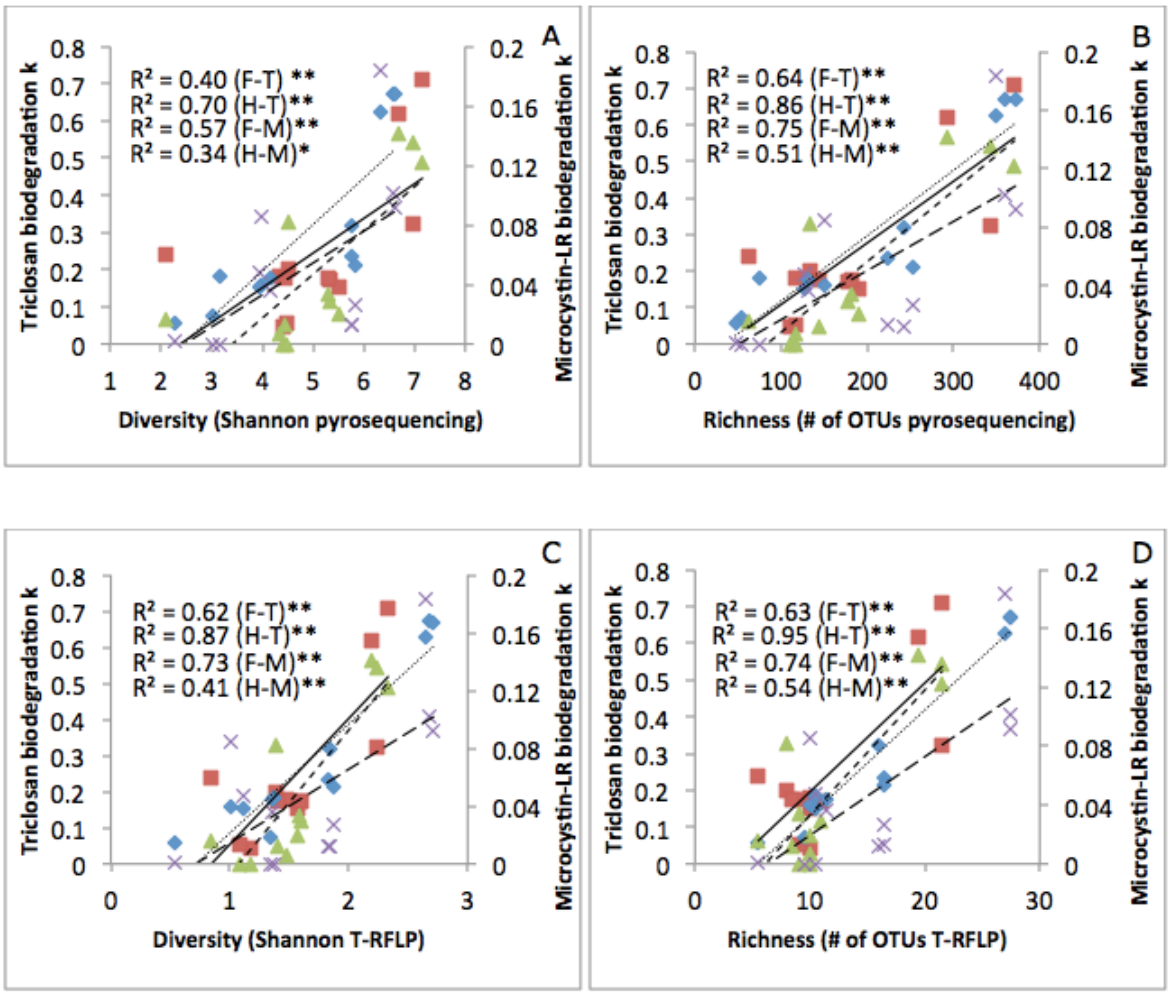

Figure 5.14 – Dependence of triclosan (T) and microcystin-LR (M) biodegradation rate constant (k) in Farmers Creek (F) and Hawkesbury River (H) on: A) bacterial diversity (Shannon) and B) bacterial richness estimated via pyrosequencing; C) bacterial diversity (Shannon) and D) bacterial richness estimated via T-RFLP. F-T=◆; H-T=■; F-M=▲; H-M=×. Continuous regression lines= F-T; dotted regression lines=H-T; short dashed regression lines= F-M; long dashed regression lines= H-M. *=P=0.002; **=P<0.001.

To simplify the reporting of the outcome of the linear regression analysis outcomes were integrated in scatter plots (Figure 5.14). In brief, for both sites considered, diversity and richness of bacterial communities, independently measured by T-RFLP or pyrosequencing, had a highly significant impact on both biodegradation rate constants of triclosan and microcystin-LR.

5.4 Discussion

5.4.1 Dilution-to-extinction, biomass recovery and diversity measures

The main aim of this work was, as per previous chapter, to study the impact of a reduction in freshwater microbial diversity on the functioning and stability of the ecosystems. In this chapter a different freshwater system was studied (i.e. river) in a different geographical location (i.e. New South Wales, Australia) and with a different set up for the dilution levels (i.e. a narrower range). Similarly to the previous chapter, a microbial diversity gradient was achieved via a dilution-to-extinction approach. The biomass recovery was monitored until it recovered in all dilution levels to ensure that the microcosms along the gradient differed in biodiversity but not in biomass (Figure 5.1, Figure 5.2). A rapid biomass recovery was achieved within three days, coherent with findings of the previous chapter (Figure 4.2) and to other studies in freshwater systems (Szabó et al., 2007). T-RFLP and pyrosequencing analysis carried out at the end of the biomass recovery period showed a significant reduction in diversity and richness as a result of the diversity manipulation via the dilution-to-extinction approach. Pyrosequencing absolute measures of richness and diversity were much higher than T-RFLP estimates. However, when comparing the percentage reduction achieved by the diversity manipulation, the two techniques performed almost equally for Shannon diversity (i.e. based on T-RFLP data 53% average overall reduction, based on pyrosequencing 52% average overall reduction). On the other hand, pyrosequencing was more informative for reduction in richness (i.e. based on T-RFLP data 60% average overall reduction, based on pyrosequencing data 77% average overall reduction). An overview of results from this chapter and from the previous chapter suggests that the dilution-to-extinction approach to manipulate

bacterial diversity is a reliable method in freshwater systems. T-RFLP analysis, despite its limitations (Blackwood et al., 2007, Bent and Forney, 2008), was shown to be a good tool in obtaining diversity and richness estimates that are a reflection of the more informative data of pyrosequencing (Figure 4.5, Figure 5.4).

5.4.2 Communities' functioning and stability along the dilution gradient

In this work, both broad (i.e. respiration) and specialised (i.e. triclosan and microcystin-LR biodegradation) communities' functions were significantly reduced along the dilution gradient. The reduction in respiration, as a proxy of overall microbial activity, along the dilution gradient was consistent with results of previous chapter (Figure 4.6). Different authors have reported no impact of diversity gradient obtained by dilution-to-extinction approach on large scale ecosystem functions in soils (Wertz et al., 2006, Griffiths et al., 2001). That has been explained assuming that a vast microbial diversity and functions potential in soil (Curtis, 2006, Singh, 2010) corresponds with a vast redundancy as many species are able to perform large scale functions such as carbon mineralisation (Nielsen et al., 2011). On the other hand, few authors (Juarez et al., 2013) have reported in soil a reduction in carbon mineralisation following erosion of diversity with a removal approach. Studies focusing on more specific functions have found, amongst others, a detrimental effect of the diversity removal via dilution on nitrification activity (Philippot et al., 2013), chitinase activity (Peter et al., 2011), resistance to invasion by bacterial pathogens (van Elsas et al., 2012). Other works reported that a diversity removal via dilution approach did not follow a significant reduction in phenol biodegradation (Szabó et al., 2007) and nitrification and denitrification (Wertz et al., 2006). The only study looking at the impact of diversity removal

on ability to degrade a man-made pollutant (Cook et al., 2006) found a positive relation between biodiversity and biodegradation along a dilution/diversity gradient but, based upon functional data of artificial communities, proposed a relationship where communities' structure is the true driver of system functionality irrespective of diversity. In both sites studied in this work, it was observed that microbial communities in most diverse treatments (i.e. undiluted) had the capacity to biodegrade 50% of the introduced toxic compound within few days. It was also observed that at the lowest diversity treatment (i.e. dilution exp^{-4}), microbial communities lost the microcystin-LR biodegradation ability while the triclosan biodegradation ability was highly reduced with an increased DT50 compared to treatments with higher diversity. Triclosan half-life in both sites and microcystin-LR half-life in Farmers Creek increased gradually along the dilution gradient, while a peculiar trend was observed for microcystin-LR half-life in the Hawkesbury River. In the Hawkesbury River microcystin-LR half-life as expected increased from the undiluted to the first dilution (exp^{-1}) but then oddly decreased from the first to second dilution (exp^{-2}). The unexpected short half-life of microcystin-LR in the exp^{-2} dilution could be explained by a possible fast growth and/or high activity of species able to degrade the cyanotoxin. Fast growth could be explained by the removal of competitors/predators present in the exp^{-1}: that would have allowed species able to carry out biodegradation to grow without disturbances. Comparing this work with literature, apart from the peculiarity of each function studied, contrasting relations observed could be explained by different initial microbial communities, stochastic species selection that occurs during dilutions and changes of communities' structure during the regrowth phase. Findings

may also be explained from interaction of environmental factors and complexity of the systems (Langenheder et al., 2010).

For Farmers Creek stability of the system increased with an exponential trend along the dilution gradient with its highest at the undiluted treatment, on the other hand, in River Hawkesbury stability had an increase along the dilution gradient until the exp^{-1} dilution followed by a decrease for the undiluted treatment. This relationship is similar to results reported in for the experiment Loch Rescobie (1) and Loch Freuchie (2) in Chapter 4 (Figure 4.6A,C). An overall increase in stability along the dilution gradient suggests there is a positive relationship between diversity and stability. However the trend of the curve that is not consistent between the different dilutions levels would suggest that not only diversity but also community structure play an important role in the stability of the system at different diversity levels. A positive but idiosyncratic relationship between diversity and stability would best describe this relation where communities' structure also plays an important role (see Figure 1.2).

Neither microcystin-LR nor triclosan had a consistent effect on respiration or stability along the dilution/diversity gradient. Results for microcystin-LR are consistent with a similar study in a different freshwater system (Chapter 4) and possible reasons for lack of effect were discussed in section 4.4.2. Triclosan is a widely used and effective antibacterial and antifungal agent (McMurry et al., 1998, Levy et al., 1999) which can be found in many environments and organisms (Adolfsson-Erici et al., 2002, Dann and Hontela, 2011) as a result of the vast use followed by environmental discharge (Bester, 2005). Due to its antimicrobial activity, triclosan could be expected to reduce

carbon mineralisation and other bacterial functions as reported by Stasinakis et al. (2008). However, the low concentration (i.e.10 ppb) of triclosan employed in this work was not sufficient to trigger a reduction in overall microbial activities in the microcosm. Possibly, for microbial communities previously exposed to triclosan, the concentration used in this study was not sufficient to affect the system and similar findings have been reported in activated sludge (Federle et al., 2002). Also, if triclosan addition is considered as a stress to the system, an actual increase in overall activity could be expected (Schimel et al., 2007).

5.4.3 Diversity and communities' functions

The gradient of diversity achieved via the dilution-to-extinction approach and measured by both T-RFLP and pyrosequencing had a highly significant impact on both broad and specialised ecosystem functions, as well as on stability of the system. Similar results were found for bacterial richness.

A strong correlation between bacterial diversity and broad ecosystem functioning is coherent with work presented in Chapter 4, where the relation was tested in different freshwater system types, geographical location and with a different setting of dilution levels. Overall respiration, as a measure of overall microbial activity, results from all pathways leading to production of carbon dioxide and is indeed a function shared by the vast majority of microorganisms and hence redundant. Even a drastic decline in microbial diversity could be expected to have no impact on a very broad and redundant function according to the insurance hypothesis (Yachi and Loreau, 1999). It is then expected that the biological parameter mainly affecting a broad and redundant function would be microbial biomass. Clearly our findings do not comply with the insurance hypothesis, but rather we have shown that a reduction in diversity has the ability to reduce both broad and specific ecosystem functions, as well as stability of the system. Although it is correct that a broad function is carried out by many different species, it is also valid that different species will perform better due to niche partitioning (Cardinale, 2011), selection and facilitation effects (Loreau and Hector, 2001). Applied to our simplified, stable and homogeneous microcosm design, niche partitioning possibly did not play the most important role but it is noteworthy to consider that various different gradients and niches are present even in such simplified microcosm design (e.g. planktonic vs sessile organisms, oxygen gradient from

water surface to bottom, water speed gradient from centre to edges of bottle). The selection effect might have also played a role for our experimental design where we manipulated diversity via a dilution-to-extinction approach as removal of key species might have occurred. But for a broad function and for the trend in carbon mineralisation observed, the selection effect is probably not the central factor in determining the gradient of function observed. We suggest that for the system and design tested in this work the complementary effect played the most important role: a high number of species resulted in a higher number of genes, enzymes, pathways and resources that facilitated the usage of a natural pool of carbon sources and hence an augmented respiration rate (Bell et al., 2005b).

A strong correlation was also found between bacterial diversity and both specialised ecosystem functions studied: triclosan and microcystin-LR biodegradation. Opposite to respiration, biodegradation of a specific compound is not likely to be carried out by the vast majority of the microorganisms present in any given environment. As for many xenobiotics, many studies have shown that the ability to degrade triclosan is not exclusive to one bacterial species but rather shared by different species belonging to different taxonomical groups such as *Alpha Proteobateria* (Lee et al., 2012), Beta *Proteobacteria* (Meade et al., 2001) and *Gamma Proteobacteria* (Gangadharan Puthiya Veetil et al., 2012). Most isolated species responsible for microcystin-LR biodegradation also belong to the *Proteobacteria* phylum (Edwards and Lawton, 2009), with few exceptions in the *Actinobacteria* (Manage et al., 2009b) and *Firmicutes* (Nybom et al., 2012). As discussed above for respiration, differential ecosystem functioning along the diversity gradient might be explained by the different mechanisms of niche partitioning, selection

and facilitation. A gradual decrease in biodegradation activity along the diversity gradient observed in this work (Figure 5.14) might not comply with the selection hypothesis where a key species is responsible for performing a major role for a determined function as in this case where a sudden drop in such activity should be recorded. It could be argued that in case species able to degrade were slow growing, then the selection hypothesis could stand where a slow growing species was gradually diluted and the function gradually lost. However, in this case the decrease in function should have been in the same order of magnitude as the dilution, while data in this work showed otherwise. Niche partitioning might have played a minor role in this system but, because information are not available for differential niches preferred from different species able to degrade triclosan, its importance is difficult to evaluate. The findings of Wohl et al. (2004) showed that, given a specific function, species redundancy (i.e. different species able to perform the same function) increases the rate at which the function will be performed through facilitation effect. Results of our study would suggest a similar mechanism: various species able to degrade the toxic compounds introduced in the system are present in the more diverse microcosms, and consequently a higher degradation rate of the pollutant is observed. In the treatments with lower diversity fewer or no species able to degrade the toxic compounds are present resulting in a lower or null degradation rate.

5.5 Conclusions

This work confirmed findings of Chapter 4 showing that, independently of the freshwater system tested, a reduction in microbial diversity triggers: (1) a decrease in a broad ecosystem function such as carbon mineralisation; (2) a decrease in system stability; (3) a decrease in specialised function such as biodegradation of microcystin-LR and triclosan.

A huge amount of triclosan is released into the environment due to human activities (Bester, 2005), and its main breakdown pathway is biodegradation. Triclosan is also a potent antimicrobial agent which has adverse effect on a number of aquatic and terrestrial organisms, including mammals (Dann and Hontela, 2011, Gee et al., 2008). Triclosan has also the potential to inhibit the ability of microbial communities to degrade other xenobiotics in the environment (Svenningsen et al., 2011). On the other hand, cyanobacterial blooms and the toxins that they produce are occurring with increasing rate worldwide mainly due to increased nutrients but also to climatic changes: both resulting from human activities (Paerl et al., 2011). Microcystin-LR, one of the most common cyanotoxins, is a potent hepatotoxin and possibly carcinogen which is stable and persistent in aquatic environment (Lahti et al., 1997). In this work it was shown that a reduction in microbial diversity triggers a decrease in microbial biodegradation ability for both triclosan and microcystin-LR. Findings of this work stress the importance of maintaining microbial biodiversity in order to sustain important ecosystem services such as freshwater purification (Cardinale et al., 2012).

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6. OVERALL CONCLUSIONS AND FUTURE WORKS

From ecological and conservation points of view, the main findings of this project suggest that both broad and specialised functions of natural microbial communities strongly depend upon their phylogenetic diversity. Of particular interest was the observation that a decrease in microbial diversity resulted in a reduced, or removed, ability of microbial communities to degrade relevant toxic compounds in freshwater environments. As eighty percent of the world's population is exposed to high levels of threat in terms of water security, results of this work resonate the importance of biodiversity for ecosystem broad functioning and stability and, more importantly, highlight the relationship between microbial biodiversity and freshwater purification potential. Relationships between microbial diversity and broad functioning and its stability were consistent in both lake and river freshwaters tested, suggesting the relevance of the relationships across systems. Relationships between microbial diversity and biodegradation of both natural and man-made toxic compounds were also consistent in river freshwaters tested.

The current debate regarding biodiversity conservation has its main focus on macro-organisms and mostly ignores microbial diversity. This has foundation in the belief that there is a huge functional redundancy among microbial communities as a result of their vast diversity. This work demonstrated that declining microbial diversity has direct consequences for ecosystem functioning. Therefore, it is important that the debate regarding biodiversity conservation explicitly considers microbial diversity.

Other findings of ecological relevancy in this work include the significant correlation found between bacterial communities' structure of natural freshwater bodies and historical exposure to cyanobacterial blooms and cyanotoxins, but also correlations found between communities' structure and half-life of microcystin-LR.

From a technical point of view, it was confirmed that a dilution-to-extinction approach is a reliable method to manipulate bacterial diversity in freshwater. It was also revealed that T-RFLP is a valid method to gain diversity estimates that are a reflection of the more informative ones resulting from pyrosequencing. Also, given the availability of limited samples sizes, it was shown that biodegradation of triclosan at environmental relevant concentrations in freshwater microcosms can be effectively followed using commercially available ELISA kit.

From a theoretical point of view, this work provides direct evidence that ecological theories developed for macro-organisms (plant and animals) can be applied to explain relationships between microbial diversity and ecosystem functioning. This knowledge can be harnessed to demonstrate microbial regulation of biogeochemical cycles at global scale in order to include microbial data in ecosystem models and, in turn, improve their predictions.

Future works should test the relationship between bacterial diversity and biodegradation with artificially assembled communities. This approach, despite its limitations, would give an insight into the mechanisms and ecological interaction responsible for the observed relationships. Also, coherent results from the two approaches (i.e. removal in microcosms and artificial

communities) could be the starting point for bioremediation strategies where microbial ability to degrade toxic compounds in the environment could be improved via methodologies that results in increased biodiversity.

On the other hand, other works should seek instances of these relationships at ecosystem level. Determining whether microbial diversity drives biodegradation potential in complex natural systems would present a number of challenges. Nevertheless, coherent results from the two approaches (i.e. removal in microcosms and observation of natural systems) would have the potential to develop strong models allowing the prediction of toxic compounds persistence in the environment based on microbial data.

Finally, future works in controlled environments should aim to explore the relationships between microbial diversity and specialised functioning along relevant environmental gradients (e.g. temperature, pH, salinity) and in climate change scenario (i.e. temperature and carbon dioxide increase, extreme weathers) in order to identify microbial diversity impacts on resilience and recovery of ecosystem functions under stress.

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