Use of stream biofilm microbial communities and associated metals as indicators of urban runoff impact on freshwater ecosystems.

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Abstract

The main focus of this project was to investigate how anthropogenic activities affect stream biofilm microbial communities and to assess the potential uses of biofilms as indicators of freshwater ecosystem health. The work improves our understanding of the impact of urbanisation on stream ecosystems and provides innovative techniques to assess this impact more accurately.

Stream biofilms are the complex aggregation of microorganisms, covering almost every surface in freshwater environments. They play key roles in stream ecosystems and are grazed by many other organisms. Therefore, the potential impact of urbanisation on biofilm microbial communities could alter the functions of biofilms and affect the whole stream ecosystem. A wide range of experiments was conducted in both flow chamber microcosms and natural stream environments to investigate the impact of urban runoff on microbial community structure and composition. Using community fingerprinting techniques such as Terminal-Restriction Fragment Length Polymorphism and Automated Ribosomal Intergenic Spacer Analysis as well as sequencing of bacterial 16S rRNA genes, we investigated changes occurring in biofilm bacterial and ciliate protozoan communities under exposure to urban contaminants. Flow chamber experiments revealed that significant differences in the structure of the microbial community could be detected within only a few days of exposure to urban runoff contaminants and remained detectable weeks after transfer to uncontaminated water. The rapid and persistent changes highlight the sensitivity of microbial communities and suggest that natural biofilm communities frequently exposed to urban runoff are significantly altered. Experiments carried out in natural streams confirmed the differences in composition and tolerance to contaminants between biofilm communities from preserved and impacted streams, suggesting critical implications for the whole stream ecosystem. The structure and composition of microbial communities associated with biofilms result from the influence of past and present environmental conditions and therefore constitute potential integrative indicators of stream health. Rapid changes in bacterial and ciliate community structure during exposure to metal contaminants demonstrated the sensitivity of these communities and their potential use as indicators of the influence of urban areas on stream ecosystem. Additional investigation conducted in natural streams indicated that bacterial and ciliate communities can reveal changes occurring along an urbanisation gradient and confirmed their reliable use as ecological bio-indicators. These novel indicators were implemented successfully to

investigate the efficiency of an enclosed stormwater treatment system where traditional biological indicators such as benthic macroinvertebrates are not available. Results revealed the successful improvement of water through the treatment train and the minimal impact on the receiving creek.

As biofilms are sessile structures, they may also accumulate urban contaminants and could form a critical link in the movement of urban contaminants from abiotic to biotic components of the stream. Therefore, the absorption and release rates of metals (Zn, Cu and Pb) in biofilms were investigated. Results revealed the fast accumulation of metals in the matrix during the first few days. High enrichment factors were recorded between the biofilm wet weight and the water, reaching 500:1 for zinc, 1500:1 for copper and 6000:1 for lead after 21 days of exposure. During recovery in uncontaminated water, metals were retained in the biofilm and 10 to 16 % of zinc and copper accumulated during exposure still remained in the biofilm after 14 days of recovery. The release of lead was even slower and more than 35 % of accumulated lead remained in the biofilm after 14 days of recovery. Investigation in natural streams confirmed the accumulation of metals in biofilms from urban streams and highlighted the potential risk for organisms at a higher trophic level. These results suggested that metals associated with biofilms could provide a highly relevant indicator of the presence of metals in freshwater systems at concentrations detrimental to aquatic biota. Using biofilm bacterial, ciliate and macro-invertebrate communities as bio-indicators, the relevance of biofilm associated metals (As, Cd, Cr, Cu, Pb, Ni and Zn) was investigated and compared to sediment associated metals. A greater proportion of the changes occurring in each of the communities could be explained by metal concentrations in biofilm than in sediments confirming the ecological relevance of biofilm associated metals.

This project provides an insight into the effect of urban contaminants on stream microorganisms and contributes to the understanding of how urbanisation affects the entire stream ecosystem. Novel tools emerging from the use of biofilm as an indicator of stream health will assist land planners, aquatic resource managers and decision makers by facilitating better assessment of urban influences on aquatic environments.

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CHAPTER 1

1 Introduction

As the human population continues to grow, the effect of urbanisation on freshwater ecosystems is becoming increasingly important. Almost all global population growth in the next 40 years is expected to occur by expansion of existing urban areas (United-Nations, 2009). Rural lands are progressively converted into urban areas, continuously increasing the anthropogenic impact on freshwater bodies. It is therefore crucial to improve our understanding of the effect urbanisation has on freshwater ecosystems and to provide aquatic resource managers and land planners with tools to assess the stress experienced by aquatic organisms.

1.1 Effect of urbanisation on freshwater ecosystems

The expansion of urban areas and the increase of human density cause multiple and complex impacts on freshwater ecosystems. The geomorphology of the streams is generally simplified and the riparian vegetation removed (Paul & Meyer, 2001). These changes profoundly modify the stream habitats and affect aquatic organisms (Allan, 2004). As rural land is converted into urban areas, the hydrology of the stream is also altered. The increase of impervious surfaces reduces considerably the extent of soil infiltration and the vegetation is generally replaced by buildings and roads, decreasing the amount of evapotranspiration (Paul & Meyer, 2001; Walsh *et al.*, 2005). Therefore, urbanisation leads to higher peak discharges; the stream flow is greater during storm events and carry higher sediment loads (Nelson & Booth, 2002), causing floods and increasing stream erosion (Bernhardt & Palmer, 2007).

Qualitatively, urbanisation also increases concentrations of contaminants entering streams. When flowing onto impervious surfaces, rainwater accumulates contaminants such as suspended sediments, heavy metals, hydrocarbons and oil, organic chemicals, nutrients and litter (Burton & Pitt, 2002). Those contaminants can accumulate in urban runoff in two ways. First, impervious surfaces are recipients for the deposition of urban pollutants that can be carried by the rainfall (wet deposition) or by the wind (dry deposition) (Davis & McCuen, 2005). These pollutants can come from motor vehicle exhaust gases or a wide range of other sources in the adjacent urban environment. Secondly, every surface on which the water flows adds its own set of contaminants by dissolution. For example, gutters are a source of copper,

road bitumen and galvanised roofs a source of zinc and painted walls a source of lead (Davis & Burns, 1999; Davis *et al.*, 2001; Kennedy & Gadd, 2000).

1.1.1 Heavy metals in urban runoff

Metals are naturally present in aquatic ecosystems as a result of weathering of soil and rocks (Sigee, 2005). However, whereas low concentrations of some trace metals can occur naturally in freshwater, human activities like industry and mining (Douglas & Beveridge, 1998) as well as urbanisation (Bernhardt & Palmer, 2007; Chalmers *et al.*, 2007; Ward & Trimble, 2003) have considerably increased metal concentrations in surface waters. All metals can be toxic to aquatic organisms if exposure levels are sufficiently high (Laws, 1993) and in urban catchments, metal concentrations frequently reach toxic levels that constitute a threat to aquatic ecosystems (Förstner & Wittmann, 1981; Kennedy, 1999; Mance, 1987; Rice, 1999).

Metals pollutants are very diverse, the Australian and New Zealand Environment and Conservation Council (ANZECC) water quality guidelines identify 26 common metals that constitute a potential threat for freshwater ecosystems (ANZECC & ARMCANZ, 2000). They can also have different valences and be present in many different forms which influence highly their toxicity (Sterritt & Lester, 1980; Tessier & Turner, 1995). In contrast with organic pollutants, metals are not biodegradable. Therefore, when entering into a stream, metals can either be washed further downstream, or accumulate in the stream ecosystem. Generally, primary sources of contaminants in urbanised areas are cars and their tyres (rubber contains metal in significant proportions), industries, paints, road bitumen and galvanised roofs (Davis *et al.*, 2001; Kennedy & Gadd, 2000; Laws, 1993).

Metal ions bind easily with particles present in the water such as silt or organic matter. Depending on the characteristics of each element and on the amount and the nature of particles transported by the water, a significant proportion of the contaminants can be transported in association with particles (Bibby & Webster-Brown, 2006). This can highly influence the toxicity as well as the accumulation of metals. Table 1.1 shows the average proportion of particulate and dissolved contaminants for zinc, copper and lead in stormwater obtained from different studies carried out in New Zealand.

Table 1.1: Average partitioning between dissolved and particulate zinc, copper and lead in urban runoff. The data are averages obtained from multiple studies (16, 14 and 14 for zinc, copper and lead, respectively) carried out in New Zealand (ARC, 2004a).

Zinc		Copper		Lead	
dissolved (%)	particulate (%)	dissolved (%)	particulate (%)	dissolved (%)	particulate (%)
48.1	51.9	33.8	66.2	7.1	92.9

There are large gaps in the understanding of where contaminants originate (Kennedy & Gadd, 2001) and each geographic area has its own specificities. Within New Zealand the influence of local geology on element concentrations in water can be important because of the volcanic origins of the country. In the Auckland area, the volcanic parent materials have an important influence on background concentrations of copper, cadmium, chromium, cobalt, nickel, vanadium and zinc in soils (ARC, 2001).

In urban areas, the concentration of metals is highly variable but a range of elements are of particular concern because of their frequent presence in high concentrations and their hazard for the environment. For these reasons, zinc, copper and lead will be specifically investigated in this project (ANZECC & ARMCANZ, 2000; ARC, 2007b; Kayhanian *et al.*, 2007; Kennedy, 1999; Mance, 1987). The concentrations for zinc, copper and lead measured in stormwater by different studies carried out in New Zealand are given in Table 1.2.

Table 1.2 : Concentrations of zinc, copper and lead present in different types of urban runoff. All values are given in µg l^{-1}.

Site	Zinc	Copper	Lead
Residential area, New Zealand [1]	251	17	34
Commercial area, New Zealand [1]	302	23	55
Industrial area, New Zealand [1]	717	47	95
Pakuranga (Residential) [2]	444	15	55
Auckland SH-1 Motorway [2]	159	53	108
Southdown (Industrial) [2]	466	42	82
Urban (10%ile) [3]	90	15	60
Urban (50%ile) [3]	260	110	190

[1] Average of medians for nine different studies (where data available) (ARC, 2004a).

[2] ARC 1992 in Kennedy & Gadd (2001).

[3] (Williamson, 1993) The percentile (%ile) is the value of a variable below which a certain percent of observations fall.

1.1.2 Effects of metals on aquatic life

The mechanisms by which metals are toxic to organisms are very complex and have been subject to much study. Toxic reactivity of metals is mainly a consequence of their specific chemical properties, including reduction/oxidation potential, acid/base chemistry and structural or ligand coordination properties (Zalups & Koropatnick, 2000). Excessive concentrations of metal ions may affect cellular metabolism in a myriad of ways, including disruption of cell membrane integrity, alteration of the cellular redox state, as well as inhibition of respiration, DNA replication and protein synthesis (Agarwal *et al.*, 1989; Jensen & Winge, 2000). These toxic effects can arise from direct inhibition of protein functions or indirect mechanisms such as metal induced free radical reactions. The high reactivity of biomolecules to radicals can result in damage to DNA, RNA, proteins and lipids (Jensen & Winge, 2000).

A large number of factors can influence the toxicity of metals. Not only because there is a wide range of metals potentially harmful for the environment but also because each of them has different possible valences and can be present in many different forms that can strongly influence their toxicity (Allen, 1993; Sterritt & Lester, 1980). Several environmental conditions also affect the form in which the metals are present and thus their potential toxicity, these include pH, concentrations of chelating agents, concentrations of inorganic anions and presence of other cations (Campbell *et al.*, 1997; Sterritt & Lester, 1980; Worms *et al.*, 2006). Bioaccumulation can also be an important aspect of the metal toxicity and mobilisation through food chain (Rainbow, 2007; Sterritt & Lester, 1980). Many studies have focused on the effects of metals on aquatic organisms, especially fish and benthic macroinvertebrates. Most of them have assessed the acute concentrations of metals for different species leading to their quick death (Mance, 1987). Those concentrations are generally high but other studies have also revealed that much lower concentrations can have a long term effect on the growth of organisms or on their fertility. Often the effects are not seen before adulthood and are therefore difficult to assess (Mason, 1996).

Chemistry and toxicity of zinc, copper and lead

Zinc

In water, zinc exists mainly as Zn^{2+}. It forms the complexes $[ZnOH]^{+}$, $[Zn(OH)_3]^{-}$ and $[Zn(OH)_4]^{2-}$ in waters with pH values above 7 and low alkalinity whereas in waters with high alkalinity, complexes with carbonate are formed such as $[ZnCO_{3(aq)}]^{0}$. Zinc also easily forms complexes with organic compounds (Dojlido & Best, 1993). Although zinc is an essential element for living organisms mainly for enzymatic processes (Stillman & Presta, 2000), at high concentrations it becomes toxic for all aquatic organisms. Reported acute concentrations for fish and invertebrates can vary from 0.1 mg l^{-1} to a few tens of mg l^{-1} depending on the species and other factors such as temperature and water hardness (Mance, 1987).

Copper

In water, most copper is of valence $^{2+}$ and the cupric salts rapidly dissolve to form an aquo complex $Cu(H_2O)_4^{2+}$. Water molecules can also be replaced by a variety of ligands to form different complexes (Dojlido & Best, 1993). Dissolved copper in surface waters exists mainly as the complex $[CuCO_{3(aq)}]$ and partly as Cu^{2+} (Dojlido & Best, 1993). Small amounts of copper are essential to life but large doses are toxic, particularly to plants (Dojlido & Best, 1993). Biological chemistry of Cu is dominated by participation in redox reactions, playing vital roles in many enzymatic processes (Sigee, 2005; Stillman & Presta, 2000). There is a considerable range of levels of toxicity both within and among taxa and the organism life stage is generally a determinant factor for toxicity (Mance, 1987). Acute concentrations for fish and invertebrates range from 0.005 to a few tens of mg l^{-1} (Mance, 1987).

Lead

Lead is rarely found naturally as a free metal in freshwater, most of it comes from human activities. In surface waters, lead is present as hydrated Pb^{2+} or $[PbCO_{3(aq)}]^{0}$. The concentration of lead in water is usually limited by the solubility of $PbCO_3$ and by its adsorption onto particulate matter. Lead does not appear to be an essential element for life for any organism (Dojlido & Best, 1993). Concentrations between 0.07 and several tens of mg l^{-1} have been reported as acutely toxic for fish and invertebrates (Mance, 1987).

1.2 Stream biofilms

Stream biofilms are a complex aggregation of microbial communities adhering to almost every surface in freshwater. Microbial communities include bacteria, archea, fungi, and algae all embedded or associated with a polymeric matrix produced by the constituent organisms. On and within biofilms live protozoa, rotifers, nematodes and insect larvae that shelter or graze on the polymer slime (Lamberti, 1996; Parry, 2004). Being at the bottom of the stream food web, biofilm organisms are crucial primary producers for higher trophic levels. They also play key roles in cycling nutrients, organic compound degradation, water quality remediation and suspended sediment removal (Fischer, 2003; Takada *et al.*, 1994). Consequently, the disruption of stream biofilms may have consequences for the entire stream ecosystem.

1.2.1 Biofilm development and structure

Biofilm constituting microorganisms can live either free floating in the water or in a film attached to surfaces. The formation of stream biofilms therefore requires microorganisms to change from a planktonic mode of life (in the water column) to a biofilm mode of life (as sessile communities). There is evidence that bacteria can regulate the formation of stable cell-surface interactions in response to environmental factors, however the change of behaviour from planktonic to sessile is complex and involves species specific mechanisms that are not well understood (Stanley & Lazazzera, 2004).

Biofilm formation begins with an initial adhesion of bacteria on the substratum surface (Figure 1.1). This adhesion can occur on a wide range of physical interfaces in freshwater including air/water boundaries, water/biomass such as algae, zooplankton and submerged higher plants or water/solid surfaces such as suspended particulate material, sediments and large rocks and stones. Bacterial twitching or flagellar motility is important for the initial attachment, allowing the cells to position themselves and contact the substratum (O'Toole & Kolter, 1998; Toutain *et al.*, 2007). However, sedimentation, Brownian motion or water flow can also bring the cells in contact with the surfaces (Palmer *et al.*, 2007). The adhesion is initially reversible but becomes less reversible within minutes due to the progressive removal of water from in-between the interacting surfaces and the production of extracellular

polymers (Marshall *et al.*, 1971; Stoodley *et al.*, 2002). Reversible attachment of pioneering bacteria to surfaces enables them to review the suitability of a particular micro-environment before committing to irreversible colonization of an unknown habitat.

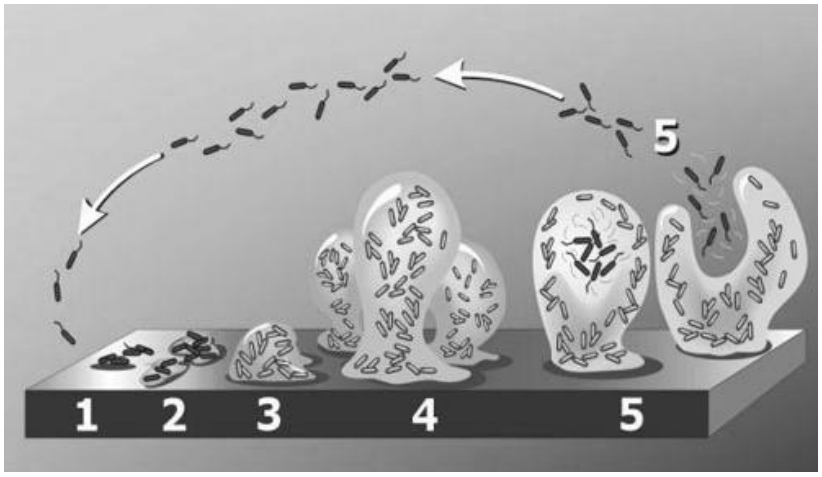

Figure 1.1: Biofilm development. The development is presented in five stages: 1: initial attachment of cells to the surface, 2: production of extracellular polymeric substances (EPS) and transition to irreversible attachment, 3: development of three dimensional structures, 4: maturation of biofilm, 5: detachment of cells from the biofilm. Source: Stoodley *et al*. (2002).

Once the primary colonizers have adhered, secondary colonizers can attach in a second layer resulting in the formation of a multispecies biofilm. The maturation of the biofilm involves an increase in microbial biomass and the production of the extracellular matrix. Mature stream biofilms are highly complex both in terms of microbial diversity and three dimensional structure (Battin *et al.*, 2003b). They include bacteria, archea, fungi, filamentous and single cell algae all dispersed among a polymeric matrix produced by the constituent organisms. Their structure is heterogeneous and includes channels and voids which facilitate the diffusion of substances through the biofilm (Costerton *et al.*, 1995). The structure of biofilms is stabilised by the extracellular polymeric substances (EPS) that are predominantly composed of polysaccharides as well as proteins, nucleic acids and lipids (Neu & Lawrence, 2009; Stoodley *et al.*, 2002; Sutherland, 2001b). Microorganisms can also be released by the matrix and revert to the planktonic mode of growth, completing the biofilm developmental life cycle (Figure 1.1) (Stoodley *et al.*, 2002).

The reasons why bacteria form biofilms are not clear but there are a numerous benefits that could motivate them to choose a sessile mode of life. First, mature biofilms are resistant to

physical forces and can tolerate antimicrobial agents at concentrations much higher than planktonic bacteria (Jefferson, 2004; Lewis, 2001). Bacteria within biofilms can withstand nutrient deprivation, pH changes and oxygen radicals better than planktonic bacteria (Jefferson, 2004). Biofilms also present a number of benefits due to the communal behaviour of constituting organisms. When bacteria are forming biofilms, changes in gene expression can result in phenotypic heterogeneity of a single species within the biofilm. This suggests that bacteria can specialize and divide the labour so that biofilms can be seen as interactive communities. Within multispecies complexes, microorganisms also interact; often forming cooperative communities with a shared metabolism (Shapiro, 1998). This includes cross feeding, where one organism utilizes the metabolic waste of another, or the co-degradation of complex molecules (Wolfaardt *et al.*, 1994). The proximity of organisms in biofilms also enables higher rates of gene transfer (Jefferson, 2004). Finally, the formation of biofilms enables bacteria to remain in a favourable environmental niche. For instance, in stream systems which flow continuously, microorganisms need to remain in the same place and avoid being washed away. In freshwater ecosystems most microorganisms live in biofilms, an alternative view is therefore to consider biofilms as the default mode of growth, which leads to the question of what triggers the planktonic mode rather than what motivates the biofilm mode of growth (Jefferson, 2004).

1.2.2 Biofilms and stream ecology

Benthic microbial populations within stream ecosystems are both numerically and metabolically dominant over the planktonic microbial populations (Edwards *et al.*, 1990; Fischer & Pusch, 2001; Geesey *et al.*, 1978). Organisms within the biofilm recycle organic detritus in the stream, use dissolved organic matter, fix nitrogen or recycle organic nitrogen, and fix energy and carbon by photosynthesis and chemosynthesis.

Within the stream food web, biofilms are intensively grazed by protozoa, invertebrates and fish (Lamberti, 1996; Parry, 2004). All invertebrate feeding groups consume biofilm in some form by either grazing the biofilm directly or filtering and collecting particles that have been dislodged from the biofilm. As biofilm composition is high in lipids and proteins compared to detritus (Lamberti, 1996), invertebrates feeding on decaying leaves may also obtain a significant proportion of nutritional components from the biofilm growing on the leaves. This

is supported by a study of Hall & Meyer (1998) showing that more than 20 % of the carbon in most invertebrate taxa is derived from bacteria, irrespective of functional feeding groups. In consequence, stream biofilms play a major role in the bottom up supply of nutrients to higher trophic levels of the food web.

Stream water quality is also regulated by biofilms. Constituent microbes can fix dissolved carbon, nitrogen and phosphorus, decreasing their concentrations in the water (Fischer, 2003) and can help to degrade toxic contaminants (Takada *et al.*, 1994). Biofilms also modify stream hydrodynamics and increase the deposition of suspended particles (Battin *et al.*, 2003a).

Although biofilms are sessile, their structure and composition are highly dynamic and reflect the complex interactions of environmental factors including temperature, light, shear forces, nutrients and contaminants (Gantzer *et al.*, 1991; Lawrence *et al.*, 2004; Lear *et al.*, 2008b). Changes in any environmental factor can affect the structure and composition of biofilms which may in turn alter the functions of biofilms and influence the whole stream ecosystem (Sheldon & Walker, 1997). As anthropogenic activities influence freshwater environments in multiple ways, they also affect biofilm structure and functions (Findlay & Sinsabaugh, 2006; Lear & Lewis, 2009) which may in turn result in the degradation of the health of the whole stream ecosystem.

1.2.3 Influence of metals on biofilm microbial communities

Numerous studies have documented the negative impacts of dissolved stormwater metal pollutants on freshwater fish and macro-invertebrates (e.g. Kiffney & Clements, 2003; Mance, 1987). In contrast, little is known about their effects on biofilm microbial communities (Serra *et al.*, 2009).

As biofilm microorganisms are more resistant to various stress factors than planktonic cells, they may be more resistant to high concentrations of trace metals. This was suggested by Teitzel & Parsek (2003) who demonstrated that a biofilm of *Pseudomonas aeruginosa* was 2 to 600 times more resistant to Zn, Cu and Pb than free living cells. Their capacity for adsorbing metals in the EPS and for precipitating metal salts outside the cells (see § 1.3.1.1)

may explain the tolerance of biofilms to high metal concentrations (Douglas & Beveridge, 1998; Holding *et al.*, 2003; van Hullebusch *et al.*, 2003).

Despite the increased tolerance of biofilm communities to metals, several studies have highlighted that depending on the concentration and the time of exposure, metal contaminants can modify the structure and the composition of embedded microbial communities. In their experiment, Admiraal *et al.* (1999) grew biofilms at three different sites within the same river, each experiencing different levels of metal pollution. By measuring the metabolic response of biofilm communities under metal exposure, they revealed that the biofilm coming from the most polluted site was less affected by high concentrations of zinc. This suggested an adaptation of algal and bacterial populations towards metal tolerant communities. Later, Mahmoud *et al.* (2005) compared the epilithic bacterial community from a zinc contaminated river to a community from a reference site. They observed similarities in abundance and activity but also differences in community composition, suggesting that a metal tolerant bacterial community had developed at the zinc contaminated site. Using Denaturing Gradient Gel Electrophoresis, Massieux *et al.* (2004) also revealed a change in bacterial community structure following exposure to copper.

Algae represent another important component of freshwater biofilms and have been subject to numerous studies because of the ease with which they can be observed. Gold *et al.* (2002) transferred biofilm from a reference site to a site polluted by heavy metals and observed a shift in diatom community structure and taxonomy. Soldo and Behra (2000) showed a shift in algal biofilm communities and an increase of their tolerance to metals after long-term exposure to high copper concentrations. Guasch *et al.* (2003) revealed the decrease of biofilm photosynthesis under zinc exposure.

As stream biofilms play a key role in the transfer of energy to organisms feeding on them and in the cycling of nutrients, shifts in microbial populations induced by increased metal concentrations could alter the nutritional function of biofilms and affect organisms at a higher trophic level. It is therefore important to develop a better understanding of the extent to which metals affect biofilm microbial communities.

1.3 Monitoring the impact of urbanisation on freshwater ecosystems

Despite the increasing importance and awareness of the impacts caused by humans on freshwater ecosystems, the effects of the various anthropogenic influences remain difficult to assess. The nature and the intensity of human disturbances are highly variable and often involve physical, chemical and biological components of the ecosystem. Modifications can occur in the hydrology and the morphology of the streams, changing stream habitats and flow regimes. Water temperature, dissolved oxygen, suspended sediments, nutrients and contaminants are also affected by urbanisation. To assess the extent to which aquatic ecosystems are impacted by urbanisation, various indicators can be used. A common approach involves the use of a suite of physico-chemical measurements which include dissolved oxygen, nitrogen and phosphorus, turbidity and pH (Tsegaye *et al.*, 2006). However, these environmental parameters can be highly variable in time and require repeated measurements. In contrast, some indicators are influenced by past and present conditions and therefore represent an integrative measurement of the human impact. In this project, the accumulation of metals in biofilm and sediments was used as an integrative indicator of stream contamination. Biofilm bacterial and protozoan communities were also used as integrative biological indicators of stream health.

1.3.1 Monitoring metal accumulation in streams

As described previously (see § 1.1.1), urbanisation has considerably increased metal concentrations in surface waters (Bernhardt & Palmer, 2007; Chalmers *et al.*, 2007; Ward & Trimble, 2003). In urban areas, metal concentrations frequently reach toxic levels and constitute a threat to aquatic ecosystems (Förstner & Wittmann, 1981; Kennedy, 1999; Mance, 1987; Rice, 1999). To assess whether heavy metals are present in the ecosystem at concentrations that are detrimental to aquatic biota, sediments are commonly analysed for metal concentrations (ANZECC & ARMCANZ, 2000; USEPA, 2005). Metal ions have a strong affinity with suspended particles present in the water such as silt or organic matter (Bibby & Webster-Brown, 2006). Depending on the characteristics of each element and on the amount and the nature of particles transported by the water, a significant proportion of the contaminants can be transported in association with particles. As sediments are formed by the particles settling out of the water column, they accumulate contaminants associated with the

particles and therefore constitute an integrative measurement of the contaminants present in the water.

Although biofilms are constantly growing to regenerate parts that have been grazed by organisms feeding on them or stripped off by the water flow, they are relatively sessile structures and have been suggested to be an alternative indicator of metal contamination (Mages *et al.*, 2004; Ramelow *et al.*, 1987). Biofilms retain suspended particles and accumulate particulate contaminants comparatively to sediments. However, concentrations of trace metals associated with freshwater biofilms and stream bed sediments can differ significantly (Farag *et al.*, 1998; Farag *et al.*, 2007; Holding *et al.*, 2003), revealing differences in the composition of biofilms and sediments and in their affinity for metal.

1.3.1.1 Accumulation of metals in biofilms

Biofilms present different adhesive properties that make them a good substrate for binding metals. The accumulation of metals in biofilms is probably due to the cumulative effects of those properties.

Microorganisms in biofilms are embedded in a matrix of extracellular polymeric substances (EPS) that they produce. Those substances are mainly exopolyssacharides associated with other molecules such as proteins, nucleic acids, lipids and humic substances (Flemming, 1995; Sutherland, 2001a). The composition of EPS is complex and may vary greatly depending on the bacterial strain and the environmental conditions (Sutherland, 2001a; van Hullebusch *et al.*, 2003) but typical constituents of EPS include ionisable functional groups such as carboxyl, phosphoryl, amino and hydroxyl groups that enable EPS to fix metals (Jang *et al.*, 2001; van Hullebusch *et al.*, 2003).

Inhabiting microbes themselves exhibit several characteristics that make them ideal agents for mineral binding. Their very high surface-volume ratio and the presence of charged chemical groups, such as carboxyl, phosphoryl and amino, on their cell surfaces provide a vast area with an overall anionic charge ideal for binding metals (Douglas & Beveridge, 1998). In addition, the binding of metals by bacterial cells can lead to the precipitation of insoluble metal rich phases closely associated with cell surfaces (Douglas & Beveridge,

1998; Wuertz *et al.*, 2000). To a lesser extent, metals may also enter the cell and form complexes or chelates in the bacterial cytoplasm (Sterritt & Lester, 1980; van Hullebusch *et al.*, 2003).

1.3.1.2 Ecological relevance of metal concentrations in biofilms

Within stream food webs, biofilms are intensively grazed by protozoa, benthic invertebrates and fish and may play a role in the accumulation of metals in all components of the food web. This is supported by the strong correlation between metal concentrations (especially zinc, copper, lead) found in biofilms and invertebrates (Farag *et al.*, 2007; Rhea *et al.*, 2006) that suggests a direct transfer of metals from biofilms throughout the food chain. The accumulation of metals in benthic macroinvertebrates also depends on functional feeding groups. Shredders-scrapers feeding directly on biofilm accumulate the largest concentrations of metals (Farag *et al.*, 1998) which confirms the transfer of metals from biofilms to the organisms feeding on them. At a higher trophic level, a study by Patrick and Loutit (1978) revealed an increase of metal concentrations in fish feeding on worms that had been fed on metal-enriched bacteria.

As aquatic organisms are affected by metal concentrations in both their surrounding water and their food (Clearwater *et al.*, 2002; Courtney & Clements, 2002), the binding and the accumulation of trace metals in biofilms may form a critical link in the movement of metals from abiotic to biotic components in the stream. Analyses of metals associated with biofilms may therefore constitute a more relevant indicator of the potential threat to aquatic organisms than analyses of metal associated with sediments.

1.3.2 Bio-indicators of stream health

The health of stream ecosystems can be assessed by the quality of the water and the types of habitats available for aquatic organisms. However, all physical and chemical measurements such as flow rate, temperature, dissolved oxygen and turbidity can vary greatly in time and a detailed assessment would require a large number of repeated measurements. Therefore, biological indicators are generally preferred as they are influenced by past and present

condition and provide an integrative measure of stream health (e.g. Kelly *et al.*, 2008; Victor & Dickson, 1985).

1.3.2.1 Current bio-indicators of stream health

All types of organisms are affected by their environment and can respond to various physical, chemical and biological stresses. Therefore most major groups of organisms known to inhabit freshwater ecosystems have been investigated as biological indicators.

Communities of macro-invertebrates are the most commonly used biological indicators of stream health (Boothroyd & Stark, 2000) and their response to environmental changes has been well described (Rosenberg & Resh, 1993; Roy *et al.*, 2003). Other organisms such as fish (Walters *et al.*, 2009), macrophytes (Thiebaut *et al.*, 2002), algae (Dokulil *et al.*, 2003) and more specifically diatoms (Wu, 1999) are also frequently used as indicators of stream environmental conditions.

In contrast, few studies have examined the use of microbial communities as indicators of health of aquatic ecosystems. Although extensive research has focused on monitoring specific groups of indicator microbes (such as faecal coliforms), these studies are specifically conducted to assess the presence of pathogenic bacteria and the implications for human health. Microbial diversity studies were limited in the past by the lack of methodological tools but recent advances in molecular microbiology now enable a detailed description of microbial communities. Fingerprinting techniques such as Automated Ribosomal Intergenic Spacer Analysis (ARISA), Terminal Restriction Fragment Length Polymorphism (T-RFLP) and Denaturing Gradient Gel Electrophoresis (DGGE) are rapid, cost effective and can be used to screen a large number of samples to monitor changes in community structure.

The use of biofilm microbial populations as a bio-indicator has the advantage of being widely applicable because of the ubiquity of biofilms in freshwater environments. Furthermore, microorganisms living in association with the biofilm represent some of the most diverse and abundant types of organisms in terms of species (Fierer & Jackson, 2006; Heywood & Watson, 1995) and genomic diversity (Torsvik *et al.*, 2002; Venter *et al.*, 2004). Microbes also respond very rapidly to changes in their environment due to rapid population dynamics (Paerl & Pinckney, 1996).

Two types of microorganisms will be investigated in the present research project: (i) bacteria that are crucial for the development and the structure of biofilms and play key roles in the cycling of nutrient and the degradation of contaminants and (ii) protozoa that feed on biofilms and have an essential role in nutrients cycling and energy transfer throughout the ecosystem.

The use of bacterial communities as indicators of stream health has received very little attention (Lear *et al.*, 2009) probably because of their small size and the absence of distinguishing phenotypic characters. Protozoa in contrast have very diverse phenotypes and have been used as indicators of the efficiency of a wastewater treatment system (Luna-Pabello *et al.*, 1990) or as a biotic index to assess freshwater pollution (Foissner, 1999; Jiang, 2006).

1.3.2.2 Molecular methods to monitor changes in microbial populations

Although microorganisms can have distinguishing features such as cell shape, size, presence or absence of organelles, flagella and cilia, their identification by microscopy is not always possible and requires a high level of knowledge. However, recent developments in molecular based techniques have enabled the detailed evaluation of the structure and composition of microbial communities (Theron & Cloete, 2008). Most of these techniques involve an initial amplification by Polymerase Chain Reaction (PCR) of a target DNA sequence (Dorigo *et al.*, 2005). The variations in the sequence are then detected either by sequencing or by electrophoresis analysis. The most common techniques are presented here with particular emphasis on RFLP and T-RFLP, RISA and ARISA and DNA sequencing of clone libraries.

Initial amplification by PCR

The polymerase chain reaction is a process in which a selected sequence is amplified to produce large quantities of DNA fragments. The reaction takes place in a three step process repeated several times to form an exponential number of copies of the target sequence (Figure 1.2). During the first step called “denaturation”, the heat separates the 2 strands of DNA. The temperature is then lowered and the primers (oligonucleotides used as starting points for

DNA synthesis) can anneal on both sides of the targeted sequence. Finally the DNA polymerase extends the primers and synthesizes copies of the target DNA sequence (McPherson & Moller, 2000; Mullis *et al.*, 1994).

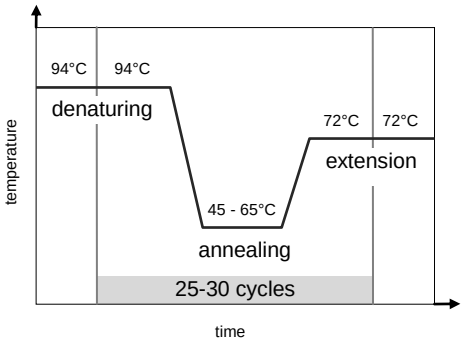

Figure 1.2: Polymerase Chain Reaction (PCR). The reaction includes three main temperature controlled steps repeated over time: denaturing, annealing and extension.

Replicated sequences act as new templates during the following cycles and therefore the number of sequences increases exponentially. Oligonucleotides used as primers in the reaction can be designed to target DNA sequences present only in specific taxa. Primers can in theory target any classification level so that a PCR can be used to detect a specific species or target the complete community of a type of organism such as bacteria (Blackwood *et al.*, 2005).

RISA and ARISA

Ribosomal Intergenic Spacer Analysis (RISA) is based on the amplification by PCR of the intergenic spacer located between the 16S and 23R rRNA genes of the bacterial genome. The length of the intergenic region is highly variable among bacterial species (Fisher & Triplett, 1999; Ranjard *et al.*, 2001) and therefore the PCR results in a mixture of DNA fragments of different length. Fragments can then be separated by electrophoresis to produce a community profile where each band corresponds in theory to a single taxa. In the automated version (ARISA), fluorescently labelled primers are used during PCR amplification. The fragments are then separated by automated capillary electrophoresis and the fluorescence monitored. This results in a profile of the bacterial community where each peak of fluorescence corresponds to a single species and the intensity to the relative representation of the species in

the community. Although different intergenic spacer lengths are assigned to different species, it is known that multiple species can produce bands of identical lengths or that some bacteria can have intergenic spacer regions of different length (Fisher & Triplett, 1999). Nevertheless, ARISA is a quick and reproducible technique to effectively differentiate bacterial communities in terms of their structure (Fisher & Triplett, 1999; Ranjard *et al.*, 2001). ARISA has been extensively used to describe bacterial communities and has been adapted for the analysis of fungal communities (Ranjard *et al.*, 2001). However, it is not commonly used for eukaryotes due to the highly variable size of the spacer even within species (Moos & Stefanovsky, 1995).

RFLP and T-RFLP

Both Restriction Fragment Length Polymorphism (RFLP) and Terminal Restriction Fragment Length Polymorphism (T-RFLP) involve an initial PCR targeting one of the rRNA genes. The genes coding for ribosomal RNA are more conserved than intergenic spacers and their sequence varies only slightly in length. Therefore, the PCR product is cut at specific recognition sites by restriction endonucleases to enable the differentiation of the species based on the sequence of the DNA. For the RFLP technique, the resulting fragments are then separated by agarose gel electrophoresis, stained and visualised. RFLP patterns obtained from complex environmental samples generally exhibit a great number of bands that reflect the genetic diversity present in the sample and can be compared between samples. RFLP analysis of ribosomal DNA sequences is also called ARDRA (Amplified Ribosomal DNA Restriction Analysis). An automated method named Terminal Restriction Fragment Length Polymorphism (T-RFLP) has also been developed in which one or both PCR primers are fluorescently labelled (Liu *et al.*, 1997). The digestion of the sequences produces a mixture of fluorescently labelled terminal fragments of different length that can be separated by automated capillary electrophoresis. This results in a community profile where peaks of fluorescence correspond to different species and the intensity of the peaks to the relative representation of the species in the community. Recognition site positions are often conserved between species and one or more species may produce fragments of identical length (Theron & Cloete, 2008). However, these techniques have been used efficiently to compare microbial communities (Dorigo *et al.*, 2005; Liu *et al.*, 1997; Marsh, 1999).

Ribosomal RNA gene sequencing

The sequencing of rRNA genes enables the identification of organisms by assessing the genetic similarity between species. In a first step, the gene sequences from the total community DNA are amplified by PCR using specific primers. To isolate the different sequences and enable their sequencing, the PCR product is inserted into bacterial cells and cloned. This results in a library of bacterial colonies, each containing a single sequence of the targeted rRNA gene. A second PCR then amplifies the isolated sequences to produce a suitable amount of DNA for sequencing. The sequences obtained can be compared to genomic databases (e.g. the National Center for Biotechnology Information [NCBI] database) and the species identified by similarity. To reduce the number of fragments to analyse by sequencing, an RFLP analysis is sometimes used prior to sequencing. Only one copy of each different RFLP pattern is then sequenced. The sequencing of rRNA genes has been extensively used to describe communities of both bacteria using the 16S rRNA gene (e.g. Eiler & Bertilsson, 2004; Glockner *et al.*, 2000) and eukaryotes using the 18S rRNA gene (e.g. Diez *et al.*, 2001; Lopez-Garcia *et al.*, 2001). Unlike (A)RISA and (T)RFLP, this approach provides an insight into the species composition of communities. However, the construction of clone libraries is both time-consuming and costly which prevent their use for the analysis of large numbers of samples.

Other methods

Other molecular methods can be used to assess the composition or the structure of microbial communities. Denaturing Gradient Gel Electrophoresis (DGGE) is based on the separation of rRNA gene amplicons by a chemical denaturing gradient that separates products based on the differences in nucleotide content (Muyzer & Smalla, 1998). Double stranded DNA fragments denature at different points on the gel depending on their A-T and G-C content resulting in a profile of bands representing different species. Temperature Gradient Gel Electrophoresis (TGGE) is a variant based on a gradient of temperature to denature DNA fragments (Muyzer & Smalla, 1998). Finally, in the Single Strand Conformation Polymorphism (SSCP) analysis, fragments are separated by electrophoresis depending on the three-dimensional conformation of their single stranded DNA (Lee *et al.*, 1996).

1.4 Research aims and objectives

This project aimed to both improve our understanding of the impact of urban runoff on stream ecosystems and develop new techniques to assess this impact more accurately. Although extensive research on the effect of urbanisation has focused on fish or macro-invertebrates, very little is yet known about the impact on freshwater microorganisms. The main focus of this project was to investigate the effect of urbanisation on stream biofilms and assess their potential uses as indicators of the influences of urban developments on freshwater ecosystems.

Stream biofilms play key roles in stream ecosystems and are grazed by many other organisms. As a result, the potential impact of urbanisation on biofilm microbial communities could alter the functions of biofilms and affect the whole stream ecosystem. To improve our understanding of the complex effects of anthropogenic activities on freshwater ecosystems, it is crucial to assess their influence on microbial communities and the potential implications for organisms at a higher trophic level. Therefore, the first objective of this project was to investigate how urban runoff contaminants affect biofilm microbial communities. The effect of common storwater contaminants (Zn, Cu & Pb) on bacterial and protozoan communities was first investigated in controlled environments. This allowed a better control of environmental factors such as flow rate and incident light and provided a higher level of replication. The impact of actual urban runoff was then investigated in controlled environments and natural streams.

Organisms embedded in biofilm constitute potential integrative ecological indicators of stream health and recent developments in molecular biology have enabled their rapid monitoring. The second objective of this project was therefore to investigate if biofilm microbial communities could indeed provide a reliable indication of stream health. Several molecular techniques were tested and optimised and the sensitivity of microorganisms to urban contaminants was assessed. Biofilm bacterial and ciliate communities were used as bio-indicators and compared with macro-invertebrates to investigate the changes occurring along an urbanisation gradient. These novel indicators were then implemented to assess the efficacy of a stormwater treatment system.

In addition to their potential effect on microbial communities, urban runoff contaminants could also accumulate in biofilms. As biofilms are intensively grazed by many organisms, biofilms may form a critical link in the movement of urban contaminants from abiotic to biotic components of the stream. The third objective of this project was to investigate the accumulation of metals in biofilm and the potential implications for organisms grazing on them.

To assess whether heavy metals are present in the ecosystem at concentrations that are detrimental to aquatic biota, metals associated with sediments are commonly analysed. However, if biofilms accumulate contaminants and transfer them to higher trophic levels, the contaminants associated with biofilms may constitute a more relevant indicator of ecological risk. Therefore, the fourth and last objective of this project was to investigate the use of biofilm associated metals as ecological indicators of stream metal contamination. The relevance of both sediment and biofilm associated metals was assessed and compared. Biofilm associated metals were then used to investigate metal contamination in streams progressively impacted by urbanisation and to assess the efficacy of a stormwater treatment system in removing heavy metals.

In order to reach these objectives, the research project included the following steps:

- The sensitivity of microbial communities to urban runoff contaminants and the suitability of molecular techniques to reveal the changes occurring in the community structure were assessed. Zinc, copper and lead were used as model contaminants and their effect on microbial communities was investigated in a controlled environment (chapter 3).

- Simultaneously, the dynamics of absorption and desorption of metal contaminants (zinc, copper and lead) in biofilm were monitored (chapter 3).

- The ecological relevance of metal concentrations present in biofilms was investigated using both microbial communities (chapter 4) and macro-invertebrates (chapter 7) as bio-indicators.

- To investigate whether urban runoff permanently affect microbial communities in receiving ecosystems, biofilm was collected from streams variously impacted by anthropogenic activities and the tolerance of embedded bacterial communities to zinc, copper and lead was investigated (chapter 5).

- The effect of urban runoff on aquatic biota results from the combined action of numerous contaminants. To extend the conclusions of chapters 3 and 5 which were based on the model contaminants (Zn, Cu and Pb), we assessed how actual urban runoff collected from different urban areas affected biofilm microbial communities (chapter 6).

- In order to compare the newly developed indicators of urban impact on stream ecosystems with other indicators available, the changes occurring in streams progressively impacted by urbanisation were monitored using traditional (i.e. macro-invertebrate communities and sediment associated metals) and newly developed indicators (i.e. biofilm bacterial communities, ciliate communities and biofilm associated metals) (chapter 7).

- Finally, the new indicators developed were implemented to assess the efficiency of a stormwater treatment train where traditional indicators are not available (chapter 8). This practical application demonstrates actual opportunities for the use of biofilm as indicators of urban impact on freshwater ecosystems.

By providing an insight into how urban runoff affects aquatic microorganisms this study improves our understanding of the general impact of anthropogenic activities on freshwater ecosystems. Innovative tools developed throughout the project will facilitate the assessment of urban influences on aquatic environments and assist land planners in their attempts to restore urban streams.

CHAPTER 2

2 Materials and methods

This research project consisted of several related experiments which aimed to understand how urban runoff affects aquatic microbial communities and to investigate the potential uses of stream biofilm as an indicator of the impact of urbanisation on freshwater ecosystems. Experiments were conducted in both flow chamber microcosms and in natural streams. Although all experiments followed different protocols to reach their specific aims, some techniques were used repetitively in several experiments. In this chapter, the different experimental systems used throughout the project and the main techniques used to analyse the samples are presented. Further details about each specific experimental design are given in the following chapters.

2.1 Experimental systems

Natural biofilms are generally complex associations of various species and types of organisms. Stream biofilms represent one of the most diverse forms of biofilms as they include numerous species of several types of organisms, including bacteria, algae and fungi. To investigate ecological processes occurring in biofilms, most research has so far looked at simplified biofilm systems comprised of one or a small number of species (O'Toole *et al.*, 2000). In this project however, experiments were always carried out on complex biofilm communities directly obtained from natural streams or grown from the communities present in stream water.

Bacterial communities in stream biofilms are responsive to a wide range of environmental parameters (Besemer *et al.*, 2007; Lear *et al.*, 2008a; Lear & Lewis, 2009). In natural stream systems, factors such as flow rate, incident light and substrate type can vary across very small scales, providing a large diversity of microhabitats (Soininen, 2005). This can influence microbial communities and blur the results. Therefore, to allow a better control of environmental variables and provide a higher level of replication, several experiments were conducted in flow chamber microcosms, mainly in the first part of the project. Subsequent experiments were then carried out in actual streams to test the initial conclusions in the natural environment.

2.1.1 Flow chamber microcosms

Three types of flow chamber were used in the project.

The high capacity flow chamber system was filled with a relatively large volume of water (~400 litres) and included three parallel flow channels (200 cm [l] x 18 cm [w] x 11 cm [h]) in which a stream flow was simulated (Figure 2.1 & Figure 2.2). The water was collected in a large reservoir and circulated by a single water pump (Dynapond 7000, Davey Inc.). Because the water from all three channels was collected into the same tank and circulated by a single pump, this system did not allow the application of different chemical treatments. This system was used for growing homogeneous biofilm and investigating the recovery of bacterial populations after exposure to water contaminants. Further details about the use of the flow chambers will be given in the subsequent chapters.

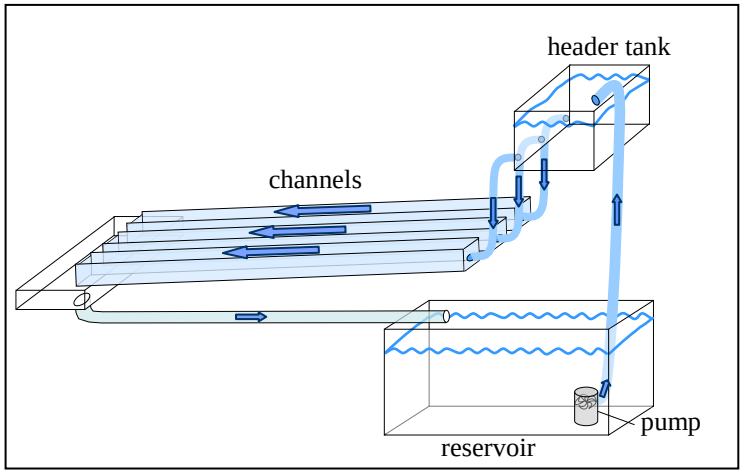

Figure 2.1: High capacity flow chamber. The system included three parallel channels in which a stream flow was simulated. The water was collected into a large reservoir and circulated through a header tank by a single water pump. The total volume of water in the system is approximately 400 litres. Arrows show the direction of the water flow.

Figure 2.2: High capacity flow chamber, showing channels and header tank.

To apply different chemical treatments, two systems of 10 separate low capacity flow chambers were used. In the first system, each flow chamber was filled with 17 litres of water and consisted of two interconnected tanks (Figure 2.3 & Figure 2.4). One tank (reservoir) contained a small pump (Watermaster pro, White International) that circulated the water through the system while the other (test tank) included an arrangement of grids and walls creating an homogeneous flow over a raised platform (21 cm x 14 cm). That area could hold three glass slides (6.5 cm x 14 cm) used as a substrate to grow biofilm.

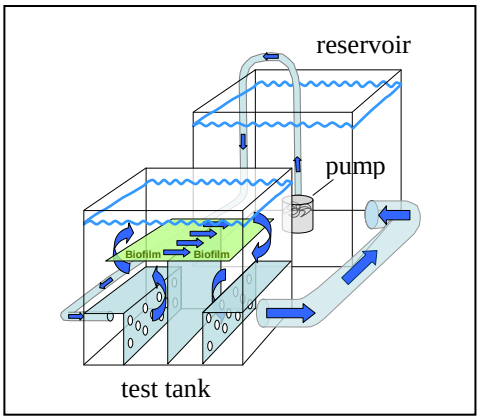

Figure 2.3: Low capacity flow chamber, first setup. Arrows show the direction of the water flow.

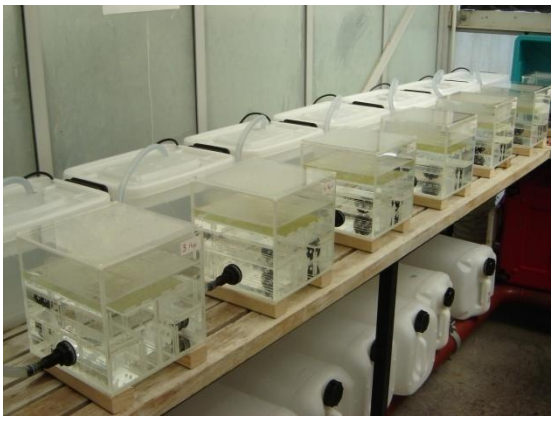

Figure 2.4: Low capacity flow chambers, first setup, in operation.

A second system of 10 separate low capacity flow chambers consisted of a flow channel (150 cm [l] x 10 cm [w] x 7 cm [h]) and a single tank hosting a small water pump (Watermaster Pro, White International) (Figure 2.5 and Figure 2.6). Each chamber contained 18 litres of water. This setup provided the advantage of fitting 6 slides per chamber instead of 3 in the previous system. The channels could also host rocks that were directly collected from a stream, allowing the analysis of biofilm communities established on natural stream substrate.

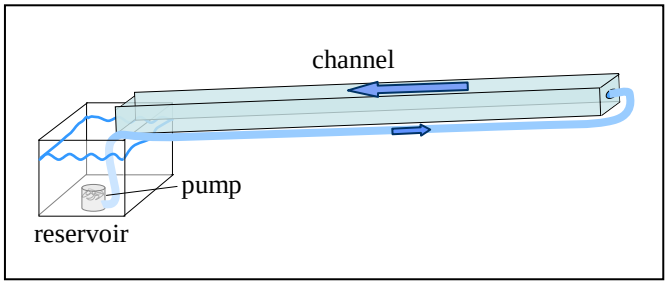

Figure 2.5: Low capacity flow chamber, second setup. Arrows indicate the direction of the water flow.

Figure 2.6: Low capacity flow chambers, second setup containing natural substrates.

Between successive experiments, to ensure no transfer of contaminants or bacteria, the flow chambers were washed twice, first with liquid detergent then with sodium hypochlorite (5%), rinsed twice and dried. Glass slides used as substrate to grow biofilm were also washed twice, soaked in 5M HNO_3 for 24 h, rinsed and dried.

2.1.2 Natural stream sites

Although flow chambers were initially used to enable a better control of environmental variables, further experiments conducted in natural streams were needed to investigate the actual potential of biofilm as an indicator of urban impacts on stream health. A comparative study required multiple stream sites draining catchments variously impacted by urbanisation. Auckland Region was selected for its convenience as it includes numerous low impacted streams with catchments essentially dominated by forests or farmland as well as more impacted streams with different degrees of catchment urbanisation. More than 1 400 000 people live in the Auckland Region, most of them in the urbanised areas that covers approximately 11 % of the total surface (Auckland Regional Council monitoring programme 2009, http://monitorauckland.arc.govt.nz). This high density of population causes a great impact on urban streams. In contrast, rural areas have a low population density and streams are only slightly impacted by urbanisation. In total 28 different stream sites covering all types of catchments were sampled throughout the project (Figure 2.7). Further details about the sites are presented in the relevant following chapters.

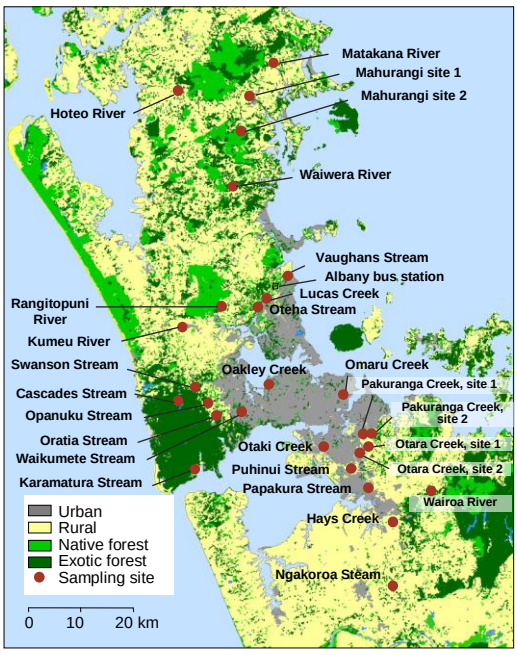

Figure 2.7: Map of the land cover in the Auckland Region and locality of sampling sites used in the project. Land cover data from NZ Ministry for the Environment, Land cover database version 2, 2004. Map produced with ArcExplorer™, ESRI Inc.

2.2 Collection and handling of samples

The sampling process differed between experiments conducted in flow chamber microcosms or in actual stream sites. In flow chamber microcosms the process involved mainly collecting biofilm for molecular and metal analysis. Additional physico-chemical analyses such as pH, light and temperature were sometimes performed to confirm the homogeneity of environmental parameters in the different chambers. Water was also collected and analysed in some experiments to monitor variations in metal concentrations. In contrast, the sampling process in stream sites was usually more complex and could involve the collection of water, sediments and macro-invertebrates in addition to biofilm. Environmental parameters at each site were also monitored including light, temperature, dissolved oxygen, pH, flow rate, depth and width of the stream.

2.2.1 Biofilm sampling

For molecular analysis, biofilm samples were scraped off glass slides or individual rocks using Speci-Sponges™ (Nasco, U.S.A.). Speci-sponges™ are dry sponges exempt from any microbial DNA designed to absorb the suspension produced when rubbing wet surfaces. Sample substrates (e.g. glass slides) were carefully removed from the water before being rubbed vigorously with a Specie-Sponge. The sponges were placed into individual Whirl-Pak™ bags (Nasco, U.S.A.), which were sealed and transported to the laboratory in darkness on ice (when sampled from natural stream sites).

Sampling of biofilm for metal analysis depended on the substrate sampled. From sandblasted glass slides, the biofilm was collected by scraping the slide with a sterile plastic stick (SteriClin, BioLab, New Zealand). Biofilm samples were transferred to separate 50 ml Teflon® beakers. Beakers were previously washed twice and soaked in 5M HNO_3 for 24 h to remove any residual metals, then dried and weighed (± 0.05 mg). When the biofilm was collected directly from rocks, Speci-sponges™ were used for metal analysis in a similar manner as for molecular analysis. To increase the biomass obtained and improve the precision of metal analysis, multiple sponges were often obtained from the same stream location. Speci-sponges™ are not designed for metal analysis, but clean sponges were processed in parallel and confirmed their negligible influence on metal concentrations (< 0.055 µg As, 0.0026 µg Cd, 0.00164 µg Cr, 0.0076 µg Cu, 0.00036 µg Pb, 0.0025 µg Ni and 0.0019 µg Zn yielded per clean sponge processed).

2.2.2 Sediments and water sampling

Sediment samples were obtained by scooping approximately 30 cm^3 of the top two centimetres of stream bed sediments into a 50 ml Falcon™ tube (BD Biosciences, USA). Sediments were stored in the tubes, transported to the laboratory on ice and kept at 4°C until drying prior to the digestion and the analysis of metal concentrations.
Water was either (i) collected in 50 ml Falcon tubes when sampled for analysis of total metal concentration or (ii) filtered on site through a 0.45 µm membrane filter (Millipore, USA) and stored in 50 ml Falcon tubes when sampled for analysis of dissolved metal concentrations. Four drops of 35 % UltraPure HNO_3 (Merck Inc.) were directly added to the Falcon tubes, to prevent metals from binding to the walls of the tubes.

2.2.3 Macro-invertebrate sampling

Benthic macroinvertebrates were sampled with a triangular net of 0.5 mm mesh size (provided by D. Jenkinson, University of Auckland) using a standard procedure (Stark *et al.*, 2001). At each site, the sampling was performed in a 30 m reach beginning at the downstream end. The substrate sampled was chosen among rocks, gravel, roots, branches, leafs or detritus in proportion to their relative presence at the site. Attention was also given to cover a variety of velocity regimes. The net was placed directly downstream of the substrate and an area of approximately 0.1 m^2 was disturbed by hand to dislodge all the invertebrates. This process was repeated 10 times to sample a total area of approximately 1 m^2 per site. The material collected in the net was carefully transferred to a quick seal plastic bag and submerged with 70 % ethanol to preserve the invertebrates until sorting and identification.

2.2.4 Monitoring of environmental variables

The light was measured using a photometer (Li185B, LiCor Inc.) above the water in various locations across the stream and/or in the water above each rock sampled for biofilm analysis. Temperature, pH, dissolved oxygen and conductivity were monitored at approximately 10 cm below the water level with a multimeter (Multi350i, WTW, Germany). Flow rate and depth were monitored at various locations across the stream and/or above each rock sampled for biofilm analysis using a flow meter (FP101, Total Lab Systems Ltd., New Zealand).

2.3 Analysis of metal concentrations

Metal concentrations were analysed in biofilm, sediment, invertebrate and water samples. Initially only zinc, copper and lead were analysed and the whole process including drying, digestion and analysis by Flame or Graphite Furnace Atomic Adsorption Spectrophotometry was performed at the University of Auckland. Later in the project, samples were dried and if required sieved at the University of Auckland but digested and analysed by Hill Laboratories (New-Zealand) to allow the assessment of a wider range of metals (As, Cd, Cr, Cu, Pb, Ni and Zn) by Inductively Coupled Plasma Mass Spectrometry.

2.3.1 Sample preparation

Biofilm

The preparation of biofilm samples depended on the substrate sampled. When the biofilm was collected from glass slides and transferred to Teflon beakers, the beakers were simply dried at 55 °C until no change in weight could be detected within 12 hours. When the biofilm was collected from rocks, it needed first to be extracted from the Speci-Sponges™. To do so, each sponge was transferred to a stomacher bag (Seward, UK) containing 30 ml of deionised and microfiltered Milli-Q® water (Millipore, USA). If multiple sponges had been obtained from the same stream location and needed to be pooled together, sponges were transferred to a single stomacher bag containing 20 ml of water per sponge. The bag was macerated for 120 s at maximum speed with a laboratory stomacher (Stomacher 400, Seward, UK) and sponges were squeezed to remove the biofilm material. The suspension was then filtered through a 250 µm nylon mesh. The biofilm suspension obtained was dried a 55°C until no change in weight could be detected within 12 hours.

Sediments

Sediment samples were dried at 55°C for 12 to 24 hours. Samples were then crushed gently in a mortar and sieved through a 250 µm mesh. Sediments were then further dried at 55°C until no change in weight could be detected within 12 hours.

Invertebrates

Invertebrates were quickly drained on a paper towel and frozen at -80°C. Samples were freeze dried (BTK, VirTis, USA) until all the water had evaporated and crushed gently in a

mortar. Samples were then dried at 55°C until no change in weight could be detected within 12 hours.

Water

Water samples were acidified and if required filtered directly during the sampling process (see § 2.2.2). No further preparation was needed.

2.3.2 Sample digestion for metal analysis

Biofilm and sediment samples processed at the University of Auckland were digested by hot nitric acid digestion with a modified method of Hseu (2004). Briefly a solution (10 ml) of 69% trace pure HNO_3 (Merck Ltd, New Zealand) was added to each dry biofilm or sediment sample and boiled for 30 min whilst covered. Lids were then removed and the samples kept boiling until only 2 ml remained. A solution of 0.2 M HNO_3 (10 ml) was added to each sample and again boiled until only 2 ml remained. Samples were then diluted in 0.2 M HNO_3 to a final volume of 10 ml and stored at 4°C until analysis. Three certified reference materials (tomato leaves, 1573a (NIST, USA) and lake sediments, lksd1 & lksd2 (NRC, Canada)) were similarly processed to test the efficiency of the extraction process.

Biofilm, sediment and invertebrate samples analysed by Hill Laboratories (Hamilton, New Zealand), were digested by nitric/hydrochloric acid following US EPA 200.2 protocol.

No digestion was required before the analysis of dissolved metal concentrations in water. However, to analyse the total concentration of recoverable metals, water samples were extracted with nitric/hydrochloric acid at 85°C for 2 h 45 min by Hill Laboratories following US EPA 1638 protocol.

2.3.3 Metal analysis

At the University of Auckland, concentrations of zinc, copper and lead in water and in the product of biofilm digestions were assessed using Flame Atomic Adsorption Spectrophotometry (Model 933 AAS, GBC Scientific Equipment Ltd., Australia) or Graphite Furnace Atomic Adsorption Spectrophotometry (Model 3000, GBS Scientific Equipment Ltd., Australia) when metal concentrations were below detection using the former method.

The three certified reference materials confirmed the efficiency of the extraction process (> 85% of recovery).

At Hill laboratories, concentrations of arsenic, chromium, cadmium, copper, nickel, lead and zinc were all analysed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (for more details about ICP-MS refer to Nelms, S. [2004] Inductively Coupled Plasma Mass Spectrometry Handbook, Oxford, Blackwell).

2.4 Molecular analysis of microbial community structure and composition

Microbial community structure and composition were analysed on biofilm samples from flow chambers and natural streams. Depending on the experiments, bacteria and ciliate protozoa were investigated by ARISA, RFLP, T-RFLP or ribosomal RNA gene sequencing.

2.4.1 Sample preparation and DNA extraction

All biofilm samples collected for molecular analysis were obtained using Speci-Sponges™. Each sponge was transferred to a separate stomacher bag (Seward, UK) containing 30 ml sterile water and macerated for 120 s at maximum speed with a laboratory stomacher (Stomacher 400, Seward, UK). Sponges were then squeezed to remove the material and this biofilm suspension centrifuged (8000 x g, 20 min) in 50 ml centrifuge tubes. Each pellet was transferred to a separate 1.5 ml microcentrifuge tube and stored at -80°C until analysis.

DNA extraction

Biofilm samples contain a mixture of materials which must be separated from the DNA before amplification by PCR. The DNA from all biofilm samples was extracted using a method adapted from Miller *et al.* (1999) which combines bead-beating, sodium dodecyl sulphate (SDS) lysis, chloroform extraction, ammonium acetate precipitation and finally DNA precipitation with isopropanol.

First, 300 µl of phosphate buffer and 330 µl of SDS lysis buffer were added to each tube containing the pelleted biofilm (for buffer composition, see Table 2.1). The mixture was transferred to a 2 ml bead-beating vial (Global Science & Technology, New Zealand) containing 0.5 g of 0.1 mm zirconia/silica beads and 0.5 g of 2.3 mm zirconia/silica beads (BioSpec, Inc.). Chloroform stabilized with isoamyl alcohol (24:1) (300 µl) was added before the tube was shaken (FastPrep FP120, Bio101Savant, Global Science, New Zealand) at 4 m s^{-1} for 80 seconds. Samples were incubated at room temperature for 2 minutes, then centrifuged (13 400 x g) for 5 minutes to pellet debris. The supernatant (650 µl) was transferred to a new 1.5 ml microcentrifige tube and 360 µl of 7M ammonium acetate (Merck, Inc.) were added. Tubes were shaken by hand for 15 seconds and centrifuged at 13 400 x g for 5 minutes. This resulted in a separation of two phases. The clear upper phase (580 µl) was transferred to a new 1.5 ml microcentrifuge tube and 315 µl of ice cold isopropanol were added. Tubes were briefly mixed by hand and incubated at 4°C for at least 3 hours to help the DNA precipitate. After incubation, the tubes were centrifuged (15 700 x g) for 10 minutes at 8°C in a temperature controlled centrifuge. The supernatant was discarded, the pellet of DNA (most of the time invisible to the naked eye) was washed with 1 ml of 70% ethanol (Merck, Inc.) and the sample centrifuged again at 15 700 x g for 5 minutes. The supernatant was removed and the pellet dried in a vacuum desiccator. When dry, the DNA was resuspended in 50 µl of UltraPure™ water (Invitrogen, USA).

Table 2.1: Composition of phosphate and SDS lysis buffers used in the DNA extraction process.

	Constituent	Quantity	Supplier
Phosphate buffer	$NaHPO_4$ 1 M	93.2 ml	BDH, UK
	NaH_2PO_4 1 M	6.8 ml	BDH, UK
	Made up with water to a volume of 1 l		
SDS lysis buffer	SDS	3 g	BDH, UK
	10% NaCl 1 M,	6 ml	BDH, UK
	Tris 0.5M (pH 8)	15 ml	Invitrogen, USA
	Made up with water to a final volume of 30 ml		

2.4.2 General PCR method and electrophoresis of PCR product

All PCRs were carried out in a total volume of 50 µl using GoTaq® Green Master Mix (Promega, USA) (Table 2.2). This master mix contains Taq DNA Polymerase, dNTPs, $MgCl_2$ and reaction buffers at optimal concentrations. All primers were ordered from Invitrogen (New Zealand). Bovine serum albumin (BSA) (Invitrogen, USA) was added to all reactions. All PCRs were carried out with an Eppendorf (Inc.) Mastercycler® thermal cycler. The sequence of the primers, the temperatures used and the number of cycles varied and are detailed in the subsequent sections.

Table 2.2: Composition of PCR mixes

Constituent	Volume
GoTaq® Green Master Mix	25 µl
Forward primer (10 µM)	2.5 µl
Reverse primer (10 µM)	2.5 µl
BSA 10%	2 µl
Sample (DNA template)	2 µl
H_2O	16 µl
Total volume	50 µl

The success of PCR amplifications was assessed by electrophoresis of the PCR product on agarose gels. Gels were made from 1 % ultrapure agarose (Invitrogen, USA) and 1 x Tris/Borate/EDTA (TBE) buffer (see Table 2.3). PCR product was loaded directly onto the gel as Go Taq® master mix contains a loading dye. A ladder (1 kb+, Invitrogen, USA) was used for size determination. The gel was electrophoresed for approximately 30 minutes at 90 Volts. Visualisation was achieved through staining of gels in a solution of ethidium bromide (1 mg l^{-1}) for 15 minutes. Stained gels were rinsed with running water for 1 minute and viewed under UV light in a Gel Doc Molecular Imager (Bio-Rad, USA).

Table 2.3: TBE buffer composition for agarose and polyacrylamide gels

	Constituent	Quantity	Supplier
10 x TBE buffer	Tris base	108 g	Invitrogen, USA
	Boric acid	55 g	Merck, Inc.
	EDTA 0.5M (pH 8)	40 ml	BDH, UK
	Made up with water to a final volume of 1 l		

2.4.3 Automated Ribosomal Intergenic Spacer Analysis

Changes in bacterial community structure were assessed by Automated Ribosomal Intergenic Spacer Analysis (ARISA). This technique exploits the length polymorphism of the DNA sequence located between 16S and 23S rRNA genes. By replicating the sequence and analysing the length of the fragments, ARISA provides a detailed profile of the community structure (see § 1.3.2.2 for more details).

The 16S-23S intergenic spacer region of the bacterial rRNA gene was amplified by PCR (95 ^{o}C for 5 min; 30 cycles of 95 ^{o}C for 30 s, 61.5 ^{o}C for 30 s, 72 ^{o}C for 90 s, and then 72 ^{o}C for 10 min) using the primers SDBact (5' TGCGGCTGGATCCCCTCCTT 3') and LDBact (5' CCGGGTTTCCCCATTCGG 3') (Ranjard *et al.*, 2001). The primer SDBact was labelled at the 5' end with a HEX (6-carboxyhexafluorescein) fluorochrome (Molecular Probes, Invitrogen, New Zealand) to enable the detection by an automated analyser. PCR products were purified (ZR-96 DNA Clean & Concentrator™ Kit, Zymo Research, USA) following the manufacturer's protocol, quantified using a NanoDrop™ spectrophotometer (ND8000, Thermo Scientific, USA) and subsequently standardised to provide 30 ng of DNA per sample. The purified product was then run on a 3130XL Capillary Genetic Analyser (Applied Biosystems, New Zealand) together with a DNA size standard ladder LIZ1200 (Applied Biosystems, New Zealand) using a 50 cm capillary and standard GeneMapper® (v 3.7; Applied Biosystems, New Zealand) protocol, but with an increased run time (15kV, 65 000s). Peaks of fluorescence were monitored with GeneMapper® software.

The size of DNA fragments for each peak was rounded to the nearest whole number and fragments less than 200 b.p. or greater than 1000 b.p. were excluded from analysis. Peaks of weak fluorescence (≤ 100 units) were also excluded to remove background fluorescence. The area under the peaks was normalised for subsequent analysis. Similarities among bacterial ARISA data were assessed by creating a matrix of Manhattan distances using PRIMER© statistical software (v.6.1.11, PRIMER-E Ltd, UK). Data were visualised by plotting the distances on a nonmetric Multidimensional Scaling (MDS) plot where points located closer together represent similar datasets. If required, permutational ANOVA (PERMANOVA) was performed using the software PRIMER© with the add-on PERMANOVA+ (PRIMER-E Ltd, UK) to assess if the differences were significant between treatments and sampling dates. Monte-Carlo P values were stated rather than the PERMANOVA P values when the number of possible permutations was too low (< 50) (Anderson *et al.*, 2008).

2.4.4 Restriction Fragment Length Polymorphism and Terminal Restriction Fragment Length Polymorphism

Changes in ciliate community structure were assessed by either RFLP or T-RFLP. Both techniques are based on the digestion of rRNA genes by restriction endonucleases after their amplification by PCR. The analysis of the fragment length then provides a profile of the community structure (see § 1.3.2.2 for more details).

For RFLP, the 18S rRNA gene sequence was first amplified by PCR (94^{o}C for 5 min; 30 cycles of 94^{o}C for 45 s, 55^{o}C for 60 s, 72^{o}C for 90 s, and then 72^{o}C for 7 min) using the primers 384F (5' YTB GAT GGT AGT GTA TTG GA 3') and 1147R (5' GAC GGT ATC TRA TCG TCT TT 3') targeting a ~700 b.p. fragment (Dopheide *et al.*, 2008). The PCR product was purified (PureLink™ PCR DNA purification kit, Invitrogen, USA), quantified using a NanoDrop™ spectrophotometer (ND8000, Thermo Scientific, USA) and subsequently standardised to a concentration of 20 ng of DNA μl^{-1}. The PCR product was then digested with the restriction endonuclease HaeIII (Invitrogen, USA) in an overnight incubation at 37°C. Approximately 175 ng of purified amplicon was digested in a 10 µl mixture containing 2 U of the enzyme and 1 µl of reaction buffer (Invitrogen, USA).

Separation of RFLP fragments was performed by electrophoresis on a 6% polyacrylamide gel (see Table 2.4). After incubation with restriction enzymes, 5 µl of the product was mixed with 2 µl of loading dye (see Table 2.5) and loaded onto a vertical gel alongside with a 1 kb+ ladder (Invitrogen, USA) and run at 100 Volts for approximately 2 hours. Restriction fragments were visualised after staining of the gel in a solution of ethidium bromide (1 mg l^{-1}) under UV light in a Gel Doc Molecular Imager (Bio-Rad, USA).

Table 2.4: Polyacrylamide gel composition for electrophoresis of Restriction Fragment Length Polymorphism fragments.

	Constituent	Quantity	Supplier
6% polyacrylamide gel	30% acrylamide	8 ml	See below
	10 x TBE buffer	4 ml	See Table 2.3
	20 % ammonium persulphate	200 µl	Bio-Rad, Inc.
	Tetramethylethylenediamine (TEMED)	40 µl	Gibco, USA
	Water	28 ml	
	Mixed and poured immediately		
30% acrylamide	acrylamide	29 g	BDH, UK
	N, N'-methylene bisacrylamide	1 g	Bio-Rad, Inc.
	Made up with water to 100 ml		

Table 2.5: Loading dye composition for electrophoresis of Restriction Fragment Length Polymorphism fragments.

	Constituent	Quantity	Supplier
Loading dye	Ficoll 25%	1.2 ml	Sigma, Inc.
	10 x TBE buffer	200 µl	See Table 2.3
	Bromophenol blue	10 µl	Pharmacia, Inc.
	Xylene Cyanol	10 µl	BDH, UK
	Made up with water to 2 ml		

The amplification of the 18S rRNA gene sequence for T-RFLP was identical to the amplification for RFLP except for the primers 384F and 1147R that were labelled respectively with HEX and FAM fluorochromes (Molecular Probes, Invitogen, New Zealand).

In chapter 3, little DNA could be obtained from a single stage PCR, therefore a nested approach was used. In this process, a second PCR amplifies the product of the first one with primers annealing to a region within the first amplification product (Robene-Soustrade *et al.*, 2006). The initial PCR was performed using the primers 121F (5' CTG CGA ATG GCT CAT TAM AA 3') and a combination of 2755R (5' CGT TSA WGA TCY ANA ATT NCA AAG 3') and 2824R (5' CAG GGA CKT ART CAR TGC AA 3') (Primer 2755R and 2824R target different classes from the ciliate phyla, providing a complementary coverage of the phyla) (Dopheide *et al.*, 2008). In the second stage of the nested PCR, the product of the first stage was amplified using the labelled primers 384F and 1147R.

PCR products were purified (ZR Clean & Concentrator Kit™, Zymo Research, USA), quantified using a NanoDrop™ spectrophotometer (ND8000, Thermo Scientific, USA) and subsequently standardised to a concentration of 20 ng of DNA μl^{-1}. PCR products were then digested with the restriction endonucleases HaeIII and RsaI (Invitrogen, USA) in an overnight incubation at 37°C. Approximately 175 ng of purified amplicon was digested in a 10 µl mixture containing 1 U of each of the enzymes and 1 µl of reaction buffer (Invitrogen, USA). After incubation, 3 µl of the digestion product was electrophoresed on a 3130XL Capillary Genetic Analyser (Applied Biosystems, New Zealand) using the same method as for ARISA (see § 2.4.3).

The size of terminal fragments for each peak was rounded to the nearest whole number and fragments less than 20 b.p. or greater than 800 b.p. were excluded from analysis. Peaks of weak fluorescence (≤ 100 units) were also excluded to remove background fluorescence. The area under the peaks was normalised for subsequent analysis. Similarities among ciliate communities were assessed by creating a matrix of Manhattan distances using PRIMER© statistical software (v.6.1.11, PRIMER-E Ltd., UK). Data were visualised by plotting the distances on a Multidimensional Scaling (MDS) plot where points located closer together represent similar datasets. If required permutational ANOVA (PERMANOVA) was performed using the software PRIMER© with the add-on PERMANOVA+ (PRIMER-E Ltd., UK) to assess if the differences were significant between treatments and sampling dates.

2.4.5 Sequencing of bacterial 16S rRNA genes

The sequencing of bacterial 16 rRNA genes provides an insight into the species composition of bacterial communities. It enables the identification of bacteria present in the community based on their 16S rRNA gene sequence. The process requires the isolation of the genes and their amplification before sequencing (see § 1.3.2.2 for more details).

The bacterial 16S rRNA gene was amplified from the biofilm DNA extract by PCR (94°C for 3 min; 30 cycles of 94°C for 45 s, 55°C for 1 min, 72°C for 2 min, and then 72°C for 7 min) using primers Pb36 (5' AGR GTT TGA TCM TGG CTC AG 3') and Pb38 (5' GKT ACC TTG TTA CGA CTT 3') corresponding to *Escherichia coli* positions 8-27 and 1492-1509 (Saul *et al.*, 2005). Products were purified (PureLink™ PCR DNA purification kit, Invitrogen, USA) then inserted and cloned in *E. coli* competent cells (One Shot® TOP10, Invitrogen, USA) using a pGEM®-T cloning kit (Promega, USA) according to the manufacturer's instructions. *E. coli* colonies (~120 per clone library) were picked and subcultured in Luria-Bertoni (LB) broth (Difco™, USA) at 37°C for 24 h. Cells were lysed by heating at 95°C for 10 min. The insert was then amplified by PCR (94°C for 3 min; 25 cycles of 94°C for 45 s, 55°C for 45 s, 72°C for 90 s, and then 72°C for 10 min) using the primers pGEM-F (5' GCC GCG GGA ATT CGA TT 3') and pGEM-R (5' CGA ATT CAC TAG TGA TT 3') which target the sequences located on each side of the insert.

The diversity of isolated 16S rRNA genes was then assessed by RFLP of the cloned sequences. Aliquots (8 µl) of the product were digested by the restriction endonuclease HaeIII (Invitrogen, USA) at 37°C for 2 h. Restriction Fragment Length Polymorphism (RFLP) profiles were then visualised by electrophoresis on a 6% poly-acrylamide gel after staining with ethidium bromide (1 mg l^{-1}). Similar RFLP patterns were identified by aligning them with the software GelCompare II™ (Applied Maths NV, Belgium). PCR product was purified and sequenced by Macrogen Inc. (Seoul, South Korea) for at least one clone of each different RFLP pattern (with one more clone analysed every 5 similar patterns). Sequence data were then blasted against the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/) to assess the identity of the cloned DNA.

2.5 Quantification of biofilm associated protozoa

As an alternative to molecular techniques to investigate ciliate communities associated with biofilm, the number of protozoa associated with the biofilm was also quantified by analysis under the microscope. To do so, an area of 1 cm^2 of the biofilm growing on glass slides was disturbed using a plastic stick (SteriClin, BioLab, New-Zealand). The solution obtained was then transferred to a volumetric microscope slide. A volume of 0.1 mm^3 was then observed under a bright field microscope at 400 x magnification (DMRE Compound Microscope, Leica Microsystems, Germany) and all the moving protozoa of length > 10 µm were counted.

2.6 Identification of macro-invertebrate species

Samples were transferred to a sieve (0.5 mm mesh) and rinsed with water to remove the preserving ethanol. The sample was then transferred to a white tray, using water to fully rinse all the content into the tray. All the invertebrates were collected, counted and identified for each sample. Exception was made when a taxa included more than 100 individuals in one sample. In that case, the first 100 were counted and the total number within that community was estimated from the proportion of sample processed. Identification was made at the lowest taxonomic level achievable, especially trying to reach the recommended minimum level of identification required for the macro-invertebrate community index (Winterbourn *et al.*, 2006).

CHAPTER 3

3 Flow chamber experiments to investigate the effect of urban runoff metal contaminants on biofilm microbial communities and their accumulation in the biofilm matrix.

Abstract:

This study investigates the absorption rates of zinc, copper and lead in freshwater biofilm and assesses whether biofilm microbial communities are affected by exposure to environmentally relevant concentrations of these metals. Seven experiments were undertaken in flow chamber microcosms in which mature biofilm was exposed to different concentrations of either zinc, copper or lead, or to a combination of these metals. Changes in bacterial populations were assessed by ARISA and 16S rRNA gene clone libraries over different periods of exposure and recovery. Changes in ciliate populations were monitored by RFLP of 18S rRNA genes and protozoa were counted by microscopy. Absorption/desorption rates of metal in the biofilm were analysed by atomic absorption spectrometry after hot nitric acid digestion of the samples. Significant differences in bacterial community structure occurred within only one to three days of exposure to metals and remained detectable at least 14 days after transfer to uncontaminated water. Clear differences in ciliate and more globally protozoan populations were also observed within a few days of exposure to metals. Analysis of metal concentrations revealed a rapid uptake of stormwater-associated metals and their retention in the biofilm highlighting the potential role of biofilms in the transfer of metals to organisms at higher trophic levels. The sensitivity of stream biofilm microbial populations to metal exposure supports their use as a sensitive indicator of stream ecological health.

3.1 Introduction

The effective use of organisms as bio-indicators to monitor the impact of urbanisation on freshwater ecosystems relies on their sensitivity to the contaminants contained within urban runoff. Therefore, the first set of experiments aimed to assess whether biofilm microbial communities are affected by exposure to environmentally relevant concentrations of some common urban contaminants; zinc, copper and lead. In addition, the absorption and desorption rates of these metals in biofilm was monitored to investigate their use as an indicator of metal contamination in streams.

To allow the adequate control of environmental variables and to provide a higher level of replication, all experiments in this chapter were conducted in laboratory flow chamber microcosms in which mature biofilm was exposed to known concentrations of zinc, copper and lead for various periods of time. Several techniques were tested to monitor the changes occurring in biofilm microbial community structure and composition. Automated Ribosomal Intergenic Spacer Analysis (ARISA) and 16S rRNA gene clone libraries were used to investigate changes in bacterial populations. Protozoa were quantified by microscopy and ciliates in particular, were monitored by Restriction Fragment Length Polymorphism (RFLP) and Terminal-Fragment Length Polymorphism (T-RFLP) of the 18S rRNA gene. The absorption and desorption rates of metals in biofilm were also investigated by Atomic Absorption Spectrometry.

3.2 Experimental design

Seven separate experiments were sequentially undertaken in flow chamber microcosms. The first three experiments investigated the effect of moderately or highly contaminated synthetic urban runoff on biofilm microbial communities by exposing biofilm to a mixture of zinc, copper and lead for various periods of time. Two complementary experiments were then undertaken to investigate the effect of lower concentrations of these contaminants for various periods of exposure. The final two experiments were carried out to investigate the effect of the different metals included in the synthetic urban runoff, as individual contaminants. This chapter focuses specifically on the effects of zinc, copper and lead because of their common

abundance in urban runoff and their environmental significance in aquatic environments (see § 1.1.1 for more details).

To develop relatively uniform, mature biofilm for use in each experiment, 30 or 33 sandblasted glass slides (65 mm x 140 mm) were incubated for 28 days in the high capacity flow chamber (see § 2.1.1 for more details). This flow chamber contained water from Opanuku Stream (Latitude 36°53'42 S, Longitude 174°35'44 E, see Figure 2.7). For the first three experiments only, the water from Opanuku Stream was diluted (1:1) with dechlorinated tap water to increase the liquid volume. Opanuku Stream was chosen as it is highly characterised and located in a relatively unmodified catchment, that is unlikely to have been exposed to significant concentrations of urban stormwater contaminants (for details about catchment and stream water characteristics, refer to ARC (2004b)).

At the beginning of each experiment, three glass slides covered with mature biofilm were collected from the high capacity flow chamber and the remaining 27 or 30 were transferred to 9 or 10 low capacity flow chambers (3 per flow chamber). Each of the flow chambers was filled beforehand with 17 l of water from the high capacity flow chamber. Zinc, copper and lead (as $ZnCl_2$, $CuCl_2$ and $Pb(NO_3)_2$, respectively) were added 30 min before the slides were transferred to ensure that metals were homogeneously distributed in the water before the biofilm was introduced.

Slides were exposed to the different treatments in the low capacity flow chambers for various periods of time and eventually transferred to clean water in the high capacity flow chamber to monitor the recovery of microbial populations and the release of metals. When ready for sampling, slides were carefully removed from the flow chambers and sampled for DNA and metal analyses. The biofilm was removed from half the surface of the slide using a Speci-Sponge (see § 2.2.1 for more details) and the DNA was extracted for molecular analysis of microbial communities following the standard protocol (see § 2.4.1). The remaining biofilm was scraped off the slide using a sterile plastic stick, transferred to a separate Teflon® beaker (see § 2.2.1 for more details) and processed for metal analyses following the standard protocol (see § 2.3).

Changes in bacterial community structure between samples and treatments were investigated by ARISA. Similarities among bacterial ARISA data were assessed by Manhattan distances

and visualised on a MDS plot. Permutational ANOVA (PERMANOVA) was also performed to assess if the differences were significant between treatments and sampling dates. Monte-Carlo P values are stated in the results section rather than PERMANOVA P values as the small number of samples limits the number of possible permutations (see § 2.4.3 for more details).

The structure of the ciliate community associated with the biofilm was investigated by RFLP or T-RFLP depending on the particular experiment. For the first 3 experiments, the structure of the ciliate community was successfully investigated by RFLP after amplification of the 18S rRNA gene using the primers 384F and 1147R and digestion of the product with the restriction endonuclease HaeIII (see § 2.4.4 for more details). For the other experiments the structure of the ciliate community was investigated by T-RFLP. Little DNA could be obtained from a single stage PCR using the labelled primers 384F and 1147R therefore a nested approach was performed (see § 2.4.4 for more details). The product of the nested PCR was for most samples very concentrated while the remaining samples could not be amplified. This suggests that the copy number of ciliate 18S rRNA genes may be too small to be amplified in some samples. Also, after digestion of the PCR product and processing for fluorescence analysis, the profiles obtained contained very few peaks. Unsuccessful DNA amplification and the small number of peaks decreased the reliability of the statistical analysis. For this reason, the results will not be presented. As an alternative, biofilm associated protozoa (including ciliates, flagellates and amoeba) were observed and quantified by microscopy (see § 2.5 for more details). Microscopic observations generally revealed a strong dominance of flagellates in the protozoan population and confirmed the small number of ciliates present in the biofilm.

3.2.1 Synthetic urban runoff moderately or highly contaminated

The first experiments investigated the effect of moderately and highly contaminated synthetic urban runoff on biofilm bacterial and ciliate communities and the absorption and desorption rates of zinc, copper and lead in the biofilm matrix. Three separate experiments (hereafter referred to as ‘short term’, ‘long term’ and ‘recovery’, see Table 3.1) were sequentially undertaken in which mature biofilm was exposed to three different treatments; (a) a control treatment with no added metals; (b) a moderately contaminated urban runoff, simulated by

addition of 500µg/l of zinc, 50 µg/l of copper and 50 µg/l of lead, and (c) a highly contaminated urban runoff simulated by addition of 1000 µg/l of zinc, 100 µg/l of copper and 100 µg/l of lead (Table 3.1). Metal concentrations were chosen based on the data from Kayhanian *et al.* (2007), Kennedy (1999), and Williamson (1993).

Table 3.1: Experimental design of short term, long term and recovery experiments investigating the effect of synthetic urban runoff moderately or highly contaminated. Experiments were undertaken in flow chamber microcosms, in which biofilm was exposed to a moderately contaminated synthetic stormwater, a highly contaminated synthetic stormwater and a control treatment with no metals added. Biofilm was sampled before each experiment (day 0) and after different periods of exposure to metals (▮), and recovery (▯) in which metal exposed biofilms were transferred to non-contaminated water.

Experiment	Treatments	Metal concentrations (µg/l)	Sampling times
short term	Control Synthetic urban runoff, moderately contaminated Synthetic urban runoff, highly contaminated	No metals added Zn 500 + Cu 50 + Pb 50 Zn 1000 + Cu 100 + Pb 100	1 day 3 days 5 days ↓ ↓ ↓ ▮▮▮▮▮▶
long term	Control Synthetic urban runoff, moderately contaminated Synthetic urban runoff, highly contaminated	No metals added Zn 500 + Cu 50 + Pb 50 Zn 1000 + Cu 100 + Pb 100	7 days 14 days 21 days ↓ ↓ ↓ ▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▮▶
recovery	Control Synthetic urban runoff, moderately contaminated Synthetic urban runoff, highly contaminated	No metals added Zn 500 + Cu 50 + Pb 50 Zn 1000 + Cu 100 + Pb 100	7 days 14 days 21 days ↓ ↓ ↓ ▮▮▮▮▮▮▮▮▮▯▯▯▯▯▯▯▯▯▯▯▶

For the short and long term experiments, one slide from each flow chamber was sampled after 1, 3 and 5 days or 7, 14 and 21 days, respectively. For the recovery experiment, one slide from each microcosm was sampled after 7 days of exposure to metals, while the two remaining slides were transferred back into uncontaminated water within the high capacity flow chamber. Slides were then sampled after 7 days and 14 days of recovery in uncontaminated water. This enabled the recovery of the bacterial populations as well as the release of metals to be monitored.

All samples were assessed by ARISA to monitor the changes in bacterial community structure. In addition, bacterial community composition was assessed by sequencing of bacterial 16S rRNA genes extracted from samples at 0 and 7 days. To reduce the number of samples, the DNA extracted from replicate samples was pooled. Sequences obtained from the clone libraries of the 16S rRNA genes were blasted against the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/) to assess the identity of the cloned DNA. The sequences obtained in this study have been deposited in the NCBI nucleotide database under accession numbers FJ662584-FJ662761.

3.2.2 Synthetic urban runoff with low level of contamination

Concentrations of metals present in urban runoff are highly variable. To investigate the effect of a broader range of concentrations and assess the minimal concentrations at which changes in microbial communities could be detected, two complementary experiments (hereafter referred to as 'short term at low concentrations' and 'long term at low concentrations', see Table 3.2) were undertaken. Using a similar protocol, three different treatments were applied; (a) a control treatment with no added metals; (b) a synthetic urban runoff with low level of contamination, simulated by addition of 100 µg/l of zinc, 10 µg/l of copper and 10 µg/l of lead, and (c) a highly contaminated urban runoff simulated by addition of 1000 µg/l of zinc, 100 µg/l of copper and 100 µg/l of lead (Table 3.2).

Table 3.2: Experimental design of the short term and long term experiments at low concentrations. Two experiments were undertaken in flow chamber microcosms in which biofilm was exposed to a synthetic stormwater with low level of contamination, a highly contaminated synthetic stormwater and a control treatment with no metals added. Biofilm was sampled before each experiment (day 0) and after 1, 3 and 5 or 7, 14 and 21 days of exposure to metals.

Experiment	Treatments	Metal concentrations (µg/l)	Sampling times
short term	Control Synthetic urban runoff with low level of contamination Synthetic urban runoff highly contaminated	No metals added Zn 100 + Cu 10 + Pb 10 Zn 1000 + Cu 100 + Pb 100	1 day 3 days 5 days ↓ ↓ ↓
long term	Control Synthetic urban runoff with low level of contamination Synthetic urban runoff highly contaminated	No metals added Zn 100 + Cu 10 + Pb 10 Zn 1000 + Cu 100 + Pb 100	7 days 14 days 21 days ↓ ↓ ↓

One slide from each flow chamber was sampled after 1, 3 and 5 days during the short term experiment and 7, 14 and 21 days during the long term experiment. Changes in bacterial community structure were assessed for all samples by ARISA and protozoa were counted by microscopy. The concentrations of metals in biofilm samples from the short term experiment were also analysed.

3.2.3 Zinc, copper and lead as individual contaminants

In all the previous experiments, zinc, copper and lead were always combined together to simulate a complex urban runoff. In the last two experiments of this chapter (hereafter referred to as 'short term individual metals' and 'long term individual metals', see Table 3.3), mature biofilm was exposed to zinc, copper and lead as individual contaminants as well as combined together. This enabled us to differentiate the effect of each of the three metals and

to compare with the combined effect. Five different treatments were applied; a control treatment with no added metals; three treatments with addition of one metal each (1000 µg/l of zinc, 100 µg/l of copper or 100 µg/l of lead, respectively) and a highly contaminated synthetic urban runoff containing 1000 µg/l of zinc, 100 µg/l of copper and 100 µg/l of lead (Table 3.3).

Table 3.3: Experimental design of short term and long term individual metal experiments. Two experiments were undertaken, in which biofilm was exposed to zinc, copper and lead as individual contaminants or combined together. Biofilm was sampled before each experiment (day 0) and after 1, 3 and 5 or 7, 14 and 21 days of exposure to metals, for the short term and long term experiments, respectively.

Experiment	Treatments	Metal concentrations (µg/l)	Sampling times
Short term individual metals	Control	No metals added	1 day 3 days 5 days
	Zinc only	Zn 1000	
	Copper only	Cu 100	
	Lead only	Pb 100	
	Synthetic urban runoff, highly contaminated	Zn 1000 + Cu 100 + Pb 100	
Long term Individual metals	Control	No metals added	7 days 14 days 21 days
	Zinc only	Zn 1000	
	Copper only	Cu 100	
	Lead only	Pb 100	
	Synthetic urban runoff, highly contaminated	Zn 1000 + Cu 100 + Pb 100	

One slide from each flow chamber was sampled after 1, 3 and 5 days during the short term experiment and 7, 14 and 21 days for the long term experiment. Changes in bacterial community structure were assessed by ARISA and protozoa were quantified by microscopy. The accumulation of metals in all biofilm samples of the short term experiment was also monitored to investigate the competition between ions for binding sites.

3.3 Results

3.3.1 Synthetic urban runoff moderately or highly contaminated.

In the first three experiments, biofilm was exposed to synthetic urban runoff moderately or highly contaminated and the changes monitored after different periods of exposure and recovery (refer to Table 3.1 for more details).

Changes in bacterial community structure revealed by ARISA

Similarities among bacterial ARISA data were assessed by creating a matrix of Manhattan distances. Data can be visualised by plotting the distances on a nonmetric Multidimensional

Scaling (MDS) plot where points located closer together represent more similar datasets (Figure 3.1). The plot of the short term experiment (Figure 3.1 a) reveals rapid changes in bacterial community structure following exposure to separate treatments. Monte Carlo P-values (Table 3.4) also confirm that the differences between metal exposed and unexposed populations became significant within 3 days of exposure.

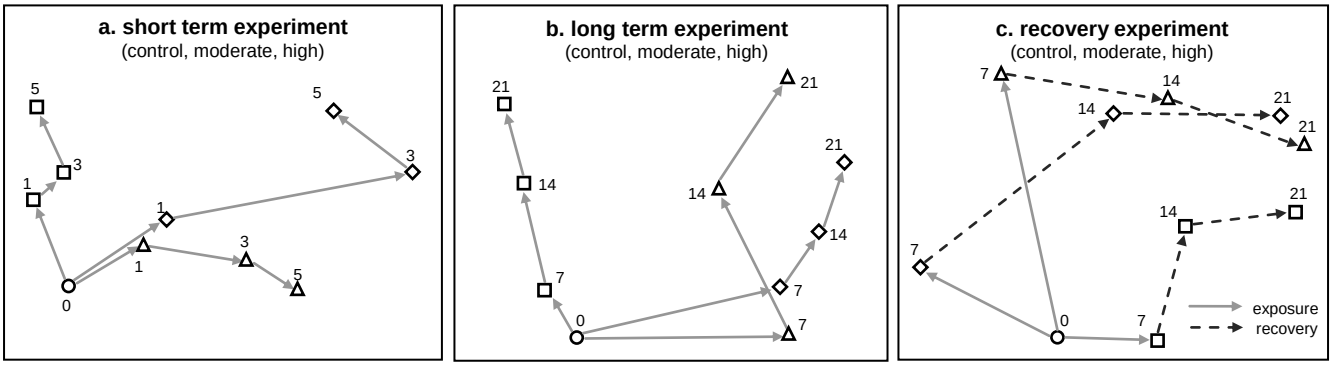

Figure 3.1: Nonmetric Multidimensional Scaling plot of Manhattan distances between bacterial ARISA data within (a) the short term experiment, (b) the long term experiment, and (c) the recovery experiment (treatments: control, synthetic urban runoff moderately contaminated and synthetic urban runoff highly contaminated). 2D stress values are 0.06, 0.05 and 0.1, respectively. Three replicates were averaged before analysis. The trajectory shows the movement of data points over time starting from the original population (○) prior to the construction of individual treatment microcosms. Treatments are (□) control, (△) synthetic urban runoff moderately contaminated and (◇) synthetic urban runoff highly contaminated. Numbers on plot refer to days as separate treatments. For further details of individual treatments, refer to Table 3.1.

Both medium and high concentrations of metals caused a similar shift in bacterial populations during the first day of exposure but differences between the two treatments became more apparent after 3 to 5 days of exposure. Based on the results of the long term experiment (Figure 3.1 b), differences continued to increase until the longest period of exposure observed in this set of experiments (21 days). During the long term experiment, significant differences between exposed and unexposed populations were consistent throughout the 21 days of exposure (Table 3.4).

Table 3.4: Pairwise comparisons of bacterial ARISA data between treatments by experiment and sampling date. Data are Monte Carlo P values (MCP) of PERMANOVA analysis based on Manhattan distances. Treatments are control, synthetic urban runoff moderately contaminated (moderate) and synthetic urban runoff highly contaminated (high). * indicates significant differences (MCP < 0.05).

	Short term experiment			Long term experiment			Recovery experiment		
	1 d	**3 d**	**5 d**	**7 d**	**14 d**	**21 d**	**7 d**	**14 d**	**21 d**
control vs moderate	0.129	0.045*	0.067	0.008*	0.021*	0.049*	0.019*	0.026*	0.114
control vs high	0.082	0.038*	0.038*	0.006*	0.009*	0.033*	0.024*	0.09	0.156
moderate vs high	0.682	0.071	0.206	0.066	0.037*	0.111	0.015*	0.251	0.612

When the biofilm was transferred back to clean water (recovery experiment, Figure 3.1 c), bacterial populations exposed to metal progressively became more similar to the unexposed populations but differences between them remained detectable after the longest period of recovery observed in this experiment at 21 days.

The analysis of ARISA profiles uses a large number of variables (800 ARISA peaks) and the technique has been shown to be highly sensitive to population changes (Jones *et al.*, 2007; Lear *et al.*, 2008a). However, the visualisation of differences using MDS plots as well as the statistical analysis are relative, not quantitative and therefore cannot provide any insight into the true extent to which bacterial populations are modified. For instance, if replicates are very similar, significant differences observed could originate from minor variations in a small number of peaks within the total population. To evaluate the extent to which changes occur, the different ARISA profiles can be compared visually. Figure 3.2 presents the profiles after 7 days of exposure as well as the profile obtained from the initial population (0 days) of the long term experiment.

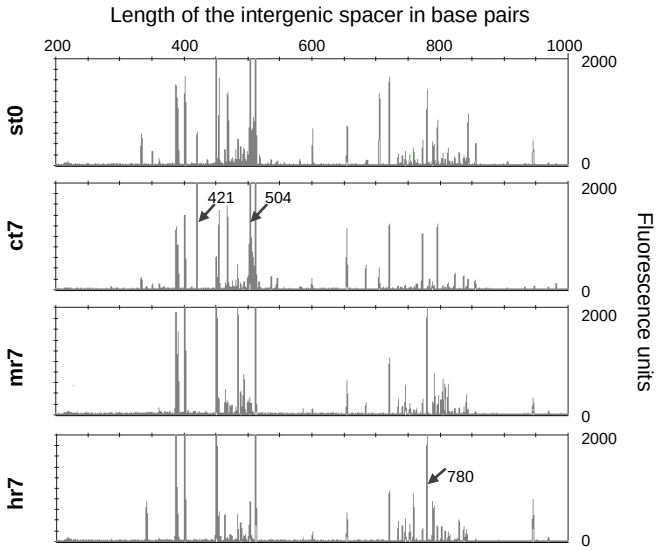

Figure 3.2: Bacterial ARISA profiles of the initial biofilm community (st0), and of each of the treatments, control (ct7), synthetic urban runoff moderately contaminated (mr7) and synthetic urban runoff highly contaminated (hr7) after 7 days of exposure. Data are from the long term experiment and profiles of three replicates are overlaid. Highlighted peaks show some examples of differences among treatments.

Although on this graph, differences can be easily pinpointed (for instance, peaks at 421 and 504 base pairs are highly represented in the control population but not in metal exposed populations and a peak at 780 base pairs becomes much more represented under metal stress), it appears that the overall structure of the community was not intensively altered as a great proportion of the peaks are retained.

Changes in bacterial community composition revealed by clone libraries of bacterial 16S rRNA genes

Sequenced 16S rRNA gene clone libraries complete the analysis of bacterial community structure by providing an insight into the changes occurring in the composition of the community. Samples analysed are the initial biofilm of the long term experiment and the biofilm from each of the treatments after 7 days of exposure. The proportion of Alphaproteobacteria is very constant among treatments (35 % to 38 %) whereas proportions of Beta- and Gammaproteobacteria fluctuate more between treatments (Figure 3.3).

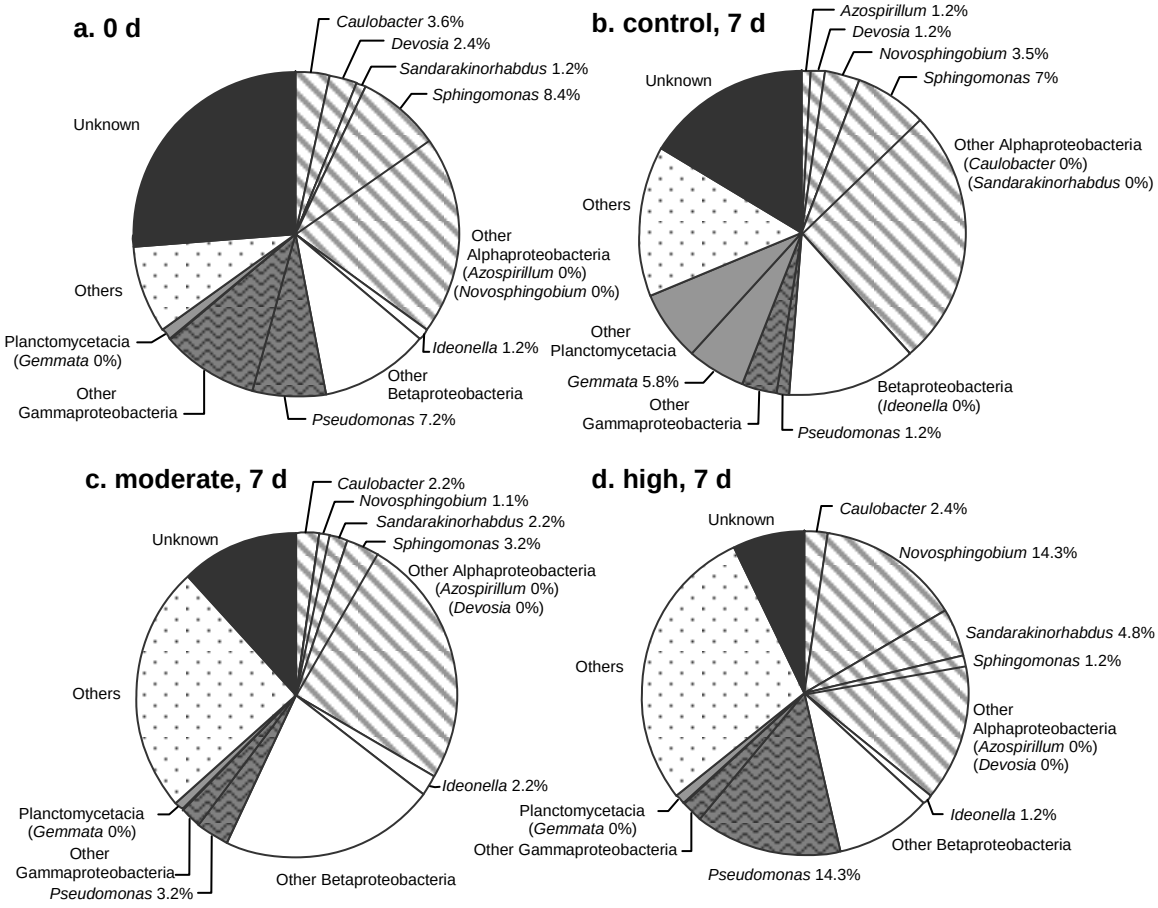

Figure 3.3: Comparison of bacterial communities based on cloned 16S rRNA gene sequences before (a) and after 7 days of all treatments: (b) control, (c) synthetic urban runoff moderately concentrated and (d) synthetic urban runoff highly contaminated. Libraries are based on the sequence of 83, 86, 93 and 84 clones, respectively. Data is presented at both genus (identified on graphs) and class levels: (▨) Alphaproteobacteria, (□) Betaproteobacteria, (▩) Gammaproteobacteria, (■) Planctomycetacia, (⬚) Others, (■) Unknown. Only the genera that appear to be influenced by the treatments are represented, for the complete list of genera, see appendix App-1.

Betaproteobacteria seem to proliferate in the biofilm exposed to moderate levels of metals while Gammaproteobacteria are present in similar proportion in the initial biofilm and the biofilm exposed to high levels of metals but in lower proportions in the other treatments. We also notice a much greater representation of Planctomycetacia in the control treatment than in the other samples (13 % instead of 1 %) and a decrease of unknown sequences with increasing metal concentrations. At the genus level, certain genera are more represented in some treatments than in others (Figure 3.3 and see appendices, Table App-1, for complete list of genera). The genera *Caulobacter*, *Ideonella* and *Sandrakinorhabdus* were present in all samples except the control (Figure 3.3) which may indicate a better survival of these populations under metal stress. In addition, the genera *Novosphingobium* and *Pseudomonas* proliferated in all treatments but seemed to prevail especially under high metal concentrations (Figure 3.3). Conversely, several genera were present in the control but absent (e.g., *Azospirillum, Devosia and Gemmata*) or present in lower proportion (*Sphingomonas)* in populations exposed to metals, which suggests that these may be metal intolerant taxa.

Genus accumulation curves (Figure 3.4) reveal a higher diversity of genera in the control treatment compared to communities exposed to metal contaminants. These curves also show that the clone library data has not revealed the full diversity of these bacterial communities since a plateau has not been reached.

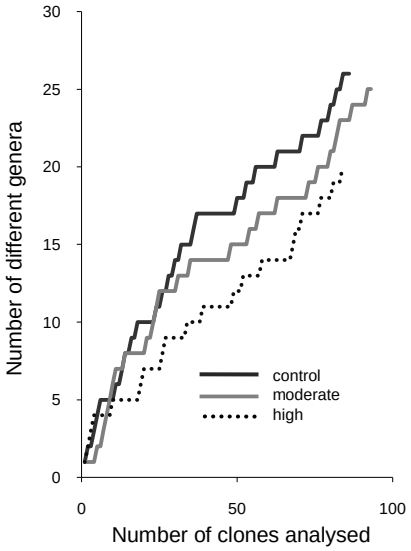

Figure 3.4: Accumulation curves of new genera identified from cloned 16S rRNA gene sequences in the long term experiment, plotted against the number of clones analysed. Data are for biofilm communities exposed to no additional metals (control); to synthetic urban runoff moderately contaminated (moderate); or to synthetic urban runoff highly contaminated (high).

Changes in ciliate community structure revealed by RFLP

The structure of ciliate communities was monitored for each sample by RFLP of 18S rRNA genes. RFLP profiles reveal that during the short term experiment, differences in ciliate communities became apparent after 5 days of exposure to metals (Figure 3.5 a). In the long term experiment, a clear change in the community structure can be seen after 7 days of exposure and remains visible until 21 days of exposure (Figure 3.5 b). Each experiment was done using a different initial biofilm and the initial ciliate populations in the recovery experiment seem less sensitive as after 7 days of exposure, differences in community structure appear moderate compared to the long term experiment (Figure 3.5 c). In all three experiments, RFLP profiles suggest that populations exposed to synthetic urban runoff (either moderately or highly contaminated) are the most similar to the initial population.

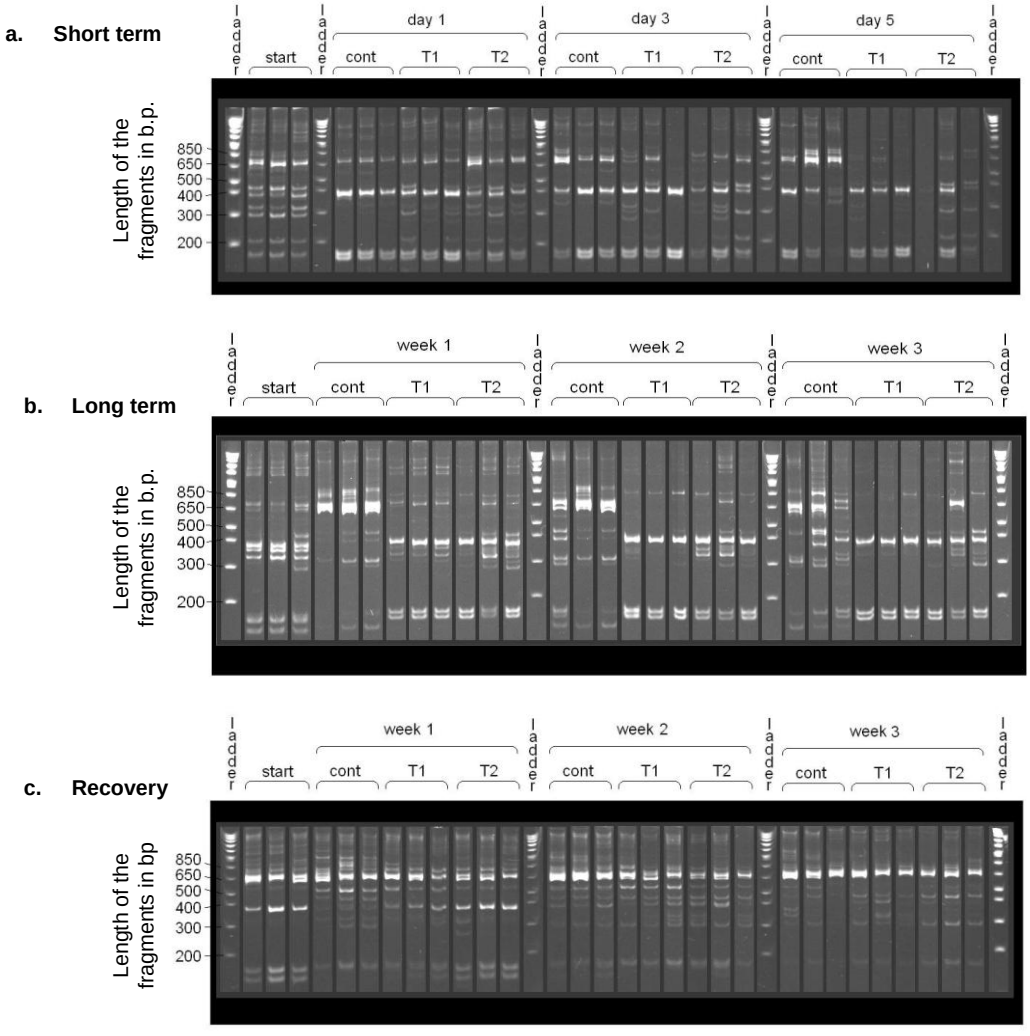

Figure 3.5: RFLP profiles of ciliate 18S rRNA genes for short term, long term and recovery experiments. PCR products were digested by restriction endonuclease HaeIII and electrophoresed on a 6% polyacrylamide gel alongside with a 1kb+ ladder (Invitrogen, USA). Treatments are: (start) before transfer to separate flow chambers, (control) with no addition of metals, (T1) synthetic urban runoff moderately concentrated and (T2) synthetic urban runoff highly contaminated. For further details about individual treatments, refer to Table 3.1.

Absorption/desorption of metals from the biofilm

The accumulation rate of metals in biofilm was maximal during the first day of exposure and decreased with time (Figure 3.6 a & b). In the short and long term experiments, the concentrations of metal absorbed in the biofilm show that the equilibrium between metals in water and in biofilm (when concentrations are in a steady state) was reached after 7 to 14 days of exposure, regardless of the metal.

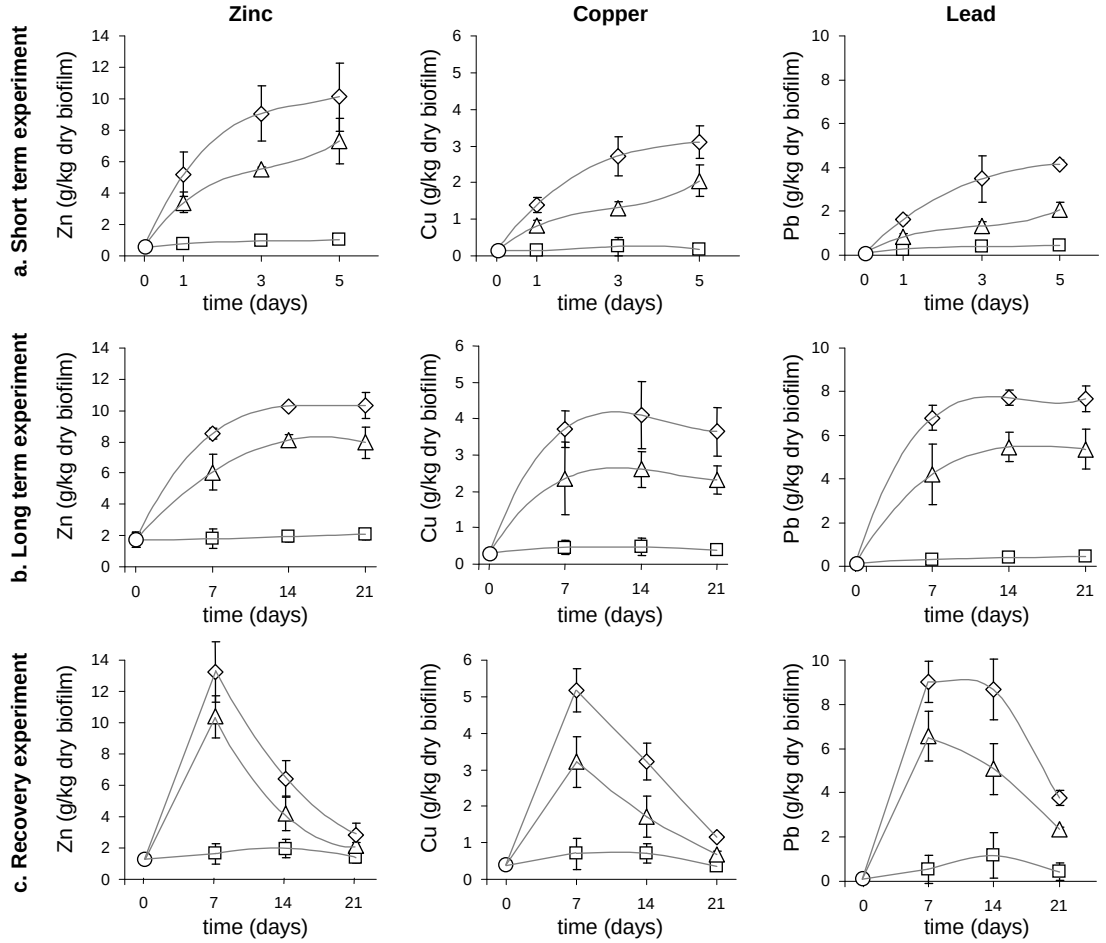

Figure 3.6: Concentrations of zinc, copper and lead in biofilms for each of the three experiments, over time. Data are (○) concentrations at the beginning of each experiment, (□) concentrations after exposure to no addition of metals, (△) concentrations after exposure to moderately contaminated synthetic urban runoff, and (◇) concentrations after exposure to highly contaminated synthetic urban runoff. For further details about individual treatments, refer to Table 3.1. Data are averages of 3 replicates and error bars are confidence intervals ($\alpha = 0.05$).

In Figure 3.7, concentrations of metals measured in biofilm on day 21 of the long term experiment were plotted against concentrations of metals in water. The graph shows that in this system, the affinity of the biofilm for lead ions is much higher than for zinc and copper.

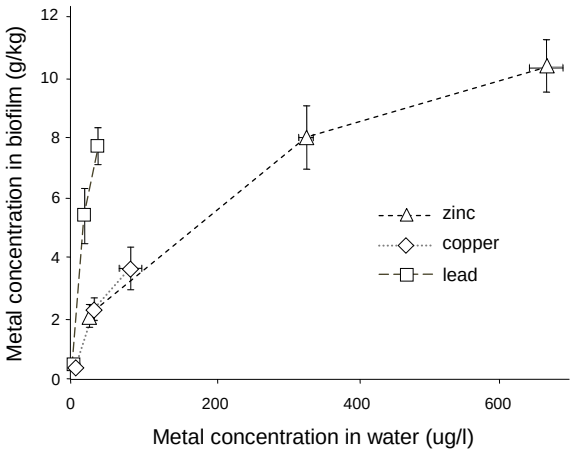

Figure 3.7: Concentrations of (△) zinc, (◇) copper and (□) lead in the biofilm (y axis) plotted against the concentration of the same element dissolved in water (x axis) after 21 days exposure in the long term experiment for each of the 3 treatments. Data are averages of 3 replicates and error bars are confidence intervals ($\alpha = 0.05$).

Metals accumulated to very high concentrations in the biofilm and enrichment factors up to 500:1 for zinc, 1500:1 for copper and 6000:1 for lead were measured between the biofilm wet weight and the water (long term experiment after 21 days of exposure under synthetic urban runoff, moderately contaminated) (Table 3.5).

Table 3.5: Concentrations of zinc, copper and lead in biofilm (mg/kg wet weight) and in water (mg/l) and enrichment factors of the metals in the biofilm compared to the water after 21 days of exposure (long term experiment). Treatments are synthetic urban runoff moderately contaminated (moderate) and highly contaminated (high). For further details about individual treatments refer to Table 3.1.

	Concentrations of zinc		Concentrations of copper		Concentrations of lead	
	moderate	high	moderate	high	moderate	high
Biofilm (mg/kg wet weight)	166.0	204.3	48.0	74.6	102.0	153.4
Water (mg/l)	0.331	0.671	0.032	0.083	0.017	0.036
Enrichment factor	501.3	304.5	1500.7	899.0	6001.8	4261.1

When after 7 days of exposure to different treatments, the biofilm was returned to uncontaminated water (Figure 3.6 c), metals were released steadily but at a slower rate than they accumulated. After 14 days of recovery in clean water, 10 to 16 % of zinc and copper accumulated during exposure still remained in the biofilm. The release of lead was even slower and more than 35 % of accumulated lead remained in the biofilm after 14 days of recovery. The percentage of Zn, Cu and Pb remaining in biofilm was similar in both high and moderate treatments (13, 16 and 41 % for the high treatment against 10, 11 and 45 % for the moderate treatment, respectively).

3.3.2 Synthetic urban runoff with low level of contamination

To investigate the effect of a broader range of concentrations and assess the minimal concentrations at which changes in microbial communities could be detected, two complementary experiments were undertaken. In these experiments, the effect of a synthetic urban runoff with low level of contamination (100 µg/l of zinc, 10 µg/l of copper and 10 µg/l of lead) was investigated and compared to the effect of synthetic urban runoff highly contaminated (1000 µg/l of zinc, 100 µg/l of copper and 100 µg/l of lead) (refer to Table 3.2 for more details).

Changes in bacterial community structure

Nonmetric Multidimensional Scaling (MDS) plots of Manhattan distances between bacterial communities reveal rapid changes occurring under exposure to highly contaminated synthetic stormwater (Figure 3.8 a & b), consistent with the observations from previous experiments (see § 3.3.1). After 1 day of exposure to separate treatments, Monte Carlo P values reveal a significant change in bacterial community structure between the control and the highly contaminated treatment that remained until the longest period of exposure of 21 days (Table 3.6).

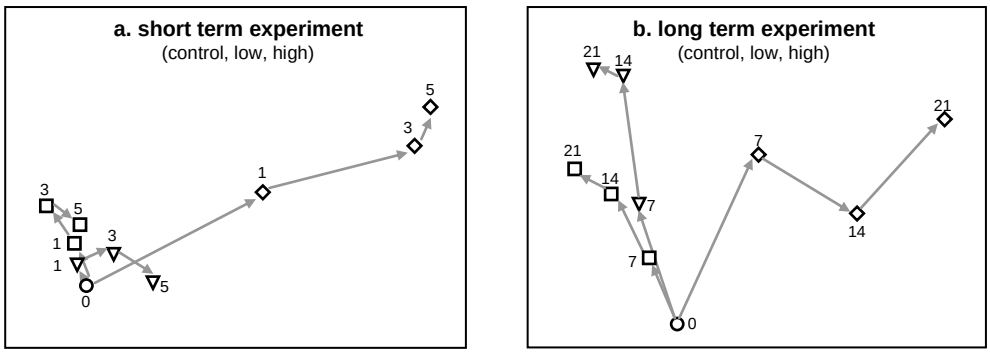

Figure 3.8: Nonmetric Multidimensional Scaling plot of Manhattan distances between bacterial ARISA data within (a) the short term and (b) the long term experiments (treatments: control, urban runoff with low level of contamination and urban runoff highly contaminated). 2D stress values are 0.04 and 0.06, respectively. For each data point three replicates were averaged before analysis. The trajectory shows the changes in bacterial community structure occurring over time starting from the original population (○) prior to the construction of individual treatment microcosms. Treatments are (□) control, (▽) synthetic urban runoff with low level of contamination and (◇) synthetic urban runoff, highly contaminated. Numbers on plot refer to days as separate treatments. For further details of individual treatments, refer to Table 3.2.

The response of bacterial communities to exposure to synthetic stormwater with low level of contamination was small in comparison with the synthetic stormwater highly contaminated. Differences are detectable and increase over time but the communities remain too similar to the control to be significantly different even after the longest period of exposure of 21 days (Table 3.6).

Table 3.6: Pairwise comparisons of bacterial ARISA data between treatments by experiment and sampling date for the short term and long term experiments. Data are Monte Carlo P values (MCP) of PERMANOVA analysis based on Manhattan distances. Treatments are control, synthetic urban runoff with low level of contamination (low) and synthetic urban runoff highly contaminated (high). * indicates significant differences (MCP < 0.05).

	Short term experiment			Long term experiment		
	1 d	**3 d**	**5 d**	**7 d**	**14 d**	**21 d**
control vs low	0.508	0.109	0.167	0.110	0.051	0.127
control vs high	0.037*	0.015*	0.019*	0.024*	0.018*	0.010*
low vs high	0.115	0.041*	0.020*	0.102	0.020*	0.007*

Changes in numbers of protozoa

Observed concentrations of protozoa decreased drastically within 1 day of exposure to highly contaminated synthetic stormwater, from approximately 300 protozoa mm^{-3} to only a few protozoa mm^{-3}. The number of protozoa remained low until the end of the short term experiment (5 days) (Figure 3.9 a) and later until the end of the long term experiment (21 days) (Figure 3.9 b). The urban runoff with low level of contamination had a more moderate impact on the number of protozoa but their concentration was less than in the control treatment on any sampling day of both short and long term experiments. The concentration of protozoa was generally highly variable between sampling dates as, even within the control treatment, an increase or a decrease by a factor of 2 could be observed in just 2 days.

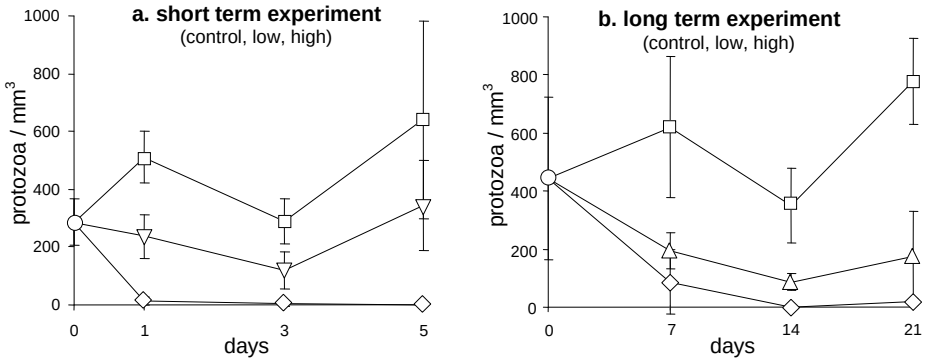

Figure 3.9: Protozoa count for the (a) short and (b) long term experiments (treatments: control, synthetic urban runoff with low level of contamination and synthetic urban runoff highly contaminated). Graphs show the concentration of protozoa in biofilm (protozoa / mm^3) over time starting from (○) the initial biofilm prior to the construction of individual treatment microcosms. Treatments are (□) control, (▽) synthetic urban runoff with low level of contamination and (◇) synthetic urban runoff highly contaminated. For further details about individual treatments refer to Table 3.2. Data are averages of 3 replicates and error bars are confidence intervals ($\alpha = 0.05$).

Absorption of metals in the biofilm

Metal concentrations in biofilm were monitored during the short term experiment. Similarly to the observations made from the previous experiments (see § 3.3.1), the absorption rate of zinc, copper and lead was maximum at the beginning and decreased with time (Figure 3.10). After 5 days of exposure, concentrations of zinc had almost reached a steady state whereas copper and lead were still accumulating.

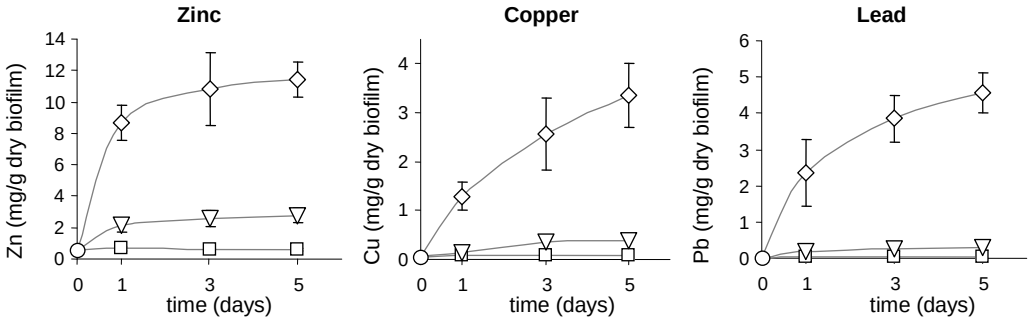

Figure 3.10: Absorption of zinc, copper and lead in biofilm for the short term experiment (treatments: control, urban runoff with low level of contamination and synthetic urban runoff highly contaminated). Data are: concentrations in biofilm (○) at the beginning of each experiment, (□) after exposure to no addition of metals, (▽) after exposure to synthetic urban runoff with low level of contamination, and (◇) after exposure to highly contaminated synthetic urban runoff. For further details about individual treatments refer to Table 3.3. Data are averages of 3 replicates and error bars are confidence intervals ($\alpha = 0.05$).

Although the amount of each metal added in the urban runoff highly contaminated treatment was 10 times greater than in the urban runoff with low level of contamination treatment, the proportion of metal in biofilm exposed to both treatments was variable. After 5 days of exposure, concentrations of zinc, copper and lead were respectively 4 times, 9 times and 15 times higher in biofilm from the highly contaminated treatment than from the low level of contamination treatment (Figure 3.10).

3.3.3 Zinc, copper and lead as individual contaminants

In the final two experiments, the effect of the different individual metals included in the synthetic urban runoff was investigated and compared with their cumulative effect. To do so, biofilm was exposed to zinc, copper and lead as individual contaminants as well as combined together for different periods of time ranging from 1 to 21 days (refer to Table 3.3 for more details).

Changes in bacterial community structure revealed by ARISA

Due to the larger number of treatments (5) applied in these two experiments, the data is plotted on a day to day basis to prevent the plots being obscured by overlapping trajectories (Figure 3.11). MDS plots reveal an increasing emergence over time of zinc and copper as the most influential treatments together with the combination of zinc, copper and lead. Lead alone has a minimal impact on the biofilm bacterial community structure as the community remains close to the control community until the longest period of exposure of 21 days.

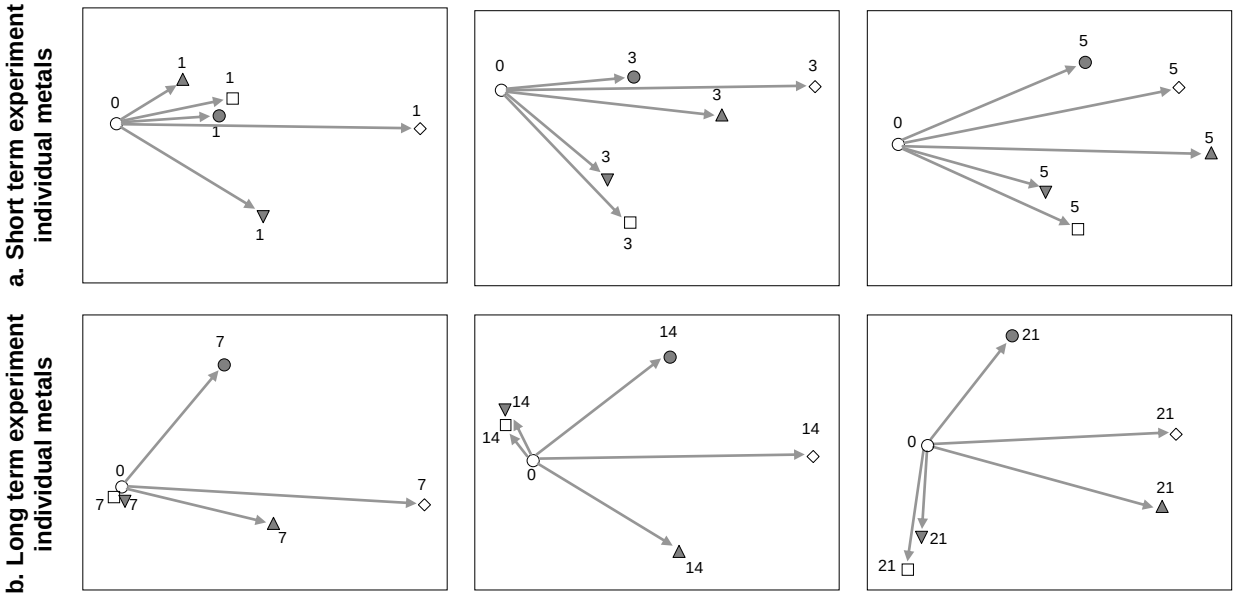

Figure 3.11: Nonmetric Multidimensional Scaling plot of Manhattan distances between bacterial ARISA data within the (a) short term (1, 3 and 5 days of exposure) and (b) long term (7, 14 and 21 days of exposure) experiments for individual metals. 2D stress values are 0 for all plots. Two replicates were averaged before analysis. The trajectory shows the movement of data points over time starting from the original population (○) prior to the construction of individual treatment microcosms. Treatments are (□) control, (▲) zinc, (▼) lead (●) copper and (◇) synthetic urban runoff highly contaminated. Numbers on plot refer to days as separate treatments. For further details of individual treatments, refer to Table 3.3.

When applied individually, zinc and copper affect the bacterial population structure in two different directions forming an acute angle on the MS plots (Figure 3.11). When zinc, copper and lead are applied together, the treatment drives the community structure in another direction which appears to be consistently within that acute angle, almost forming the angle bisector (except after 1 day of exposure). Therefore, MDS plots suggest that the effect of synthetic urban runoff highly contaminated in which zinc, copper and lead are combined results mainly from the combination of the individual effects of zinc and copper with little influence of lead. The statistical analysis of differences could not be performed as only two replicates were performed per treatment due to the limited number of microcosms available for experimentation.

Changes in numbers of protozoa

In both the short and long term experiments, the concentration of protozoa decreased drastically from hundreds of protozoa per mm^3 to almost none in just a few days of exposure to the combination of all three metals (Figure 3.12). The effect of individual metals was more

moderate as after the longest period of exposure (21 days), a significant proportion of protozoa remained in all three treatments. Again, concentrations of protozoa varied greatly between experiments and sampling dates. In addition, microscopic observation of samples from the long term experiment revealed blooms of single species after 7 days under lead exposure and 14 days of copper exposure. The consistency of these blooms between replicates and their absence in the control treatment suggest the emergence of metal resilient species.

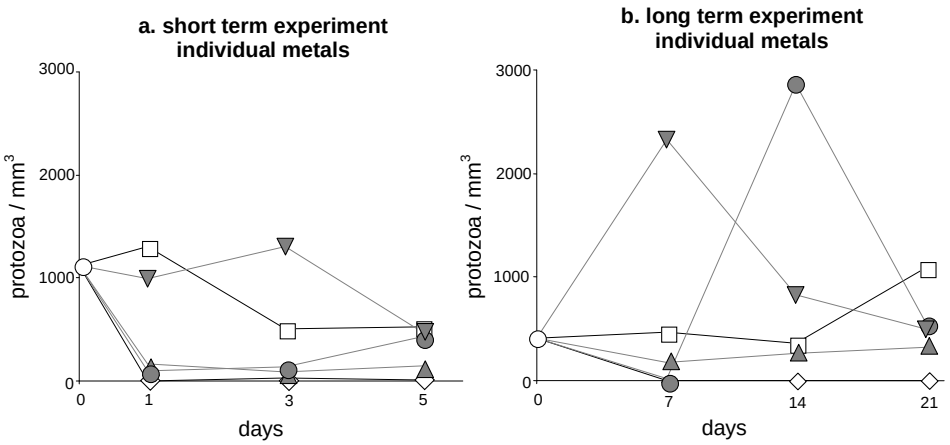

Figure 3.12: Concentrations of protozoa (protozoa / mm^3) in the biofilm for the (a) short and (b) long term experiments with individual metals. Data are concentrations of protozoa in biofilm (○) at the beginning of each experiment and in the different treatments: (□) control, (▲) zinc, (●) copper, (▽) lead and (◇) highly contaminated synthetic urban runoff containing all three metals. For further details about individual treatments refer to Table 3.3. Data points are averages of 2 replicates.

Absorption of metals from the biofilm

The comparison of the accumulation of zinc, copper and lead in biofilm when applied either individually or combined together reveals how the competition for binding sites may influence their accumulation. Figure 3.13 shows the accumulation of zinc, copper and lead in biofilm during the short term experiment when applied individually or combined with each other.

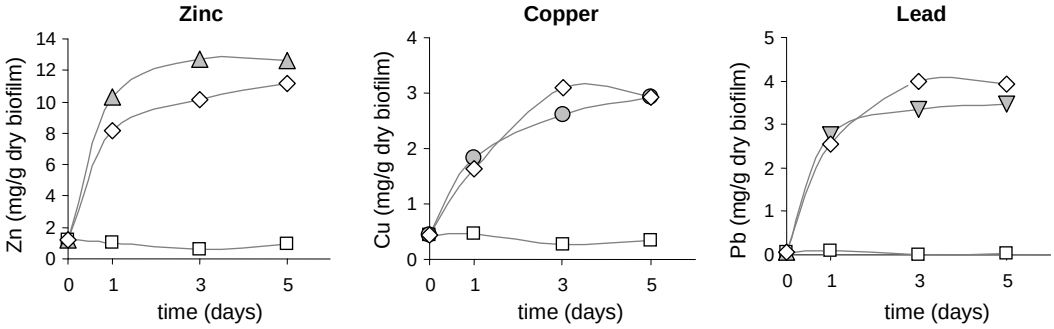

Figure 3.13: Absorption of metals in biofilm for the short term experiment with individual metals. Data are metal concentrations in biofilm (○) at the beginning of the experiment, (□) in the control treatment (no addition of metals), (△) in the treatment of zinc only, (●) in the treatment of copper only, (▽) in the treatment of lead only and (◇) in the highly contaminated synthetic urban runoff treatment (containing all three metals). For further details about individual treatments refer to Table 3.3. Data points are averages of 2 replicates.

Concentrations of zinc are consistently slightly lower in biofilm when exposed to a mixture of zinc, copper and lead than in biofilm exposed to zinc only. However, copper and lead concentrations are very similar in biofilm exposed to only one metal and in biofilm exposed to a combination of the three of them. This suggests a weak competition for binding sites and the preference for copper and lead ions compared to zinc ions.

3.4 Discussion

Experimental design

To allow a better regulation of environmental variables such as flow rate, incident light and substrate, this set of experiments was conducted in highly controlled flow chamber microcosms. This system fulfilled the requirements of this study as for each of the experiments, the biofilm obtained after 4 weeks of incubation was relatively homogeneous and remained sufficiently similar between replicates to reveal significant differences between treatments (Tables 3.4 and 3.6).

Shifts in bacterial community structure and composition

A significant variability in the response of biofilm communities originating from streams differently contaminated by heavy metal has been revealed by two previous studies (Admiraal *et al.*, 1999; Mahmoud *et al.*, 2005) and suggests the development of metal tolerant communities in permanently contaminated sites. Massieux *et al.* (2004) also revealed changes occurring in biofilm bacterial community structure under exposure to copper, using Denaturing Gradient Gel Electrophoresis (DGGE). In the present study, significant differences in bacterial community structure occurring after as little as 1 day of exposure to synthetic urban runoff, highly contaminated, confirm the rapid and high reactivity of microbial populations to metal exposure. The short lifespan of bacteria is expected to be the key contributor to the rapid modifications observed in community structure following environmental changes (Paerl & Pinckney, 1996).

In the first 3 experiments, differences in community structure initiated in the first days of metal exposure increased over time (Figure 3.1 a & b) until the longest period of exposure of 21 days. As the equilibrium between metals in water and in biofilm was reached after 7 to 14 days of exposure (Figure 3.6 a & b), differences between bacterial communities kept increasing several days after metal concentrations in biofilm had reached their maximum. Therefore, although ARISA profiles and clone libraries showed that the overall community structure and diversity were not dramatically altered after 7 days of exposure, communities subjected to different concentrations of metal continued to evolve apart. Consequently,

biofilm bacterial communities continuously exposed to urban metal contamination could potentially generate highly altered community structures with limited species diversity.

The extent of the differences in bacterial community structure between control and both moderately and highly contaminated urban runoff treatments was observed to be not simply proportional to the concentrations of metal applied (Figure 3.1). This suggested that lower concentrations of metals could trigger similar changes in bacterial community structure. However, when biofilm was exposed to a synthetic stormwater with low level of contamination (5 times less concentrated in metals than in the moderately contaminated stormwater), only little changes were observed (Figure 3.8). Therefore, all treatments suggest that there is a steep increase of toxicity between the concentrations applied in the treatments with low (100 µg/l of Zn, 10 µg/l of Cu and 10 µg/l Pb) and moderate levels of contamination (500 µg/l of Zn, 50 µg/l of Cu and 50 µg/l Pb) followed by a gentle increase of toxicity between the treatments with moderate (500 µg/l of Zn, 50 µg/l of Cu and 50 µg/l Pb) and high levels of contamination (1000 µg/l of Zn, 100 µg/l of Cu and 100 µg/l Pb).

Sequenced clone libraries of 16S rRNA gene fragments revealed the presence of both putative metal tolerant and intolerant taxa. Interestingly, the genus *Pseudomonas* which includes species that can resist much higher metal concentrations than applied in this experiment (Teitzel & Parsek, 2003) seemed to thrive under high metal concentrations (Figure 3.3). Stream biofilm provides a unique environment colonised by specific bacterial communities that have not been extensively characterized. This may explain why few other known metal tolerant taxa could be identified and that a large proportion (40 to 50%) of sequences could not be assigned to a genus. The small number of samples processed (4) and the limited number of clones analysed per sample (120) did not allow in depth analysis of the data. This highlights the use of ARISA as a convenient high throughput approach as it allows the analysis of large sample numbers and provides an extensive dataset for each of them by monitoring hundreds of potential peaks. However, besides the identification of potential sensitive and resilient bacteria, clone libraries were also adequate to reveal the decrease in bacterial diversity in the communities exposed to metals. Recent developments in high-throughput sequencing technologies could significantly increase the accuracy of this approach by facilitating the analysis of larger numbers of sequences in future studies (Mardis, 2008).

During the recovery experiment, after 7 days of exposure to metals, the biofilm was transferred back to uncontaminated water. By returning the biofilm to the high capacity flow chamber, the slides were all exposed to exactly the same physical, chemical and biological conditions. By this means, the seeding of the biofilm by lotic bacteria was the same for each slide, and bacteria that could have been excluded from the biofilm were able to return. Nevertheless, differences between exposed and unexposed populations remained visible after 14 days of recovery (Figure 3.1 c). Chemical analyses revealed that metals were released relatively slowly from the biofilm (Figure 3.6). Trapped metals remaining in the biofilm may have therefore continued to influence bacterial populations long after the slides were returned to clean water. This would suggest that stormwater contaminants have a longer lasting impact on stream ecosystems than previously considered. Alternatively, the process of succession in bacterial biofilms may be the explanation of the slow recovery. Lear *et al.* (2008a) compared established biofilm with newly forming communities in a natural stream and demonstrated that the colonising population did not develop a population structure similar to that of the mature biofilm within a period of nine weeks. In the recovery experiment, differences in bacterial populations that emerged during exposure to the metals may therefore have continued to influence subsequent bacterial growth during the period of recovery.

In stormwater receiving environments, both metal concentration in runoffs and the frequency of rain events determine the impacts on freshwater ecosystems. Considering that more than 14 days are required for the recovery of bacterial communities in that same experiment, if rain events happen more frequently, we can expect a permanent shift of bacteria towards metal tolerant populations. Furthermore the recovery rate could be overestimated in the present study. When monitoring the recovery of bacterial populations after exposure, slides were returned to a flow chamber microcosm containing water that has not previously been exposed to stormwater metals. The water was therefore likely to contain sensitive bacteria previously excluded from the exposed biofilm. In a natural stream system, the reseeding of biofilm by sensitive bacteria could be much slower due to the catchment-scale response of bacterial communities to the impacts of stormwater.

When the biofilm was exposed to zinc, copper and lead individually, ARISA data revealed the strong impact of zinc and copper on the bacterial community structure compared to a relatively small impact of lead. The effect of synthetic urban runoff containing all three metals also strongly reflected the combined effect of zinc and copper with little influence of

lead. Although lead can be toxic to freshwater fish and invertebrates (Mance, 1987), it has a greater affinity with particles than zinc and copper (Bibby & Webster-Brown, 2006). Therefore, lead ions may have quickly adsorbed on particulate matter reducing their effect on microbial community structure compared to copper and zinc ions.

The high sensitivity and the prompt response of stream biofilm bacterial populations following metal exposure support the potential use of bacterial community structure as a viable ecological indicator. In a recent study, Lear & Lewis (2009) identified differences in bacterial communities from streams impacted by different land uses (rural vs. urban). Those differences result from a cumulative effect of numerous physical and chemical parameters dictated by the catchment land use. Since trace metal concentrations are largely dependent on the degree of urbanisation in the catchment area (Chalmers *et al.*, 2007), the present study constitutes a first step in the understanding of the major drivers influencing urban stream microbial communities.

Shifts in ciliate community structure and protozoa number

RFLP profiles of ciliate 18S genes for the first three experiments (Figure 3.5) and protozoa counts for the next experiments (Figure 3.9 and Figure 3.12) consistently revealed a clear modification occurring between exposed and non-exposed populations. Even under exposure to synthetic urban runoff with low level of contamination, the number of protozoa decreased significantly compared to the control treatment within only 1 day of exposure (Figure 3.9). RFLP profiles suggested that the population exposed to a highly contaminated urban runoff remained the most similar to the initial population which may appear contradictory with microscopic observations, as they revealed a drastic decrease of protozoa under exposure to the highly contaminated treatment. However, microscopy also revealed that protozoan populations were highly dominated by flagellates and included only a small number of ciliates. This was confirmed by the small number of peaks obtained by T-RFLP (results not presented). It is therefore likely that most ciliates died quickly after exposure to higher concentrations of metals, reducing considerably the amount of DNA available for amplification. The PCR may then have amplified remains of DNA from the extinct population instead of DNA from the emerging population.

Consistent with changes occurring in bacterial communities, exposure of biofilm to individual metal contaminants suggested lead to be the less influential element as concentrations of protozoa were often higher under exposure to lead only than in the control treatment (Figure 3.12 a & b). Microscopic observation also revealed blooms of single species under exposure to copper and lead, suggesting the emergence of resilient protozoan species. Zinc, copper and lead appeared to have a strong cumulative impact on protozoan communities as they consistently caused a drastic decrease of the concentration of protozoa associated with the biofilm (Figure 3.9 a & b and Figure 3.12 a & b).

Protozoa counts helped us to interpret results obtained from the RFLP profiles and confirmed the sensitivity of protozoa to exposure to metals (Madoni & Romeo, 2006). However, the number of protozoa varied greatly between samples, sampling dates and experiments. Furthermore, blooms of single species under metal exposure suggested a deep alteration of protozoan community structure which was difficult to demonstrate from microscopic observations. Therefore, although RFLP and T-RFLP seemed to be inadequate to monitor changes in ciliate populations occurring in flow chamber microcosms, these techniques will be further investigated in following chapters as they may be more relevant for evaluation of ciliate populations in actual stream samples, as suggested in Dopheide *et al.* (2009).

Absorption/desorption of metals

In previous studies examining the sorption of metals on bacteria (Chen *et al.*, 2000; Hu *et al.*, 2005) and wastewater biofilm (Jang *et al.*, 2001), biosorption equilibrium was reached within the first few hours. In our experiments, whilst the accumulation rate was much faster in the first few days, equilibrium was not reached before 7 to 14 days. This lapse of time suggests a longer process of metal accumulation than a direct biosorption on biofilm binding sites. After exposing a thick biofilm to zinc for an hour, Hu *et al.* (2005) revealed that most of the zinc was located in a thin outer layer of 20 µm, suggesting that the penetration of metals into the deep layers of a biofilm can be retarded by binding to cells and extracellular polymers in the outer layer. Several hours or days would then be necessary for metal ions to reach the deepest binding sites. Metals may also accumulate by various slower processes of immobilization in organic or inorganic compounds and precipitation in the biofilm which could require several days to reach equilibrium (van Hullebusch *et al.*, 2003; White & Gadd, 2000).

The toxicity of trace metals depends not only on the exposure of freshwater organisms to dissolved metal ions but also on the transfer of precipitated metals throughout the food chain (Clearwater *et al.*, 2002; De Schamphelaere *et al.*, 2004). By retaining and releasing metal pollutants, biofilms play a key role in both the concentration of dissolved metals in the water column and the transfer of metal to invertebrates and fish grazing on them. In this experiment, metals accumulated to very high concentrations in the biofilm. Enrichment factors up to 500:1 for zinc, 1500:1 for copper and 6000:1 for lead were measured between the biofilm wet weight and the water (long term experiment after 21 days of exposure under synthetic urban runoff, moderately contaminated). In addition, the relatively slow release of metals during recovery in clean water supports that biofilm may have a great influence on the transfer of metals to organisms feeding on them by quickly accumulating metals during storm events and retaining them for several days after the event. These same properties support the potential use of biofilm as indicator for metal contaminants in streams (Mages *et al.*, 2004; Ramelow *et al.*, 1987). Because of the fast accumulation and the slow release of metals by biofilms, they constitute an integrative indicator of metal exposure occurring over a period of days or weeks.

3.5 Conclusion

Differences between biofilm bacterial and protozoan communities exposed to metals and control treatment became significant after only 1 to 3 days of treatment. Differences in bacterial community structure increased until the longest exposure time of 21 days and when the biofilm was returned to uncontaminated water after 7 days of exposure, differences remained detectable after 14 days of recovery. The sensitivity of stream biofilm bacterial and protozoan populations to metal exposure supports their use as an indicator of stream ecological health. The quick sorption of metals in biofilms, as well as the relatively slow release following a return to uncontaminated water, underlines the potential role biofilms could play in the retention and the release of metal contaminants in stream ecosystems and in the transfer of metals to organisms at higher trophic levels.

CHAPTER 4

4 Analysis of metal concentrations in stream biofilm and sediments and assessment of their relevance as indicators of metal pollution.

Abstract

Metals associated with sediments have traditionally been used to assess the extent of heavy metal contamination in freshwater environments. Stream biofilms present an alternative media for evaluation of metal contamination which may be more relevant to the risk incurred by the ecosystem. Zinc, copper and lead concentrations were measured in biofilms and sediments of 23 stream sites variously impacted by urbanisation. Simultaneously, biofilm was sampled and analysed for bacterial and ciliate protozoa communities as a measure of stream ecological health. Bacterial communities were analysed by ARISA and biofilm protozoan communities by T-RFLP. Statistical analysis revealed that biofilm associated metals explained a greater proportion of the variations observed in bacterial and ciliate communities than did sediment associated metals. This study suggests that the analysis of biofilm associated metals provides a better assessment of the detrimental effect of trace metals to aquatic biota.

4.1 Introduction

To assess whether heavy metals are present in the ecosystem at concentrations that are detrimental to aquatic biota, sediments are commonly analysed for metal concentrations (ANZECC & ARMCANZ, 2000; USEPA, 2005). Although less frequently used, metals associated with stream biofilms have been suggested to be an alternative indicator of metal contamination (see § 1.3.1 for more details) (Mages *et al.*, 2004; Ramelow *et al.*, 1987).

In this chapter, the difference between metal concentrations in sediments and biofilms and their respective relevance as indicators of metal impact on stream ecosystem health were investigated.

Concentrations of metals present in stream biofilm and sediments of 23 stream sites variously impacted by urbanisation were analysed and compared. As chapter 3 revealed that biofilm bacterial and ciliate communities were sensitive to metal exposure, biofilm was sampled simultaneously and analysed for bacterial and ciliate protozoa communities. Bacterial communities were investigated by ARISA and ciliate communities by T-RFLP. Bacterial and ciliate multivariate data was then statistically analysed to assess the relevance of the biofilm and sediment associated metals as indicators of the detrimental impact on aquatic biota.

4.2 Experimental design

4.2.1 Study sites

A total of twenty-three sampling sites localised in twenty different streams were identified across the Auckland Region (Figure 4.1). Sites covered all the major catchment types of the region ranging from native forest to exclusively urban catchments (See Figure 2.7 for more details). The different sites were therefore exposed to a wide range of physical and chemical parameters and the potential sources of trace metal contamination covered farming, residential areas and industry. The sites examined in this study are also part of a long-term water quality monitoring program lead by the National Institute of Water and Atmosphere (NIWA, New Zealand) for the Auckland Regional Council (ARC, New Zealand). Trends in water quality for these sites are available for 1995-2005 and provide information about catchment land use, water temperature, conductivity, pH, suspended sediments, turbidity, ammoniacal nitrogen, nitrite and nitrate, total phosphorus, dissolved reactive phosphorus and dissolved oxygen (ARC, 2007a).

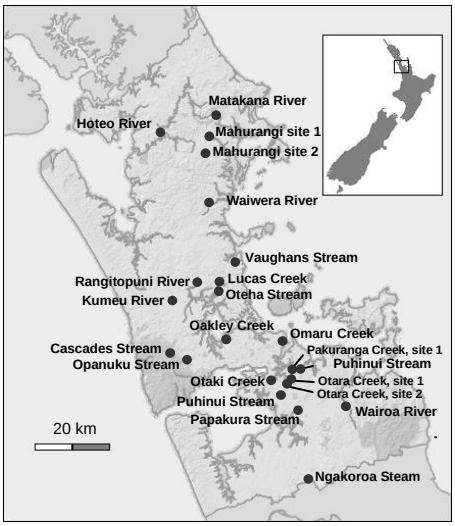

Figure 4.1: Location of sampling sites around Auckland City, New Zealand. More details about site locations can be found in ARC (2007). Mahurangi: site 1 = at Warkworth water treatment plant, site 2 = at Forestry HQ, Pakuranga: site 1 = at Greenmount Drive, site 2 = at Guy's Rd, Otara: site 1 = at Kennel Hill, site 2 = at East Tamaki Rd.

4.2.2 Sampling procedure

All sites were sampled by Dr Gavin Lear and Andrew Dopheide (University of Auckland) within one week (08.01.2008 – 11.01.2008) (Lear & Lewis, 2009). At each site, samples of both biofilm and sediments were obtained within a 30 m downstream transect. Biofilm was sampled from three rocks for metal analysis and five rocks for molecular analysis. Alternatively, when too few rocks were available, three rocks instead of five were sampled for molecular analysis. For both molecular and metal analysis, each biofilm sample was scraped off an individual rock from an area of approximately 100 cm^2 using a Speci-Sponge™ following the standard protocol (see § 2.2.1). Three samples of sediments were also obtained from each site following the standard protocol (see § 2.2.2).

4.2.3 Analysis of metal concentrations and composition

All samples for metal analysis were digested with boiling nitric acid and analysed by Flame Atomic Adsorption Spectrophotometery or Graphite Furnace Atomic Adsorption Spectrophotometery at the University of Auckland and following the standard protocol (see § 2.3.2).

To investigate the differences in composition between sediments and biofilms and their potential effect on metal accumulation, the particulate matter remaining after digestion and the proportion of it < 63 μm were quantified. After acid digestion, the remaining particulate matter of each sample was collected by filtration through a 0.45 μm cellulose membrane (Millipore Inc, USA). Using a sieve, the particles above and under 63 μm were separated. Each fraction was dried at 55°C for 24 hours and then weighed (±0.05 mg).

4.2.4 Molecular analysis

Each sample was analysed for bacterial community structure using ARISA by Dr Gavin Lear following the standard protocol (see § 2.4.3). Protozoan populations were assessed for one pooled sample per site using T-RFLP by Andrew Dopheide following the standard protocol (see § 2.4.4).

Similarities between community profiles were assessed by generating Manhattan distances between multivariate datasets. The relationship between biofilm microbial communities (bacteria and protozoa) and environmental variables was investigated by a distance-based linear model (DistLM) using PRIMER software, including the add-on PERMANOVA+ (Primer-E Ltd, UK). This model is used to provide an estimate of the influence of each environmental variable in determining the differences occurring in microbial community structure between sites.

4.3 Results

4.3.1 Comparison of metal concentrations in biofilm and sediments

Concentrations of zinc, copper and lead in both sediments and biofilms increased along the urbanisation gradient (Figure 4.2). Concentrations of zinc and copper were generally greater in biofilm than in sediments; in 18 of the 23 sites, concentrations of zinc were significantly higher in biofilms than in sediments and in 14 of the 23 sites, concentrations of copper were significantly higher in biofilms than in sediments. Lead analyses show more contrasted results as concentrations were significantly higher in biofilms than in sediments in 6 of the 23 sites but also lower in 5 of the 23 sites.

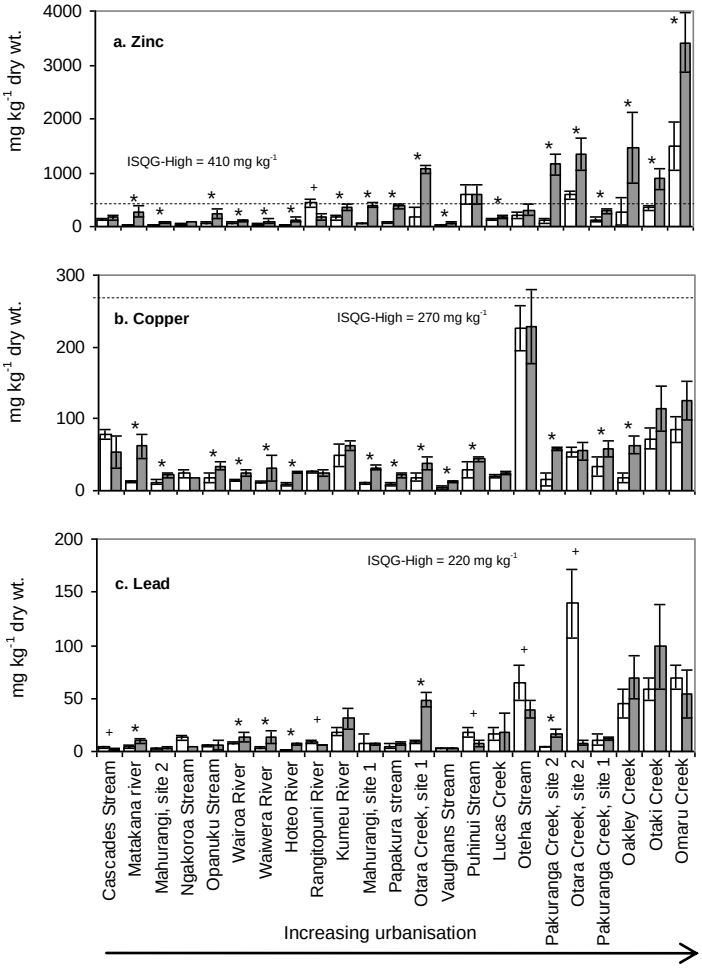

Figure 4.2: Concentrations of zinc, copper and lead (mg kg^{-1} dry wt) in (□) sediments and (■) biofilm at the 23 stream sampling sites in the Auckland Region (New Zealand). Data are averages of replicates (3) and intervals of confidence ($\alpha = 0.05$) (except for concentrations in biofilm from Ngakoroa Stream for which the data was not replicated). The dashed lines show the ANZECC high interim sediment quality guidelines (ISQG-High) which indicate possible risk to environment health (ANZECC & ARMCANZ, 2000). The urbanisation gradient is based on the proportion of catchment dedicated to urban areas (ARC, 2007). * indicates concentrations significantly higher in biofilm than in sediments and $^{+}$ indicates concentrations significantly higher in sediments than in biofilm.

The comparison of metal concentrations with ANZECC (2000) sediment quality guidelines reveals that concentrations of zinc can constitute a potential threat to aquatic ecosystems in the Auckland Region as the average concentrations in sediments were above the guideline in 4 of the 23 sites. In contrast, average concentrations of copper and lead in sediments did not exceed the guideline in any of the 23 sites.

Concentrations of all three elements in biofilms were linearly related to their concentrations in sediments (Figure 4.3). Concentrations of copper were the most strongly correlated (correlation coefficient ρ = 0.93) followed by zinc (ρ = 0.86) and lead (ρ = 0.80).

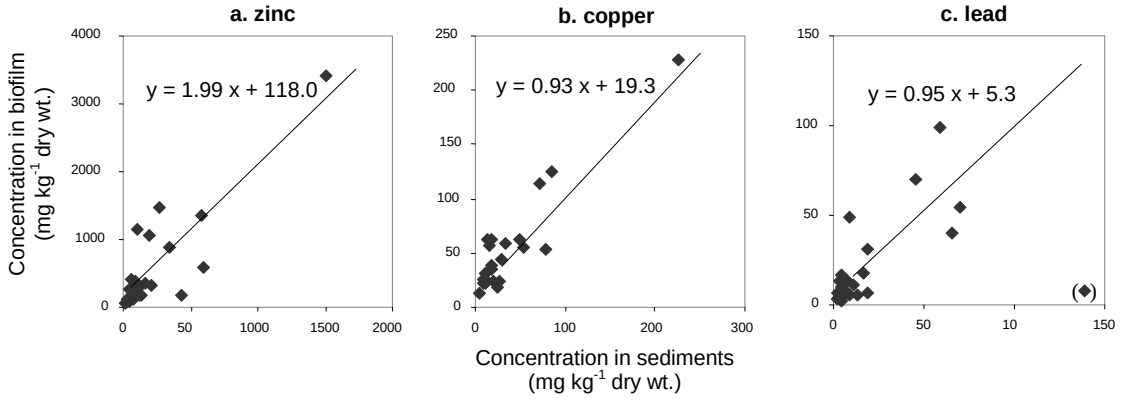

Figure 4.3: Comparison of zinc, copper and lead concentrations (mg kg^{-1} dry wt) in biofilm and in sediments. Data point in brackets was outlier and was not considered for the linear regression or for the correlation coefficient. Correlation coefficients (ρ) are: 0.86 for zinc, 0.93 for copper and 0.80 for lead.

Quantification of inorganic particulate matter remaining after acid digestion shows that biofilm samples were significantly more digested than sediment samples (student paired t-test, $p < 0.01$) (data available in appendices, Table App-2). On average, 52 % (dry wt.) of biofilm samples was lost during acid digestion compared to only 21 % of sediment samples. The proportion of fine inorganic particles < 63 μm remaining after digestion also significantly differed and constituted on average 27 % of sediment samples compared to 40 % of biofilm samples (student paired t-test $p > 0.01$). Both weight loss and particle size highlight the differences in composition between biofilm and sediment and suggest that biofilms contain more organic matter than sediments and retain more fine inorganic suspended particles.

To investigate whether the differences in composition could explain the variations in metal concentrations, the ratio (biofilm/sediments) of metal concentrations was plotted against the ratio of both weight loss by acid digestion and fine particle content for each site (Figure 4.4).

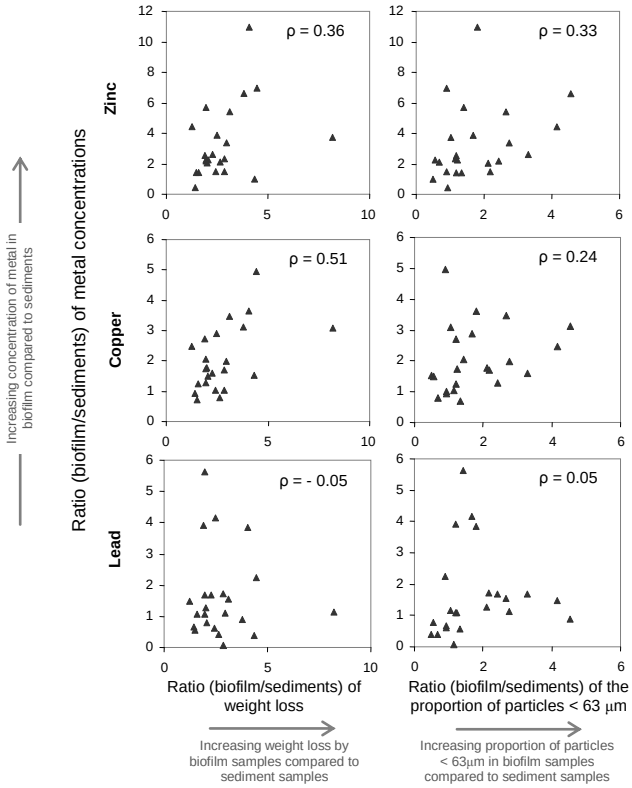

Figure 4.4: Comparison of the differences in composition between biofilm and sediments samples and the differences in metal concentrations. Y axis is the ratio between zinc, copper or lead concentrations in biofilms and their concentrations in sediments. X axis is the ratio between weight loss by biofilm samples and sediment samples (left) and between the proportion of remaining particles < 63 μm in biofilm samples and sediment samples (right) after acid digestion.

Graphs reveal that differences observed between zinc and copper concentrations in biofilm and sediments are correlated to the differences in weight loss and in fine particle (< 63 μm) content. The bigger the difference in composition between sediments and biofilm, the bigger the difference observed in metal concentrations. For both zinc and copper, the correlation is greater for weight loss than for fine particles (for zinc, ρ = respectively 0.36 and 0.33, for copper ρ = respectively 0.51 and 0.24). Again, lead showed contrasting results as no correlations were observed (ρ = - 0.05 and 0.05, respectively).

4.3.2 Influence of sediment and biofilm associated metals on microbial community structure

The ecological relevance of biofilm and sediment associated metal concentrations was assessed by their potential to explain the differences observed in biofilm bacterial and ciliate

communities using a distance based linear model (DistLM). This model provides an estimate of the influence of environmental variables (here the concentrations of metals in biofilm and sediments) in determining the variations occurring in multivariate datasets (here the structure of bacterial and ciliate communities). Results show that for all 3 elements, concentrations measured in biofilms explain a greater proportion of the variations observed in bacterial communities than concentrations measured in sediments (Table 4.1). Overall, concentrations of lead in biofilms explained the greatest proportion (8.6 %) of the differences.

Table 4.1: Relationship between differences in bacterial ARISA profiles based on Manhattan distances and concentrations of zinc, copper and lead in sediments and biofilms.

Variable	P	Proportion (%)
Zinc in sediments	0.258	5.7
Zinc in biofilm	0.068	6.6
Copper in sediments	0.665	4.5
Copper in biofilm	0.596	4.6
Lead in sediments	0.271	5.6
Lead in biofilm	0.007	8.6

Data were assessed by marginal tests using a distance based model (DistLM) and indicate the significance of the relationship (P) and the proportion of the variability in the multivariate dataset explained by the environmental variables (Proportion). Three ARISA profiles were averaged for each of the 23 sites with the exception of Vaughans Stream and Pakuranga site 2 that were excluded from the analysis because insufficient PCR product was obtained.

Similarly, a greater proportion of variation in the structure of ciliate protozoa communities could be explained by the concentrations of metals associated with biofilm than associated with sediments (Table 4.2). Concentrations of lead in biofilms again explained the greatest proportion (10.3 %) of the variations in this multivariate dataset.

Table 4.2: Relationship between differences in ciliate T-RFLP profiles based on Manhattan distances and concentrations of zinc, copper and lead in sediments and biofilms.

Variable	P	Proportion (%)
Zinc in sediments	0.516	5.6
Zinc in biofilm	0.133	7.8
Copper in sediments	0.464	5.7
Copper in biofilm	0.317	6.6
Lead in sediments	0.749	4.6
Lead in biofilm	0.025	10.3

Data were assessed by marginal tests using a distance based model (DistLM) and indicate the significance of the relationship (P) and the proportion of the variability in the multivariate dataset explained by the environmental variables (Proportion). Insufficient PCR product was obtained for Vaughans Stream, Lucas Creek, Oteha Stream, Pakuranga site 2 and Otaki Creek, which were excluded from the analysis.

Trends in water quality for the stream sites during 1995-2005 presented in ARC (2007a) were also analysed by DistLM and combined with our measurements of metal concentrations. Data show that none of the variables could explain a major proportion of the differences observed in both bacterial and ciliate communities (Table 4.3) (maximum 7.3 % of the differences in bacterial populations explained by dissolved oxygen or metals in biofilms and 10.6 % of the differences in ciliate populations explained by the catchment area). Relative to the water quality variables, the combination of zinc, copper and lead concentrations in biofilm explained a greater proportion of the differences observed in bacterial and ciliate communities (7.3 % and 8.9 % respectively) than either nitrogen, phosphorus or turbidity.

Table 4.3: Relationship between differences in bacterial and ciliate community profiles based on Manhattan distances and environmental explanatory variables.

Bacterial communities

Variable	P	Proportion (%)
Zn, Cu, Pb in biofilms	0.042	7.3
Dissolved oxygen	0.041	7.3
Water temperature	0.064	7.0
Urbanisation	0.065	6.9
Catchment area	0.146	6.1
Turbidity	0.209	5.8
Phopsphorus	0.300	5.5
Zn, Cu, Pb in sediments	0.317	5.4
pH	0.462	5.0
Conductivity	0.576	4.7
Nitrogen	0.627	4.6

Ciliate communities

Variable	P	Proportion (%)
Catchment area	0.009	10.6
Water temperature	0.044	9.7
Zn, Cu, Pb in biofilms	0.061	8.9
Nitrogen	0.176	7.4
Dissolved oxygen	0.208	7.4
Phopsphorus	0.209	7.3
pH	0.478	5.7
Zn, Cu, Pb in sediments	0.493	5.5
Urbanisation	0.635	5.1
Conductivity	0.691	5.0
Turbidity	0.733	4.6

Data were assessed by marginal tests using a distance based model (DistLM) and indicate the significance of the relationship (P) and the proportion of the variability in the multivariate dataset explained by the environmental variable (Proportion). Concentrations of zinc, copper and lead were standardised to 1 and combined in two variables; Zn, Cu, Pb in sediments and Zn, Cu, Pb in biofilms before analysis to provide overall indicators of all three metal concentrations.

4.4 Discussion

Analyses of metal concentrations revealed that zinc and copper were frequently enriched in biofilm compared to sediments. Since biofilms are intensively grazed by fish and invertebrates, this enrichment in metals could present a potential ecological threat to aquatic ecosystems that would be underestimated by the analysis of metal concentrations in sediments. These findings highlight the importance of biofilm associated metal concentrations and the need for guidelines applicable to contaminant concentrations in biofilms. The enhanced accumulation of metals in biofilms could also improve the monitoring of contaminants present at low concentrations in aquatic environments.

The quantification of particulate matter and the proportion of fine particles remaining after acid digestion suggest that biofilms generally contain more organic matter and a greater proportion of fine particles than sediments. The differences in composition could also be correlated to the differences observed in concentrations of zinc and copper, highlighting the link between composition and affinity for these metals. Differences in both organic and fine particle content may explain the enrichment of metals in biofilms. First, concentrations of metals in inorganic sediments dependent on particle size, as finer particles adsorb higher concentrations of metals (Förstner & Wittmann, 1981). A greater incorporation of fine sediments in biofilms would therefore enhance the metal retention capacity. Second, biofilm cells as well as extracellular polymeric substances (EPS) include ionisable functional groups such as carboxyl, phosphoryl, amino and hydroxyl that enable them to fix metal ions (Jang *et al.*, 2001; Sutherland, 2001a; van Hullebusch *et al.*, 2003) further enhancing the affinity of biofilms for metals.

For all three elements, zinc, copper and lead, the concentrations measured in biofilms could explain a greater proportion of the differences observed in bacterial and ciliate community structure than the concentrations measured in sediments. This confirms the greater environmental relevance of biofilm associated metals, not only for bacteria embedded in biofilms and therefore directly exposed to accumulated metals but also on mobile, ciliated protozoa that feed on biofilms. Comparison with stream water quality data reveal that although none of the parameters considered could explain a major proportion of the variations observed in bacterial and ciliate communities, the combination of metal in biofilms explained a greater proportion of the variations observed in both bacterial and ciliate

communities than commonly measured nutritional parameters such as nitrogen and phosphorus that are expected to have an important influence. This highlights the toxicity of metals in urban streams and their capacity to modify freshwater microbial community structure.

Finally, contrasting results obtained for concentrations of lead highlight the differences of behaviour between elements. Concentrations of lead were not generally higher in biofims than in sediments but they differed significantly in 12 of the 23 sites, ranging from 18 times more concentrated in sediments to 6 times more concentrated in biofilms. A great variability between concentrations in biofilm and sediments enable the DistLM analysis to differentiate more effectively the ecological relevance of each media. As the proportion of the differences observed in microbial communities explained by the concentrations of lead in biofilm was much higher than explained by the concentrations in sediments, it confirms the greater environmental relevance of metal concentrations in biofilms.

4.5 Conclusions

Concentrations of zinc, copper and lead in both sediments and biofilm increased with the degree of urbanisation in the catchment area. Significant differences were observed between concentrations of all three metals (Zn, Cu & Pb) in biofilms compared to sediments. Concentrations of zinc and copper were generally greater in biofilm than in sediments and the enrichment could be correlated to the differences in composition observed between biofilm and sediments. Concentrations of all three metals in biofilms explained a greater proportion of the variations in bacterial and ciliate protozoa communities than concentrations in sediments. This highlights the ecological importance of biofilm associated metals and suggests that their analysis provides a better assessment of the detrimental effects of heavy metals on freshwater ecosystems.

CHAPTER 5

5 Resilience of biofilm bacterial communities obtained from streams variously impacted by anthropogenic activities to common metal contaminants of urban runoff.

5.1 Abstract

In this study, I investigated whether biofilm bacterial communities obtained from impacted streams and therefore frequently exposed to anthropogenic contaminants are more resilient to exposure to metal contaminants (Zn, Cu and Pb) than communities obtained from pristine streams. Biofilm was obtained from five streams variously impacted by farming and urbanisation. Rocks covered with biofilm were collected and transferred to flow chamber microcosms in which two treatments were applied: a control treatment with no metal added and a metal contaminated treatment with addition of zinc, copper and lead at environmentally relevant concentrations. The response of biofilm bacterial communities was monitored by Automated Ribosomal Intergenic Spacer Analysis after 0, 2, 4 and 6 days of exposure to metal contaminants. Statistical analysis revealed rapid changes occurring in the structure of biofilm bacterial communities originating from the 3 least impacted streams while little changes could be detected in the biofilm obtained from the 2 most impacted streams. The results confirm the high sensitivity of biofilm bacterial communities to exposure to metal contaminants and suggest the development of more tolerant communities in streams impacted by urbanisation.

5.2 Introduction

Bacteria embedded in stream biofilms are influenced by their surrounding environment. Previous studies revealed that physical (Besemer *et al.*, 2007) and chemical (Chapters 3; Lawrence *et al.*, 2004) parameters can affect the structure of biofilm microbial communities. As human activities profoundly change stream ecosystems (Bernhardt & Palmer, 2007; Paul & Meyer, 2001), it is expected that biofilm microbial communities are also affected by these changes. In particular, anthropogenic activities often increase metal concentrations in surface water (Chalmers *et al.*, 2007; Ward & Trimble, 2003). Chapter 3 revealed that biofilm bacterial communities were affected by exposure to metal contaminants and suggested a shift towards metal tolerant communities. Therefore, in streams impacted by human activity, the frequent metal contamination may result in the development of metal tolerant biofilm bacterial communities.

The present chapter investigates further how biofilm bacterial communities are affected by urban runoff and whether the degree of human impact in the catchment of different streams influenced their resilience of to metal exposure.

Biofilm was collected from five streams variously affected by farming or urbanisation and exposed in flow chamber microcosms to two different treatments: a control treatment with no metal added and a metal contaminated treatment with addition of zinc, copper and lead. Changes in bacterial community structure in both treatments were recorded by Automated Ribosomal Intergenic Spacer Analysis (ARISA) after 0, 2, 4 and 6 days as separate treatments. Metal concentrations in biofilm and sediments from the different streams were analysed to assess the level of metal contamination in each stream. It was hypothesised that biofilm bacterial communities originating from impacted streams exhibit an increased tolerance to metal exposure.

5.3 Experimental design

Five successive experiments were undertaken in flow chamber microcosms in which biofilm originating from streams variously impacted by urbanisation and farming were exposed to heavy metals (Zn, Cu & Pb). Five streams were chosen in the Auckland Region; Cascades stream (36° 53' 12 S, 174° 31' 06" E), Karamatura Stream (37° 00' 14" S, 174° 33' 37" E), Wairoa Stream (37° 00' 46" S, 175° 03' 13" E), Waikumete Stream (36° 54' 38" S, 174° 38' 44" E) and Oakley Creek (36° 53' 02" S, 174° 42' 10" E) (for location see Figure 2.1). Cascades and Karamatura Streams are the less impacted streams with 100 % and 95 % of their catchments covered by forest, respectively. Wairoa Stream is a moderately impacted stream mainly affected by farming whereas Waikumete Stream and Oakley Creek are the most impacted streams with 71 and 98 % of their catchment covered with urban areas, respectively. Details about catchment size and land use cover are summarised in Table 5.1.

Table 5.1: Stream catchment characteristics (based on ARC, 2007a; NIWA, 2004).

		Cascades	Karamatura	Wairoa	Waikumete	Oakley
Catchment area		1454 ha	563 ha	14 820 ha	524 ha	1201 ha
Land use	% forest	100	95	43	29	2
	% farming	-	5	57	-	-
	% urban	-	-	-	71	98

At each stream location, 35 rocks (approx. 8 cm [l] x 8 cm [w] x 3 cm [h]) covered with biofilm were taken from the stream and transferred to a plastic container filled with stream water. In addition, 200 litres of stream water were collected in 30 l plastic containers. To assess the degree of metal contamination in the streams, three samples of biofilm and three samples of sediment were also obtained from each site following the standard protocol (see § 2.2.1 and § 2.2.2 respectively). Rocks, water, biofilm and sediment samples were transported to the laboratory within an hour.

In the laboratory, 10 flow chambers (low capacity, second setup, see § 2.1.1 for more details) were used to expose the biofilm to 2 different treatments: (i) a metal contaminated treatment in which of zinc, copper and lead were added to a final concentration of 200 µg/l, 20 µg/l and 20 µg/l, respectively and (ii) a control treatment with no metal added. Each flow chamber was filled with 18 l of stream water and zinc, copper and lead (as $ZnCl_2$, $CuCl_2$ and $Pb(NO_3)_2$)

were added to five randomly assigned flow chambers. After a 30 minute mixing time, three rocks covered with biofilm were transferred carefully to each flow chamber. Care was taken to keep rocks in their original upright position.

Five rocks remained unused at the beginning of each experiment and were sampled as representatives of the initial community. One rock from each flow chamber was then sampled after 2, 4 and 6 days of exposure to separate treatments. To do so, rocks were carefully removed from the water and the top was rubbed with a Speci-sponge™ following the standard protocol (see § 2.2.1). Each sample was then analysed for bacterial community structure by ARISA following the standard protocol (see § 2.4.3).

As detailed in § 2.4.3, similarities between bacterial communities were assessed by creating a matrix of Manhattan distances, visualised on a Multidimensional Scaling (MDS) plot and the significance of differences between exposed and unexposed populations was assessed by permutational ANOVA (PERMANOVA). SIMPER analysis was also performed using PRIMER© software (PRIMER-E Ltd, UK) to assess the peaks of ARISA profiles most influenced by the separate treatments.

Samples of biofilm and sediments collected for metal analysis were processed following the standard protocol (see § 2.3). Samples were pooled together to provide one sample of biofilm and one sample of sediments for each stream. Concentrations of zinc, copper and lead were then analysed by Hills Laboratories (Hamilton, New Zealand) after nitric/hydrochloric acid digestion (US EPA 200.2).

5.4 Results

5.4.1 Metal concentrations in biofilm and sediments from the streams

Concentrations of zinc, copper and lead in biofilm and sediments from each stream were analysed to assess the level of metal contamination to which inhabiting microbial communities were exposed. Results are presented in Table 5.2.

Table 5.2: Zinc, copper and lead concentrations in biofilm and sediments (mg kg^{-1} dry weight) from the five streams. Metal concentrations were analysed by ICP-MS after digestion by nitric/hydrochloric acid following US EPA 200.2 protocol.

		Cascades	Karamatura	Wairoa	Waikumete	Oakley
Biofilms	Zn (mg kg^{-1})	89	160	120	310	1100
	Cu (mg kg^{-1})	56	37	66	36	87
	Pb (mg kg^{-1})	7.6	5.7	28	52	140
Sediments	Zn (mg kg^{-1})	93	72	86	190	350
	Cu (mg kg^{-1})	58	26	22	27	25
	Pb (mg kg^{-1})	6.5	6.1	19	31	160

Concentrations of zinc, copper and lead were low in the biofilm and the sediments from the 3 least impacted streams, Cascades, Karamatura and Wairoa. The high proportion of farming in the catchment of Wairoa Stream did not seem to influence the concentrations of zinc and copper; only lead was slightly more concentrated in both biofilm and sediments from Wairoa Stream compared to Cascades and Karamatura streams. As expected, concentrations of zinc and lead were more elevated in the two streams impacted by urbanisation. In contrast, concentrations of copper were only a little more concentrated in the biofilm from Oakley Creek but did not seem to be much higher in the urban streams. Concentrations of zinc, copper and lead remained relatively low in all the streams and did not exceed ANZECC sediment quality guidelines in any of the sediment samples (ANZECC High interim sediment quality guidelines are 410 mg kg^{-1}, 270 mg kg^{-1} and 220 mg kg^{-1} for zinc copper and lead respectively (ANZECC & ARMCANZ, 2000))

5.4.2 Response of bacterial communities to exposure to zinc, copper and lead

Multidimensional Scaling (MDS) plots reveal a clear shift between the structure of exposed and unexposed biofilm bacterial communities originating from Cascades, Karamatura and Wairoa Streams after only 2 to 4 days of exposure to the individual treatments (Figure 5.1 a, b & c). In contrast, no clear distinction was apparent in biofilm bacterial communities originating from the streams with the most urbanised catchments (i.e. Waikumete Stream and Oakley Creek, Figure 5.1 d & e).

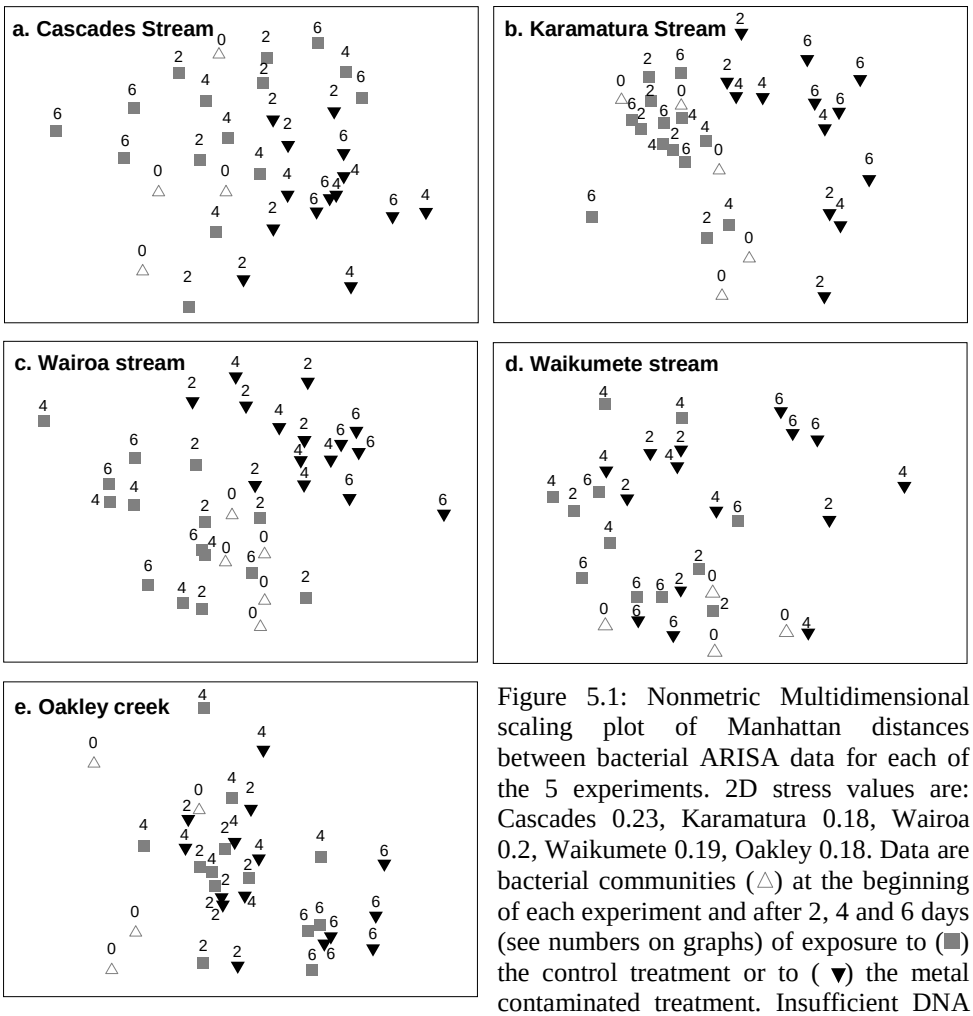

Figure 5.1: Nonmetric Multidimensional scaling plot of Manhattan distances between bacterial ARISA data for each of the 5 experiments. 2D stress values are: Cascades 0.23, Karamatura 0.18, Wairoa 0.2, Waikumete 0.19, Oakley 0.18. Data are bacterial communities (△) at the beginning of each experiment and after 2, 4 and 6 days (see numbers on graphs) of exposure to (■) the control treatment or to (▼) the metal contaminated treatment. Insufficient DNA was obtained after PCR amplification of 2 samples from Cascades Stream, 3 samples from Karamatura Stream, 4 samples from Waikumete Stream and 3 samples from Oakley Creek. Those samples were therefore excluded from the analysis.

Observations made from the MDS plots are confirmed by PERMANOVA statistical analysis (Table 5.3). Differences between exposed and unexposed communities originating from Wairoa Stream were consistently significant (P value < 0.05) from 2 to 6 days of exposure. Differences between exposed and unexposed communities originating from Cascades and

Karamatura Streams became significant after 4 days of exposure and remained significant until 6 days of exposure. In contrast, there was no significant difference (P value > 0.05) between exposed and unexposed bacterial communities originating from Waikumete Stream and Oakley Creek at any of the three exposure periods (2, 4 and 6 days).

Table 5.3: Pairwise comparison between control and metal contaminated treatment after 2, 4 and 6 days of incubation in flow chamber microcosms for each of the five streams. Data are P values from PERMANOVA analysis based on Manhattan distances between bacterial ARISA multivariate datasets. Bold characters indicate significant differences between control and exposed populations (P < 0.05).

	Cascades	Karamatura	Wairoa	Waikumete	Oakley
2 days	0.09	0.06	**0.01**	0.23	0.52
4 days	**0.01**	**0.03**	**0.01**	0.17	0.77
6 days	**0.03**	**0.01**	**0.01**	0.19	0.09

In Figure 5.2, changes in dissimilarity between exposed and unexposed communities have been represented after 2, 4 and 6 days of exposure for all five streams. The graph reveals that biofilm bacterial communities from both Wairoa and Karamatura Streams experienced the biggest modifications. Marked changes also occurred in the communities originating from Cascades Stream. Differences between exposed and unexposed bacterial communities originating from these 3 streams increased over time. A small but consistent difference between exposed and unexposed communities originating from Waikumete Stream could also be observed while communities originating from Oakley Creek remained totally unchanged.

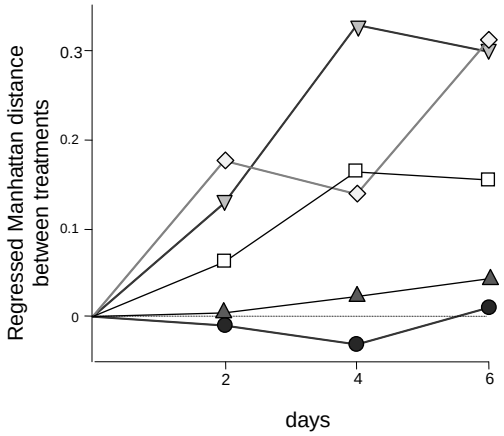

Figure 5.2: Temporal change in dissimilarity between exposed and unexposed communities for each of the 5 streams. The regressed Manhattan distance is the distance between samples exposed to different treatments reduced by the distance between samples exposed to identical treatments. The distance was calculated for each possible combination of samples and then averaged. Streams are: (□) Cascades Stream, (◇) Karamatura Stream, (▽) Wairoa Stream, (▲) Waikumete Stream and (●) Oakley Creek.

The effect of metal exposure on the diversity of biofilm bacterial communities was also investigated and results are presented in Table 5.4. The number of ARISA peaks detected increased for all five streams under exposure to metals. The evenness of the communities also generally increased except for communities originating from Karamatura Stream in which the evenness decreased only very slightly (0.849 versus 0.852) (Pielou's evenness $= \frac{H'}{H'max}$ where *H'* is the Shannon diversity index and *H'max* is the maximum value of *H'*. Values range from almost 0 [species present in very different numbers] to 1 [all species present in identical numbers]). The diversity of bacterial communities estimated by Shannon diversity index was greater under exposure to metals than in the control treatment for all five streams (Shannon diversity $H' = \sum_{i=0}^{S}(P_i \ln(P_i))$ where P_i is the relative abundance of each species or peak and S the total number of species or peaks).

Table 5.4: Diversity and evenness of biofilm bacterial communities from the five different streams at the beginning of each experiment (initial) and after exposure to the control treatment (control) or to the metal contaminated treatment (metal). Values are based on bacterial ARISA profiles and averaged for samples from three sampling dates.

Stream	Treatment	Number of peaks	Pielou's evenness	Shannon diversity index
Cascades	Initial	65	0.846	3.45
	Control	59	0.884	3.54
	Metal	65	0.891	3.71
Karamatura	Initial	114	0.835	3.96
	Control	107	0.852	3.94
	Metal	129	0.849	4.13
Wairoa	Initial	120	0.869	4.15
	Control	128	0.845	4.08
	Metal	133	0.861	4.21
Waikumete	Initial	74	0.861	3.68
	Control	65	0.836	3.48
	Metal	68	0.862	3.63
Oakley	Initial	57	0.826	3.32
	Control	70	0.891	3.77
	Metal	73	0.895	3.80

In Figure 5.3 samples from all five streams have been analysed together and represented on a unique MDS plot. This enables us to visualise the similarity between communities originating from different streams and to investigate whether the changes triggered by exposure to metals modified the communities towards a similar population profile. The plot reveals that initial bacterial communities originating from various streams were profoundly different. Communities originating from Cascades Stream and Oakley Creek appear relatively similar on the general MDS plot. However, this is mainly due to the two dimensional constraint as

communities from Cascades Stream are only slightly more similar to communities from Oakley Creek than to the communities from the 3 other streams (average Manhattan distance between Cascades and Oakley = 0.82 compared to 0.90 between Cascades and Karamatura, 0.92 between Cascades and Wairoa and 0.89 between Cascades and Waikumete, the Manhattan distance going from 0 [identical communities] to 1 [communities totally different]). To visualise the differences between the communities from Cascades Stream and Oakley Creek, the samples have been re-plotted in an MDS subset, independently of the 3 other streams (Figure 5.3, MDS subset). Changes caused by exposure to metals did not appear to modify the distinct communities towards the same direction or towards a similar community to those present in more impacted streams (i.e. Waikumete Stream and Oakley Creek).

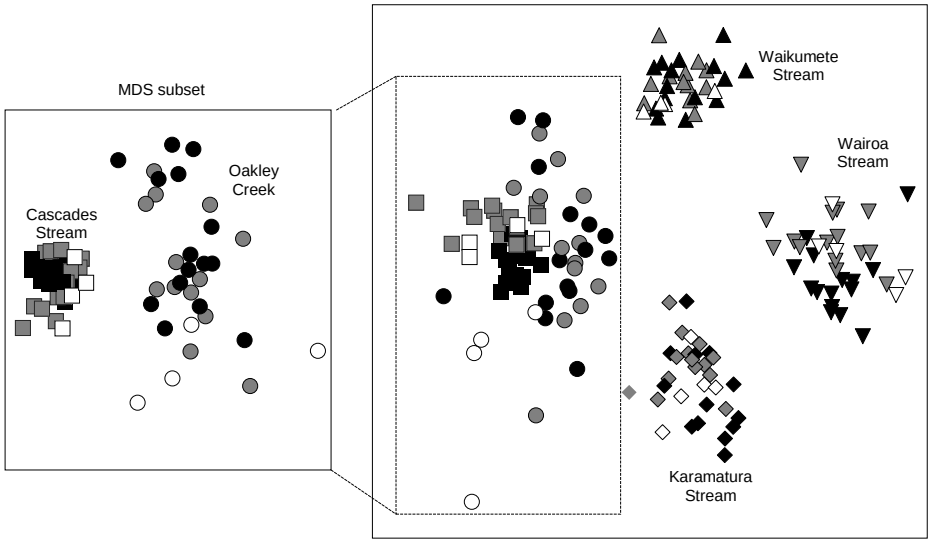

Figure 5.3: Nonmetric Multidimensional Scaling plot of Manhattan distances between bacterial ARISA data of all samples. As communities from Cascades Stream and Oakley Creek overlapped in the general MDS plot, they have been re-plotted in an MDS subset, independently of the 3 other streams. General 2D stress = 0.22 and 2D stress of MDS subset = 0.17. Data are (□) Cascades Stream, (◇) Karamatura Stream, (▽) Wairoa Stream, (△) Waikumete Stream, (○) and Oakley Creek. Colours are (□) initial community, (■) control treatment and (■) metal contaminated treatment.

5.5 Discussion

Shifts occurring in bacterial community structure originating from the three least impacted streams; Cascades, Karamatura and Wairoa Streams, confirm the sensitivity of biofilm bacteria to metal exposure as highlighted in chapter 3 and Massieux *et al.* (2004). In the present experiment, initial mature biofilm was obtained by collecting rocks from the streams

and not by incubation of artificial substrate in flow chambers like in chapter 3. As rocks extracted from the streams had various shapes and may have been located in areas under different light exposure or flow rate, a greater heterogeneity in initial biofilm bacterial communities was expected. Concentrations of zinc, copper and lead used in this experiment were also set at relatively low levels which could frequently occur in actual urban runoff. Nevertheless, significant differences could be revealed after only 2 or 4 days of exposure to individual treatments. These results confirm the high sensitivity of natural biofilm bacterial communities to urban runoff metal contaminants and support their use as bio-indicators.

The comparison of all samples on a single MDS plot (Figure 5.3) revealed that metal exposure did not drive the communities towards the same direction. This suggests that initial communities originating from the different streams contained distinct sets of metal sensitive and resilient bacteria. The great diversity of bacterial communities between streams was confirmed by their distinct clustering on the MDS plot.

Changes in bacterial community structure caused by exposure to metals appear to be relatively moderate compared to the great differences naturally existing between bacterial communities from different streams. However, the dissimilarity between bacterial populations continued to increase until the longest period of exposure applied in this experiment (6 days) (Figure 5.2). Chapter 3 also revealed that bacterial communities exposed to metals were still evolving further apart after 21 days of exposure. A continuous exposure to metal contaminants could therefore result in highly altered biofilm bacterial communities. Furthermore, zinc, copper and lead represent only a small part of common contaminants of urban runoff. In urban runoff receiving streams, the cocktail of all organic and inorganic pollutants may affect biofilm bacterial communities to a much greater extent.

The analysis of the changes reveals a small but consistent increase in diversity of bacterial populations under metal exposure. As changes occurred in bacterial community structure but did not lead to a decrease in diversity, the results suggest a reorganisation of the community and the replacement of sensitive bacteria by more tolerant bacteria. The resulting biofilm community impacted by metal exposure was therefore not just a subset of the initial community constituted of only the most resilient bacteria but a differently organised

community including previously unobserved species. As metals accumulate first in the outer layer of the biofilm (Hu *et al.*, 2005), exposure to metals could also have created a localised contamination of the biofilm with higher concentrations on the surface. This may have opened new niches for metal tolerant species in some areas of the biofilm without affecting the communities present in others.

The increase in diversity of bacterial populations under metal exposure may appear contradictory with the decrease in diversity observed in chapter 3 (see § 3.4.1). However, metal concentrations formerly used were higher (2.5 and 5 times higher) than the concentrations used in the present experiment. Therefore, the increase in diversity suggests that the relatively low concentrations of metals used in this experiment created a moderate stress on bacterial communities which might have supported the development of low number species without eradicating more sensitive species. In contrast, the decrease in diversity observed previously under higher metal concentrations suggested that the threshold of acute toxicity for multiple bacteria species was reached, leading to their quick extinction. This 'hump-backed' model of the impact of stress factors on microbial diversity has already been suggested by Giller *et al.* (2009) and Feris *et al.* (2009).

Biofilm microbial communities frequently exposed to specific contaminants are expected to exhibit a greater tolerance to these contaminants. Previous studies have revealed the increased tolerance of biofilm bacterial (Admiraal *et al.*, 1999; Lehmann *et al.*, 1999) and algal (Admiraal *et al.*, 1999; Lehmann *et al.*, 1999; Soldo & Behra, 2000) communities previously exposed to high metal concentrations by testing their metabolic response during metal exposure. However, it is still not clear whether the metabolic tolerance results from the replacement of sensitive species by more tolerant species, as suggested in chapter 3, or from other physiological and structural adaptation mechanisms. In the present experiment, although rapid changes caused by metal exposure were observed in the communities originating from Cascades, Karamatura and Wairoa Streams, no significant difference could be revealed in the communities originating from the most impacted streams; Waikumete Stream and Oakley Creek. The difference in sensitivity between distinct biofilm bacterial communities suggests that the frequent exposure to metal contaminants in impacted streams induces an increased tolerance of bacterial communities against these pollutants. These

results corroborate the findings of Admiraal *et al.* (1999) and Lehmann *et al.* (1999) that revealed the decreased metabolic response (to metal exposure) of biofilm bacterial communities previously exposed to high metal concentrations. In the present experiment, by using a molecular community fingerprinting technique (ARISA), we demonstrate that the tolerance induced is not only metabolic but also in the structure of the constituting communities.

5.6 Conclusions

Rapid changes occurring in the structure of biofilm bacterial communities obtained from the three least impacted streams confirm the sensitivity of natural biofilm communities to urban runoff contaminants and their potential use as bio-indicators. The increased tolerance of bacterial communities originating from more impacted streams also suggests that the frequent exposure to metal contaminants induces an increased tolerance of bacterial communities against these pollutants.

CHAPTER 6

6 Effect of stormwater on biofilm bacterial communities investigated in flow chamber microcosms.

Abstract

In this chapter, we investigated how stormwater affects bacterial communities embedded in stream biofilms. Stormwater was collected during heavy rainfall from different areas of Auckland City (New Zealand) and mature biofilm was exposed to the different stormwaters in flow chamber microcosms. Changes in bacterial community structure were monitored after 0, 3 and 6 days of exposure by Automated Ribosomal Intergenic Spacer Analysis. A control treatment (using the water in which the biofilm was grown) and a treatment with synthetic stormwater simulated by addition of zinc, copper and lead enabled the comparison of the changes triggered by the different treatments with previous findings. The concentrations of a wide range of contaminants present in the different stormwaters were analysed and the correlation between the response of bacterial communities and the concentrations of contaminants was investigated. Results revealed rapid changes in bacterial populations occurring under exposure to all the different stormwater treatments. Analyses of contaminants highlighted the diversity of pollutants present in urban runoff, the predominant effect of nutrients but also the complex co-influence of all the contaminants on bacterial communities.

6.1 Introduction

In urban areas, rainwater flows onto impervious surfaces before entering the stormwater system and being drained to the nearby stream. During the process, the water accumulates a wide range of pollutants from almost every point of the drainage area. These contaminants are carried by the stormwater to the receiving streams and can have detrimental effects on the ecosystem. Potential contaminants are very diverse and can originate from multiple sources (e.g. galvanised roofs, road bitumen and tyres are sources of metals, organic litter and fertilisers used in gardens are sources of nutrients). The effect of urban runoff on aquatic biota results from the combined influence of all the different contaminants present in the water.

In chapter 3, the effect of specific urban runoff contaminants (zinc, copper and lead) on biofilm microbial communities was investigated in detail. Results highlighted that exposure to environmentally relevant concentrations of these urban contaminants can modify the structure of biofilm bacterial communities. In natural streams, the effect of actual stormwater on microbial populations will result from the complex interaction of a wide range of contaminants including, but not limited to, zinc, copper and lead. Therefore, the present chapter investigates the effect of complex urban runoff on biofilm bacterial communities and enables comparison with the specific effect of zinc, copper and lead.

Stormwater was collected during heavy rainfall from streams or stormwater outlets from 8 areas of Auckland City variously impacted by urbanisation. Mature biofilm was exposed to the different stormwaters in flow chamber microcosms and changes in bacterial communities were monitored by Automated Ribosomal Intergenic Spacer Analysis (ARISA) after 0, 3 and 6 days of exposure to the treatments. A control treatment (using the water in which the biofilm was grown) and a synthetic stormwater treatment simulated by addition of zinc, copper and lead (at the same concentrations as the previous moderately contaminated treatment) enabled comparison of the observed responses. The concentrations of nitrogen and phosphorus compounds, dissolved and particulate organic carbon, hydrocarbons and metals (As, Cd, Cr, Cu, Pb, Ni and Zn) present in the stormwater samples were analysed and the correlation between the response of bacterial communities and the concentrations of contaminants was investigated statistically. Bacteria naturally present in the water were also collected and analysed by ARISA to investigate their influence on biofilm microbial communities.

6.2 Experimental design

Mature biofilm was obtained by incubation of 65 sandblasted glass slides (65 mm x 140 mm) in the high capacity flow chamber (see § 2.1.1 for more details). The flow chamber was filled with water from Opanuku Stream (Latitude 36°53'42 S, Longitude 174°35'44 E and see Figure 2.7) which is located in a relatively unmodified catchment and is unlikely to contain significant concentrations of urban runoff contaminants (for details about catchment and stream water characteristics, refer to ARC (2004b)). The incubation period for this experiment was longer than in previous experiments (34 days instead of 28 days) because of the unpredictable nature of rainfall.

Stormwater or stream water was collected from 8 sites (Table 6.1) around Auckland City on the 8th and 9th of April 2009. A total of 12 to 24 mm of rainwater fell during that period which was preceded by a period of 20 days without rainfall > 1 mm (data from Oteha, Rowe and Swanson sites, ARC, environmental data online, http://maps.auckland.govt.nz/auck-landregionviewer/?widgets=hydrotel). Twenty-five litres of water were collected from each site and stored at ambient temperature until the beginning of the experiment.

Table 6.1: Site locations and catchment characteristics.

Site	Location	Dominant land uses in catchment
Albany busway stormwater outlet	36° 43' 11" S, 174° 42' 35" E	Transport and commercial
Mission Bay stormwater outlet	36° 50' 55" S, 174° 49' 42" E	Residential
Tamaki stormwater outlet	36° 53' 26" S, 174° 52' 00" E	Residential and industrial
Sylvia Park stormwater outlet	36° 54' 59" S, 174° 51' 02" E	Industrial and commercial
Lucas Creek	36° 43' 11" S, 174° 42' 32" E	Residential and forested
Wairau Creek	36° 46' 47" S, 174° 45' 04" E	Industrial, commercial and residential
Oakley Creek	36° 53' 02" S, 174° 42' 10" E	Residential
Cascades Stream	36° 53' 12" S, 174° 31' 06" E	Forested

Ten low capacity flow chambers (second setup, see § 2.1.1 for more details) were used to expose mature biofilm to different treatments. Each of them was filled with 18 litres of storm or stream water collected from one of the 8 different sites. The 2 remaining flow chambers were used to apply a control and a synthetic stormwater treatment. Both chambers were filled with water from the high capacity flow chamber in which the slides were incubated (water originally from Opanuku Stream). The synthetic stormwater was simulated by addition of zinc, copper and lead (as $ZnCl_2$, $CuCl_2$ and $Pb(NO_3)_2$) to a final concentration of 500 µg/l, 50 µg/l and 50 µg/l, respectively.

At the beginning of the experiment, five slides were removed carefully from the high capacity flow chamber and sampled for molecular analysis. The remaining slides were distributed evenly across low capacity flow chambers (6 slides per chamber). After 3 and 6 days of exposure to the different treatments, 3 slides were sampled from each flow chamber. Biofilm was sampled using Speci-sponges™ and bacterial community structure assessed by ARISA following the standard protocol (see § 2.2.1 and § 2.4.3 for more details).

To investigate which contaminants triggered the changes in biofilm bacterial communities, the concentrations of a wide range of compounds present in each water used as a treatment were analysed. Nitrogen (nitrite, nitrate, ammonium, and organic nitrogen), phosphorus (total and dissolved reactive) and organic carbon (total and dissolved) were analysed by Hill Laboratories (New Zealand). The methods used are summarised in Table 6.2. Concentrations of recoverable As, Cd, Cr, Cu, Pb, Ni and Zn were also analysed by Hill Laboratories (see § 1.3.1 for more details). The water was collected in polyethylene bottles for nitrogen and phosphorus analyses and in glass bottles for organic carbon analysis. The pH of the water was also measured using a multimeter (Multi350i, WTW, Germany).

Storm and stream water used as treatments contained a wide range of naturally present bacteria that could potentially compete with or replace biofilm resident bacteria. To assess the extent to which they influenced biofilm bacterial communities, planktonic bacterial populations present in the water were also analysed by ARISA. Water (200 ml) from each treatment was collected and centrifuged at 8000 x g for 20 minutes. The pellet was transferred to a 1.5 ml microcentrifuge tube and processed in an identical manner to biofilm samples.

Table 6.2: Methods used for water quality analysis by Hill Laboratories (Hamilton, New Zealand, http://www.hill-laboratories.com).

Analysis	Method
Total kjeldahl nitrogen	Sulphuric acid digestion with copper sulphate catalyst Phenol/hypochlorite colorimetry
Nitrite-N and Nitrate-N	Total oxidised nitrogen with cadmium reduction and Azo dye colorimetry
Total nitrogen	Total kjeldahl nitrogen + Nitrate-N + Nitrite-N
Total ammoniacal-N	Filtration, phenol/hypochlorite colorimetry
Total phosphorus	Acid persulfate digestion, ascorbic acid colorimetry
Dissolved reactive phosphorus	Filtration, Molybdenum blue colorimetry
Dissolved organic carbon	Filtration, catalytic oxidation and infrared detection
Total organic carbon	Catalytic oxidation, infrared detection
Heavy metals	Nitric/hydrochloric acid digestion, ICP MS

As detailed in § 2.4.3, similarities between ARISA community profiles were assessed by Manhattan distances and visualised on a nonmetric Multidimensional Scaling (MDS) plot. Additional statistical analyses were also performed using PRIMER (Primer-E Ltd, UK) software to further assess the influence of the different contaminants. A distance-based linear model (DistLM) was used to investigate the relationship between biofilm bacterial communities and water analyses. The influence of bacteria present in the water was also investigated using SIMPER analysis to reveal the most influential ARISA peaks in the differentiation between groups of samples.

6.3 Results

Concentrations of contaminants

Water analysis results are presented in Table 6.3. As expected, the different types of water used as treatments contained very diverse concentrations of most contaminants and concentrations often exceeded ANZECC water quality guidelines [trigger values for New Zealand slightly disturbed ecosystems, upland rivers (ANZECC & ARMCANZ, 2000)]. For example, total nitrogen ranged from 0.11 mg/l in Cascades Stream water to 2.10 mg/l in Sylvia Park stormwater, well above the guideline of 0.295 mg/l. Total phosphorus ranged from 0.01 mg/l in Cascades Stream water to 0.36 mg/l in Tamaki stormwater, again well above the guideline set at 0.026 mg/l. Concentrations of arsenic, cadmium and nickel were always under their respective guideline but concentrations of all other metals outreached the guidelines in several types of water. Copper ranged from 0.91 µg/l in Cascades Stream water to 14 µg/l in Albany busway stormwater exceeding the guideline of 1.4 µg/l, lead varied from below detection limit in Cascades Stream water to 10 µg/l in Albany busway stormwater well above the guideline of 3.4 µg/l and zinc ranged from 2.3 µg/l in Cascades Stream water to 330 µg/l in Sylvia Park stromwater, far exceeding the guideline of 8 µg/l. The concentrations of Zn, Cu and Pb added to simulate a moderately contaminated urban runoff exceeded the concentrations found in all the different types of stormwater collected.

Table 6.3: Concentrations of contaminants in the different stormwater treatments to which biofilm was exposed in flow chamber microcosms. ANZECC trigger values (ANZECC & ARMCANZ, 2000) are also stated. Values exceeding the trigger values are shown in bold.

	Control	Cascades Stream	Zn Cu Pb [1]	Lucas Creek	Wairau Creek	Oakley Creek	Albany Busway stromwater	Mission Bay stormwater	Tamaki stormwater	Sylvia Park stormwater	Detection limit	ANZECC trigger value [2]
Total nitrogen (g/m^3)	0.23	0.11	0.23	**0.32**	**0.62**	**0.86**	**0.70**	**1.30**	**1.90**	**2.10**	0.05	0.295
Total kjeldahl nitrogen (g/m^3)	0.23	0.11	0.23	0.29	0.39	0.34	0.58	0.41	1.20	0.84	0.1	-
Nitrite-N (g/m^3)	n.d.	n.d.	n.d.	n.d.	0.010	0.009	0.012	0.024	0.023	0.020	0.002	-
Nitrate-N (g/m^3)	n.d.	n.d.	n.d.	n.d.	**0.2**	**0.5**	0.1	**0.8**	**0.7**	**1.2**	0.002	0.167 [3]
Total ammoniacal-N (g/m^3)	n.d.	n.d.	n.d.	0.010	**0.017**	**0.029**	**0.037**	**0.049**	**0.530**	**0.150**	0.010	0.010
Total phosphorus (g/m^3)	0.01	0.01	0.01	0.02	**0.06**	**0.05**	**0.09**	**0.10**	**0.36**	**0.12**	0.004	0.026
Dissolved reactive phosphorus (g/m^3)	n.d.	n.d.	n.d.	n.d.	0.02	0.01	n.d.	0.08	0.14	0.06	0.004	-
Dissolved organic carbon (g/m^3)	5.0	4.0	5.0	6.9	4.5	3.5	5.6	4.3	11.0	8.8	0.5	-
Total organic carbon (g/m^3)	5.3	5.0	5.3	6.9	7.8	4.1	11.0	5.1	13.0	11.0	0.5	-
Arsenic (µg/l)	n.d.	n.d.	n.d.	1.2	1.5	n.d.	n.d.	2.7	1.6	1.2	0.001	24
Cadmium (µg/l)	0.13	n.d.	0.12	n.d.	0.06	n.d.	n.d.	n.d.	n.d.	0.06	0.00005	0.2
Chromium (µg/l)	0.74	n.d.	0.68	0.59	**1.40**	**1.20**	**2.20**	**1.50**	1.00	0.70	0.0005	1
Copper (µg/l)	**7.3**	0.9	**55.0**	1.3	**7.6**	**5.0**	**14.0**	**9.3**	**11.0**	**5.5**	0.0005	1.4
Lead (µg/l)	0.81	n.d.	**56.00**	0.14	**4.00**	3.00	**10.00**	0.98	**4.50**	1.80	0.0001	3.4
Nickel (µg/l)	1.3	n.d.	1.5	1.8	1.3	1.1	2.4	n.d.	2.2	1.1	0.00053	11
Zinc (µg/l)	**24.0**	2.3	**470.0**	2.7	**250.0**	**25.0**	**110.0**	**19.0**	**95.0**	**330.0**	0.001	8
pH	7.38	7.82	7.38	7.55	**7.29**	7.81	**7.25**	7.45	**7.31**	7.39	-	7.38-7.82

n.d. : below detection limit.
[1] Zn Cu Pb = synthetic stormwater moderately contaminated.
[2] Trigger values for New Zealand slightly disturbed stream ecosystems, upland rivers, (ANZECC & ARMCANZ, 2000)).
[3] Trigger value for nitrate + nitrite.

Changes in bacterial community structure

The MDS plot presented in Figure 6.1 reveals the extent of the changes occurring under the different treatments and the relative direction in which the communities are modified. In addition, the average numerical distance between the initial community and the communities exposed to the different treatments are represented in Figure 6.2. Both figures demonstrate that Albany busway stormwater and Sylvia Park stromwater caused the biggest changes in bacterial community structure. Mission Bay stormwater, Tamaki stormwater and Lucas Creek water caused an intermediate impact on bacterial communities while the remaining treatments had the smallest impact. Figure 6.2 also reveals that the water collected from a pristine stream (Cascades Stream treatment) and therefore likely to contain very low levels of contaminants, caused the smallest impact on bacterial communities of all stormwater treatments but still generated a distance more than two times greater than the control treatment.

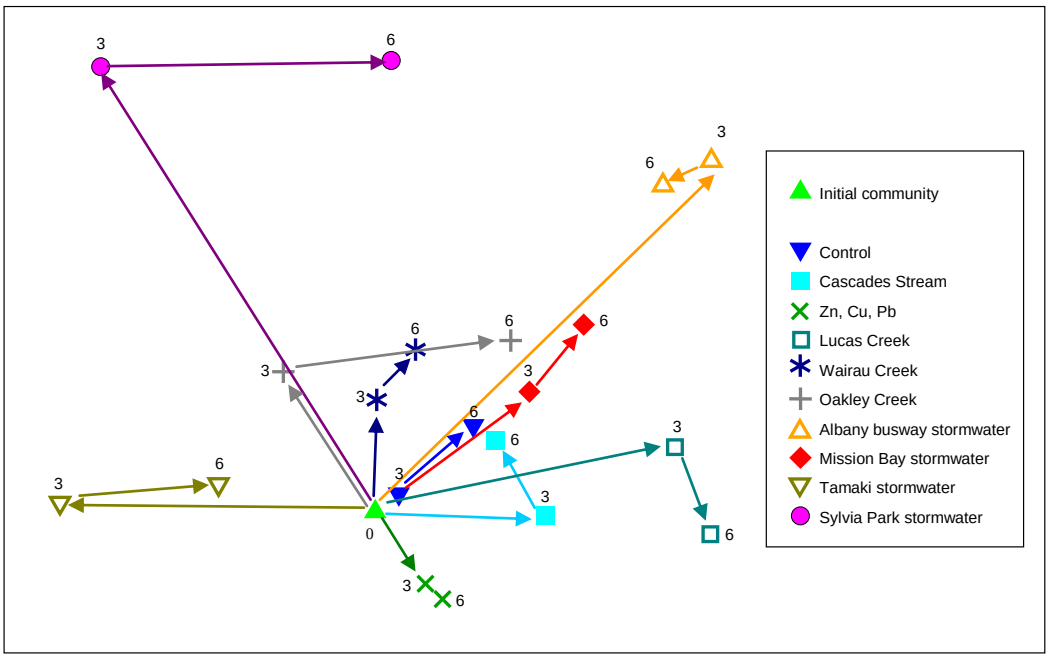

Figure 6.1: Nonmetric Multidimensional Scaling plot of Manhattan distances between bacterial ARISA profiles before and after 3 and 6 days of exposure to different stormwater treatments (see legend on graph). 2D stress value is 0.15. Three samples were averaged before analysis for each data point. Numbers on plot refer to days of exposure to separate treatments and the trajectories show the relative changes in bacterial community structure.

The direction of the changes reveals that the treatments altered the populations in very diverse ways as exposed communities are spread relatively evenly around the initial populations on the MDS plot. The treatment with synthetic urban runoff simulated by addition of Zn, Cu and Pb generated a distance similar to Cascades Stream or Wairau Creek treatments (Figure 6.2) but influenced the community structure in a different way than all other treatments (Figure 6.1).

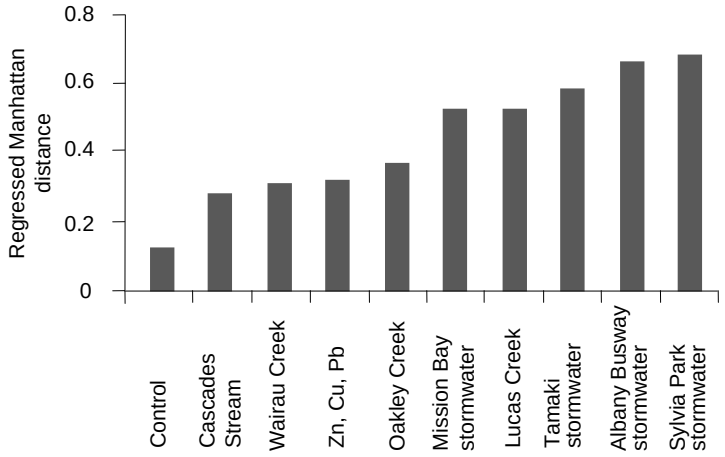

Figure 6.2: Increasing dissimilarity between initial communities and communities exposed to the different treatments. The regressed Manhattan distance is the distance between initial communities and exposed communities reduced by the distance within initial communities. The distance was calculated for each possible combination for both sampling dates (3 and 6 days) and averaged.

Relative influence of stormwater contaminants

The relationship between water analyses and the changes occurring in biofilm bacterial community structure was investigated by a distance based linear model (DistLM). The model estimates the proportion of the changes in bacterial community structure explained by the different characteristics of the water and their statistical significance (Table 6.4). As a result of the small number of samples analysed in this experiment, the proportions of the variation explained are overestimated and are therefore presented only as relative indicator. As expected, a strong correlation existed between some of the compounds present in the water (for instance between total nitrogen and total Kjeldahl nitrogen). In order to simplify the model, the highest ranked of each nitrogen, phosphorus and carbon based compounds was selected for analysis. Total nitrogen was selected to represent total Kjeldahl nitrogen, nitrite-N, nitrate-N and total ammoniacal-N, total phosphorus was selected to represent dissolved reactive phosphorus and total organic carbon was selected to represent dissolved organic carbon.

Table 6.4: Influence of water quality parameters on bacterial community structure revealed by DistLM analysis. Samples from the same treatment and sampling occasion were averaged before analysis. The proportion was then averaged from both sampling occasions (3 and 6 days). Due to the small number of samples analysed, the proportions are overestimated and are only presented as a relative indication of the influence of each factor.

Rank	Variable	Proportion (%)
1	total nitrogen	19
2	total phosphorus	16
3	total organic carbon	16
5	chromium	13
6	cadmium	12
7	zinc	10
8	lead	10
9	copper	10
10	nickel	10
11	arsenic	9
12	pH	9

DistLM analysis reveals that nitrogen, phosphorus and carbon based compounds generated the highest proportion of the changes in biofilm bacterial community structure. In comparison, metals appeared to affect bacterial communities to a smaller extent. Metals present in a wide range of concentrations and exceeding the ANZECC guidelines in some of the treatments (i.e. Cr, Cu, Pb and Zn) had a greater effect on bacterial community structure than metals present in moderate concentration and never exceeding ANZECC guidelines (i.e. As and Ni). Cadmium is an exception as it had a relatively large effect on bacterial community structure although it was never present in concentrations exceeding ANZECC guidelines. Finally the pH of the water seems to be the least influential of all the parameters. This may be explained be the small range of pH (7.25-7.82) covered in the different treatments.

Bacteria naturally present in the stream or storm water were analysed by ARISA to investigate their influence on biofilm bacterial communities. Differences between bacteria present in the water and bacteria embedded in the biofilm were assessed by Manhattan distances and visualised on a single MDS plot (Figure 6.3). The graph reveals that all communities of bacteria present in the water (including in the water of the control treatment) cluster far apart from communities embedded in the biofilm. More importantly, the treatments did not modify biofilm bacterial communities in the direction of the bacterial communities present in the water which suggests that bacteria naturally present in the water did not influence strongly biofilm bacterial populations.

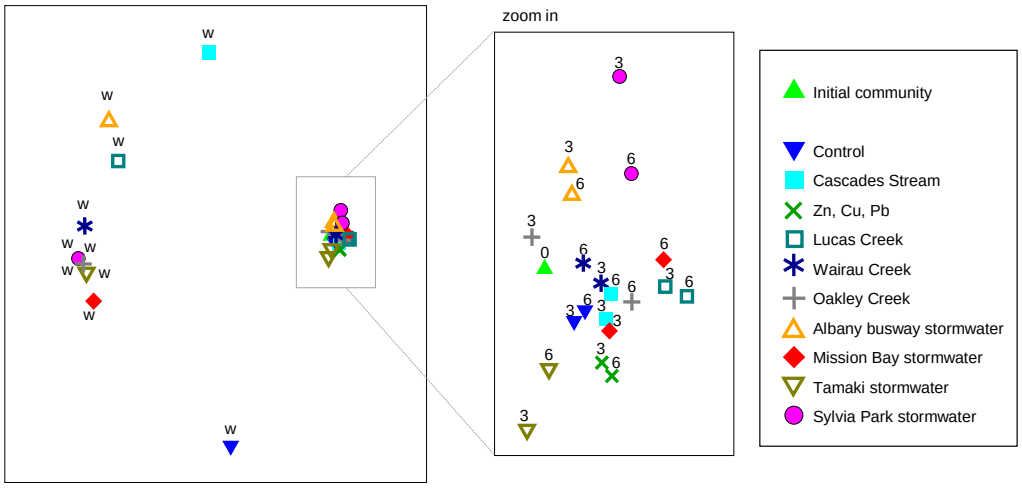

Figure 6.3: Nonmetric Multidimensional Scaling (MDS) plot of Manhattan distances between ARISA data from (w) lotic bacteria present in the different treatments at day 0 of the experiment and biofilm bacterial communities (0, 3 and 6). Numbers refer to days of exposure to separate treatments. Three samples were averaged for each biofilm data point. See legend on graph for the different treatments. 2D stress value: 0.1.

The effect of bacteria naturally present in the water was further investigated by SIMPER analysis which assesses the most variable peaks between groups of samples. In this experiment, the analysis assessed the most variable peaks between the initial biofilm bacterial communities and the communities in the different treatments. The 5 most influent peaks that were induced by each of the treatments were selected and compared to the peaks present in the water and in the initial biofilm (complete list of peaks and their respective presence/absence in the initial biofilm and the water is available in appendix 3, Table App-3). The results of the analysis revealed that 80 % of the peaks were already present in the initial biofilm highlighting that most changes occurred by re-arrangement of the initial biofilm community and not by introduction of new bacteria. Nevertheless, 10 % of the peaks were only present in water, suggesting therefore a certain influence of introduced bacteria. The remaining 10 % of peaks were not found in either the water or the initial biofilm.

6.4 Discussion

Urban runoff accumulates a wide range of contaminants to concentrations that can threaten aquatic organisms (Burton & Pitt, 2002). This complex association of contaminants has been shown to have a deleterious effect on freshwater algae, invertebrates and fish (Kayhanian *et al.*, 2008; Suren, 2000) but little is known about how they can affect microbial communities.

The fast response of biofilm bacteria observed in this project reveals that urban runoff can strongly alter the microbial community structure. In addition, bacterial communities were much more deeply affected by exposure to most types of stormwater used as treatment than by exposure to Zn, Cu and Pb at concentrations known to be toxic to aquatic organisms. This highlights the great disturbance caused by the complex association of very diverse contaminants present in urban runoff on freshwater ecosystems. Biofilm microbial populations play key roles in freshwater ecosystems. They fix dissolved carbon, nitrogen and phosphorus and provide an important source of nutrients for organisms at higher trophic levels. Therefore, the changes in bacterial community structure caused by exposure to stormwater contaminants may also alter the functions of biofilm and affect the entire stream ecosystem. As chapter 3 revealed that several weeks were needed for bacterial communities to recover after exposure to specific contaminants, in urban runoff receiving environments, the changes in biofilm bacterial communities generated during storm events could influence the ecosystem long after the rain stopped and the concentrations of contaminants returned to their normal levels.

Analyses of stormwater composition confirm the great diversity of urban contaminants and their highly variable concentrations. The stormwater collected from the different discharges into creeks (Albany busway, Mission Bay, Tamaki and Sylvia Park) contained concentrations of several contaminants exceeding the ANZECC trigger values (Table 6.3). For examples, the concentration of total nitrogen was more than 7 times above the trigger value in Sylvia Park stormwater and the concentration of total phosphorus more than 13 times the trigger value in Tamaki stormwater. Water collected from urban creeks (Lucas, Wairau and Oakley) also contained several contaminants in higher concentrations than the trigger values. Analyses of metal concentrations confirmed the frequent presence of zinc, copper and lead in concentrations exceeding ANZECC trigger values and their potential threat to urban aquatic ecosystems. All of them exceeded at least 10 times their trigger value in some types of water collected. Although present in smaller proportions, chromium also frequently exceeded its trigger value (maximum 2.2 times the value).

The extent of the changes caused by the different treatments was consistent with analyses of water composition as the stormwater collected from storwater discharges and containing high concentrations of contaminants generated the greatest changes in bacterial community structure. The stormwater collected from Sylvia Park and Tamaki that both contained high

concentrations of nitrogen and phosphorus and of some metals (mainly Zn and Cu) caused rapid and marked changes in the bacterial community structure. The stormwater collected from Albany busway that contained more moderate concentrations of nitrogen and phosphorus (which still remained above the ANZECC trigger value) but high concentrations of Cr, Cu, Pb and Zn also strongly influenced the communities.

Nitrogen, phosphorus and organic carbon generated the greatest proportion of the changes observed in biofilm bacterial community structure which confirms the strong influence of nutrient enrichment on biofilm bacterial communities revealed by previous research (Lawrence *et al.*, 2004; Olapade & Leff, 2005; Van der Gucht *et al.*, 2005). These contaminants that simulate the growth of biofilm appear to have a stronger influence on bacterial communities than heavy metals which have an inhibitory effect on sensitive species. Nevertheless, no contaminant could explain a major proportion of the changes and the impact of each treatment appeared to be the result of a complex co-influence of all the different contaminants present in the water. Analyses of water carried out in this project covered some of the most common urban contaminants but numerous other compounds could be present in the water. The report from ANZECC (2000) identifies 26 potential metal contaminants and more than 80 potential organic contaminants. It is therefore likely that contaminants not analysed in this study also influenced biofilm bacterial communities.

The great diversity of potential contaminants present in stormwater also highlights the difficulty to assess the ecological toxicity of a complex stormwater based on chemical analyses. In this experiment, biofilm bacterial communities were used as a powerful bio-indicator to assess, in flow chamber microcosms, the combined effect of all the contaminants present in the water. The analysis of planktonic bacterial communities revealed their small influence on biofilm bacterial communities, confirmed that most changes were caused by water contaminants and validated the use of biofilms as an indicator of the influence of stormwater. The relatively simple protocol used constitutes a highly interesting way to assess quickly and efficiently the ecological impact of different types of stormwater on aquatic ecosystems.

The visualisation of similarities between biofilm bacterial communities revealed that all treatments drove the populations in different ways, which indicates the co-influence of various contaminants but also suggests that each treatment influenced a different combination

of bacteria. Specific members of the bacterial community are sensitive or resistant to specific contaminants and the monitoring of their presence in biofilm could reveal the presence of specific contaminants in streams. This would significantly improve stream health bio-monitoring techniques as macro-invertebrates (traditionally used as bio-indicators) are affected by all contaminants and do not allow us to predict the presence of specific contaminants.

6.5 Conclusions

Results revealed the fast response of biofilm bacterial populations to exposure to all types of stormwater. Chemical analyses highlighted the diversity of contaminants present in urban runoff, the predominant effect of nutrients on microbial communities and the complex co-influence of all the different contaminants on bacterial communities. Results also suggest the potential use of biofilm to monitor the presence of specific contaminants and demonstrate the successful use of biofilm bacterial communities to assess the detrimental effect of complex stormwater on aquatic ecosystems.

CHAPTER 7

7 Investigation of the effect of urbanisation on stream ecosystems using biofilm microbial communities and associated metals as indicators of the changes

Abstract

In this study, both newly developed indicators (biofilm bacterial communities, ciliate communities and biofilm associated metals) and traditional indicators (macro-invertebrates communities and sediment associated metals) were used to investigate the effect of urbanisation on stream ecosystems. The ecological relevance and the reliability of these indicators were assessed and compared. Three streams progressively impacted by urbanisation as they reach Auckland City (New Zealand) were chosen and sampled in multiple locations. Biofilm bacteria, ciliates and macro-invertebrates were collected from each site and used as indicators of the changes caused by an increase of urban cover in stream catchments. In addition, concentrations of As, Cd, Cr, Cu, Pb, Ni and Zn were measured in biofilm, sediments and freshwater snails (*Potamopyrgus*) from each site as indicators of metal contamination. Results reveal that all three types of communities, biofilm bacteria, ciliate and macro-invertebrates changed significantly along the urbanisation gradient although the response of each community varied. Biofilm bacterial and macro-invertebrate communities significantly changed between sites with no urban cover in their upper catchment and sites with only 1 to 10 % of urban cover in their upper catchment whereas biofilm associated ciliate communities changed more progressively along the gradient. These results confirm the suitability of biofilm bacterial and ciliate communities as bio-indicators of the effect of urbanisation on stream ecosystems and highlight their complementary use with macro-invertebrates. The effect on the diversity of the different communities also varied; the diversity of macro-invertebrate communities decreased along the urbanisation gradient whereas no decrease in diversity could be observed for bacterial or ciliate communities. Concentrations of metals differed considerably between biofilm, sediments and freshwater snails. Concentrations of As, Pb and Zn generally increased along the urbanisation gradient, however, concentrations of metals in biofilm, sediments and freshwater snails appear to respond differently to the increase of urban cover. The ecological relevance of metal concentrations measured in biofilm, sediments and freshwater snails was assessed by investigating their correlation with changes occurring in bacterial, ciliate and macro-invertebrate communities. A greater proportion of the changes occurring in all 3 types of communities was explained by the concentrations of metals present in biofilm and freshwater snails than by the concentrations present in sediments. These results reveal that freshwater snails and biofilm are more relevant ecological indicators of the presence of metal contaminants in freshwater ecosystems than sediments.

7.1 Introduction

The impact of urbanisation on freshwater ecosystems results from the complex interactions of multiple physical, chemical and biological factors. To assess the extent to which aquatic ecosystems are affected by the multiple effects of urban runoff, various indicators can be used. A common approach consists of monitoring a suite of physico-chemical components of the water (Tsegaye *et al.*, 2006). However, the results are often highly variable in time and such methods typically require a great number of repeated measurements. An alternative is to use aquatic organisms that are influenced by past and present conditions as integrative bio-indicators.

Traditionally macro-invertebrates have been used as bio-indicators of steam health (Boothroyd & Stark, 2000). Nevertheless, recent developments in molecular biology have enabled the monitoring of microbial communities and their use as alternative or complimentary bio-indicators. Previous chapters have highlighted the potential use of biofilm bacterial and ciliate communities to monitor changes in stream environmental conditions. Chapters 3 and 6 revealed that urban runoff contaminants modify biofilm bacteria and ciliates and suggested the development of more tolerant communities. Chapter 5 confirmed the increased resistance of bacterial communities originating from impacted streams to exposure to urban contaminants.

Therefore, in the present chapter, the suitability of biofilm bacteria, biofilm associated ciliates and macro-invertebrates to reveal the changes occurring along an urbanisation gradient was investigated and compared.

Chapters 3 and 4 also revealed that some common metal contaminants of urban runoff (Zn, Cu and Pb) accumulate in biofilm. This suggests that concentrations of various biofilm associated contaminants could provide a useful indicator of the impact of urban pollutants on aquatic communities. In the present chapter, the concentrations of a wider range of metals (As, Cd, Cr, Cu, Pb, Ni and Zn) were analysed in biofilm, sediments and freshwater snails (*Potamopyrgus*). The ecological relevance of metal concentrations measured in each medium was then investigated by assessing their potential to explain the changes occurring in biofilm bacterial, biofilm associated ciliate and benthic macroinvertebrate communities.

For this project, three streams progressively impacted by urbanisation as they reach Auckland City (New Zealand) were sampled at multiple sites. Biofilm bacterial and ciliate communities

were investigated by Automated Ribosomal Intergenic Spacer Analysis (ARISA) and Terminal-Restriction Fragment Length Polymorphism (T-RFLP), respectively. Macro-invertebrates were also collected from each site and then identified and counted in the laboratory. In addition, metal concentrations in biofilm, sediments and snails (*Potamopyrgus*) were analysed at each site.

Two summer studentships were obtained to help undertake this work. Therefore, every step of this project from the planning of the experiment to the analysis of the results was aided by Max Thompson and Emma Armitage.

7.2 Experimental design

7.2.1 Study sites

Three streams progressively impacted by urbanisation were chosen in the Auckland Region (Figure 7.1). Both Oratia (Figure 7.1 a.) and Swanson (Figure 7.1 b) streams begin in the native forest of the Waitakere Ranges. The two streams then flow through farmland before reaching the urbanised areas of Auckland City. Hays Creek (Figure 7.1 c) is located South of Auckland City and originates from a lake that drains both forested and farming areas. The creek flows first through forested areas and then through a mixture of urbanised and farming areas.

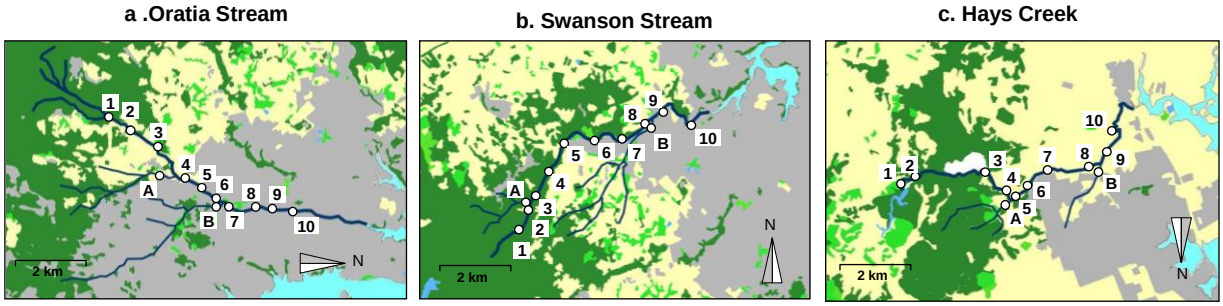

Figure 7.1: Map of sampling sites. Background is the land cover use: (■) native forest, (■) exotic forest, (□) farmland, (■) urban, (□) mining and (■) water. Land cover data from NZ Ministry for the Environment, Land cover database, version 2, 2004. Numbers (1 to 10) represent the sampling locations along the main stream and letters (A to B) the sampling locations on the main tributaries. See Figure 2.7 for broad scale location.

In total, samples were collected from 12 sites along each of the 3 streams; 10 sites were located in the main stream and 2 sites were located in the main tributaries. Sites were chosen to cover different levels of urban impact from pristine state in the head waters to deeply

impacted in the downstream locations. Sites in the tributaries were also sampled to diversify the environmental parameters. The dominant land cover proportions in the catchment area of each stream, including tributaries, is presented in Figure 7.2 and confirms the increased dominance of urban cover in the lower reaches of each stream.

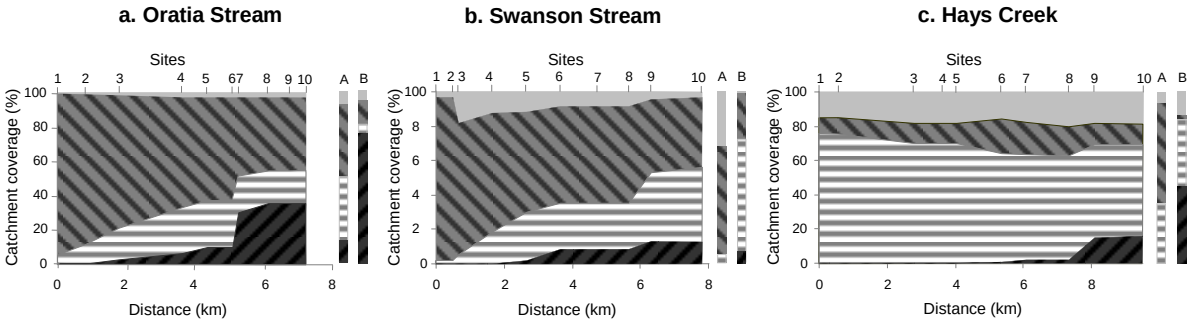

Figure 7.2 : Land cover in the catchment of Oratia Stream, Swanson Stream and Hays Creek and position of sampling sites. Data are (■) urban, (▤) farmland, (▩) forest, (□) others. Data from NIWA (2004).

7.2.2 Sampling procedure

Each stream was sampled during two consecutive days of December 2009. At each site, samples were obtained within a 30 m downstream transect. Biofilm was sampled from five rocks for metal analysis and four rocks for molecular analysis of bacterial and ciliate communities. For both molecular and metal analysis, each biofilm sample was scraped off an individual rock using a Speci-Sponge™ following the standard protocol (see § 2.2.1). Three samples of sediments for metal analysis and one sample of macro-invertebrates for community analysis were also obtained from each site following the standard protocols (see § 2.2.2 and § 2.2.3 respectively). In addition, at least 20 freshwater snails (*Potamopyrgus*) were obtained from each site and stored in a separate falcon tube for metal analysis.

Flow rate and water depth were measured at five points across the stream together with the total width of the stream to estimate the discharge of water at each site.

7.2.3 Analysis of bacterial, ciliate and macro-invertebrate communities

Biofilm was extracted from individual Speci-Sponges™ collected for molecular analysis by the standard method (see § 2.4.1). Bacterial community structure was investigated by ARISA (see § 2.4.3) and ciliate community structure by T-RFLP (see § 2.4.4). Macro-invertebrates

were identified and counted following standard protocol (see § 2.6). Similarities between bacterial and ciliate community profiles were assessed by Manhattan distances between all samples of the multivariate datasets. Similarities between macro-invertebrate communities were assessed by Bray Curtis distance after square root transformation of the dataset. Bray Curtis distance was preferred for macro-invertebrate communities because it considers the number of individuals independently of the community size whereas Manhattan distance evaluates the differences based on a normalised dataset. The relationship between biofilm microbial communities (bacteria and protozoa) and environmental variables (land cover, discharge and metals) was investigated by a distance based linear model (DistLM) using PRIMER software, including the add-on PERMANOVA+ (Primer-E Ltd, UK). This model is used to provide an estimate of the influence of each environmental variable in determining the differences occurring in the community structure between sites. SIMPER analysis was performed to investigate the most influential peaks or species and their relative contribution in the differentiation between non impacted and impacted sites.

7.2.4 Analysis of metal concentrations

Sediment samples were dried individually following the standard protocol (see § 2.3.1). After being crushed and sieved, 0.5 g of sediments from each replicate were combined to give one homogeneous sample per site. Biofilm was extracted from the Specie Sponges™ and dried following the standard protocol (see § 2.3.1). All the biofilm obtained from the replicates was pooled together to give one homogeneous sample per site. Samples of snails were prepared following the standard protocol (see § 2.3.1). All samples were sent to Hill Laboratories (Hamilton, New Zealand) and analysed for As, Cd, Cr, Cu, Pb, Ni and Zn by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) after nitric/hydrochloric acid digestion (US EPA 200.2).

7.3 Results

7.3.1 Changes in bacterial, ciliate and macro-invertebrate communities along the urbanisation gradient

Changes in bacterial, ciliate and macro-invertebrate community structures along each of the three streams are represented in Figure 7.3. Plots reveal a clear clustering of sites located in

the head waters compared to sites located further downstream for all three types of organisms. A clear gradual change is observable in the structure of macro-invertebrate communities for all streams (i.e. from the left to the right of MDS plots). A general clustering between the least and the most impacted sites can also be observed for bacterial and ciliate communities but their response to environmental changes appears to be more complex than the response of macro-invertebrate communities. This results in a more sinuous trajectory on the MDS plot and a higher stress factor, suggesting that bacterial and ciliate communities are affected by a greater number of environmental parameters. Communities of bacteria, ciliates and macro-invertebrates present in the tributaries are generally similar to the communities present in the main streams and clustered according to the level of urbanisation in their catchment.

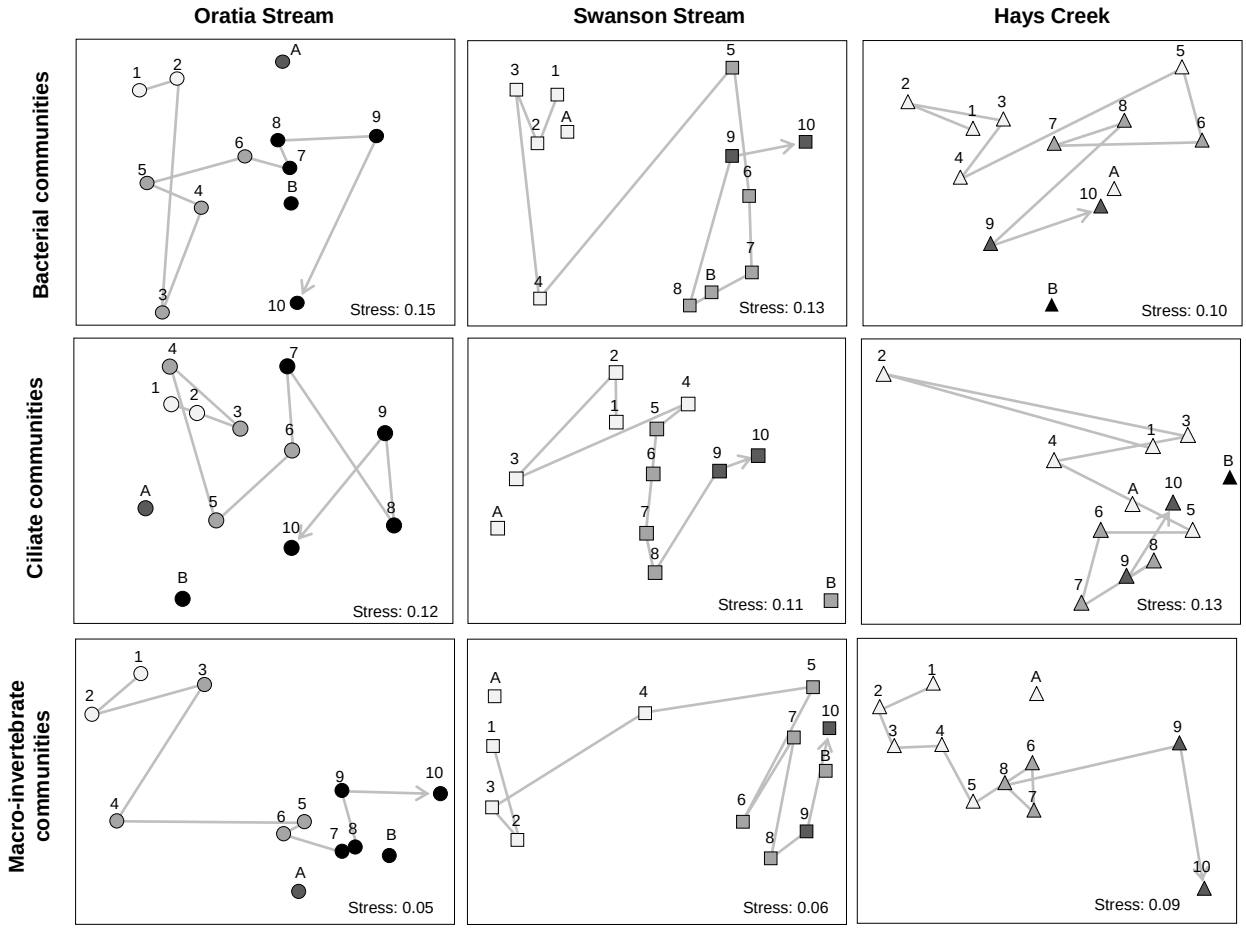

Figure 7.3: Nonmetric Multidimensional Scaling plot of similarities between bacterial, ciliate and macro-invertebrate communities in (○) Oratia Stream, (□) Swanson Stream and (△) Hays Creek. Replicates (4 for each bacterial and ciliate community data point) have been averaged before analysis. Similarities were assessed by Manhattan (bacterial and ciliate communities) or Bray Curtis (macro-invertebrate communities) distances. Darkness of the symbols represents the proportion of urban cover in the catchment area: (□) 0 %, (▨) 1 % - 10 %, (▩) 11 %- 20 %, (■) > 20 %. Letters A and B and numbers 1 to 10 represents the sampling sites (see Figure 7.1 for more details) The graph of similarities between invertebrate communities in Hays Creek excludes site B which was an outlier and clustered far from all other data in the plot.

To investigate the similarity between the changes in community structure occurring along the urbanisation gradient in the three different streams, data points from all three streams have been analysed and plotted together (Figure 7.4). The strong clustering of bacterial and invertebrate communities present in the most impacted sites from all three streams (Figure 7.4 a & c) reveals that urbanisation influences bacterial and invertebrate communities in a similar way in the three different streams. Ciliate communities present in the most impacted sites also appear to cluster together (Figure 7.4 b), but in a more moderated manner.

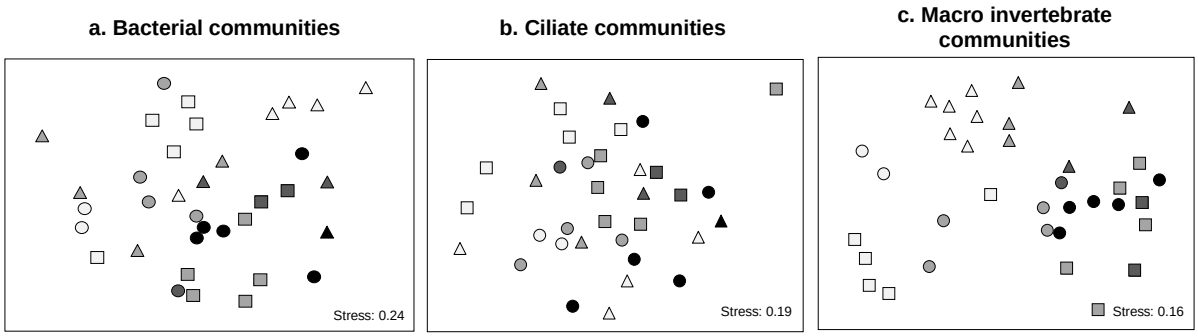

Figure 7.4: Nonmetric Multidimensional Scaling plot of similarities between bacterial, ciliate and macro-invertebrate communities in all three streams: (○) Oratia Stream, (□) Swanson Stream and (△) Hays Creek. Darkness of the symbols represents the proportion of urban cover in the catchment area: (□) 0 %, (▩) 1 % - 10 %, (▩) 11 %- 20 %, (■) > 20 %. The ciliate community in site 2 and the macro-invertebrate community in site B of Hays Creek were excluded from analysis because these data were outliers, clustering far from all other data in the plot.

The gradual influence of urbanisation on the different communities was also investigated by permutational ANOVA (PERMANOVA). To do so, samples were grouped in 4 categories: 0 % urban cover in the catchment, 1 to 10 %, 11 to 20 % and > 20 %. The data from each group were then statistically compared to each other. Permutational P values which estimate the significance of the difference between groups of samples are presented in Figure 7.5.

Results reveal a strong significant difference ($P < 0.05$) between bacterial communities present in sites with no urban cover in the upstream catchment and all the other sites including relatively preserved sites with only 1 to 10% of urbanisation in the catchment (Figure 7.5 a). There is still a significant difference between sites with 1-10% and 11-20% of urban cover in their catchment but the strength of the differentiation decreases such that no significant difference was observed between 11-20 % and > 20 % categories. This indicates a high sensitivity of bacterial communities in stream locations with little urbanisation in their upper catchment to small increases in urbanisation. In contrast, in stream locations with

greater urbanisation in their upper catchment, the effect of additional urban cover appeared to have a smaller effect on biofilm bacterial communities.

In contrast, ciliate communities do not appear to respond so intensively to small percentages of urbanisation in the catchment (Figure 7.5 b). There is no significant difference between 0 % and 1-10 % of urbanisation in the catchment or between 11-20 % and > 20 %. Between 1-10 % and 11-20 % there is a significant difference which remains moderately strong. However, there is a strong significant difference between the two most extreme categories, 0 % and > 20 %. This indicates that contrarily to bacterial communities for which most changes occur in the upper reaches, where urban cover in the catchment is still moderate, differences in ciliate communities occur progressively along the gradient of urbanisation.

Similar to bacterial communities, macro-invertebrate communities were significantly affected in the upper reaches by just a few percent of urbanisation in the catchment while further downstream the gradual increase of urbanisation did not seem to have such a strong impact (Figure 7.5 c).

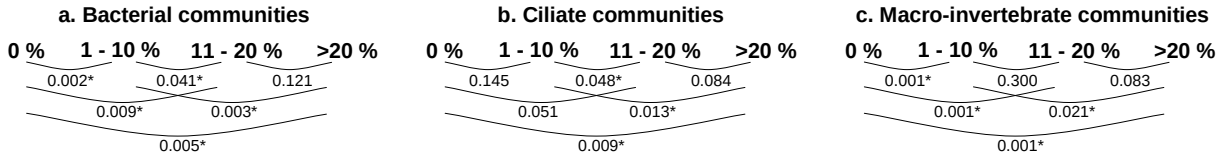

Figure 7.5: PERMANOVA P values between data gathered from stream sites with varying levels of catchment development. Percentages are the proportion of urban land cover in the catchment. Data for all 3 streams (Oratia Stream, Swanson Stream and Hays Creek) were grouped for analysis. * indicate significant differences ($P < 0.05$) between communities exposed to different levels of catchment urbanisation. Values were obtained by pairwise permutational analysis using 999 permutations.

Urbanisation is not the only factor that influences aquatic organisms. Other variables such as habitat, light and flow rate are expected to affect all types of communities. To estimate the proportion of the variation observed in each community explained by urbanisation a distance based linear model (DistLM) was used. Because of the design of the present experiment, the gradual increase of urbanisation is inevitably linked with an increase of the stream discharge (correlation coefficient (ρ) between proportion of urbanisation in the catchment and discharge = 0.29, $\rho = 0$ being completely independent and $\rho = 1$ totally correlated). Therefore, to estimate the proportion of the variation explained by urbanisation, the DistLM was processed sequentially, starting with the discharge. By this means, the discharge effect was excluded from the analysis before estimation of the effect of urban cover.

Results reveal that urbanisation significantly influenced all 3 types of communities, biofilm bacteria, ciliates and macro-invertebrates ($P < 0.05$). The proportion of urban cover in the catchment of the sites explains 4.3 % of the changes occurring in bacterial communities, 4.9 % of the changes occurring in ciliate communities and 6.4 % of the changes occurring in macro-invertebrate communities (Table 7.1).

Table 7.1: Significance (P) and proportion of the effect of the discharge and of the proportion of urban cover in the upper catchment for each of the three types of communities: bacteria, ciliates and macro-invertebrates. The relationship was investigated by sequential DistLM analysis starting with the discharge. * indicates a significant influence of the parameter ($P < 0.05$).

	Bacterial communities			Ciliate communities			Macro-invertebrate communities		
	P	Proportion (%)	Cumulative proportion (%)	P	Proportion (%)	Cumulative proportion (%)	P	Proportion (%)	Cumulative proportion (%)
Discharge	0.007*	4.8	4.8	0.010*	6.2	6.2	0.001*	14.8	14.8
Urban cover	0.002*	4.3	9.1	0.031*	4.9	11.1	0.001*	6.4	21.3

Diversity indices are frequently used to describe the response of macro-invertebrate communities to the quality of their environment (Boothroyd & Stark, 2000). In this experiment we investigated the relationship between urbanisation in the catchment of the different sampling sites and the diversity of macro-invertebrate, ciliate and bacterial communities, estimated by Shannon diversity index ($H' = \sum_{i=0}^{S}(P_i \ln(P_i))$ where P_i is the relative abundance of each species or peak and S the total number of species or peaks). Results are presented in Figure 7.6 and reveal that although a clear decrease of diversity occurred in the macro-invertebrate communities along the urbanisation gradient (Figure 7.6 c), no decrease was observed in either bacterial or ciliate communities (Figure 7.6 a & b). The diversity of both bacterial and ciliate communities appears to be particularly constant between all sites. In contrast, the diversity of macro-invertebrate communities appears to be strongly affected by only a few percent of urbanisation in the catchment which corroborates the findings of PERMANOVA analysis (see Figure 7.5).

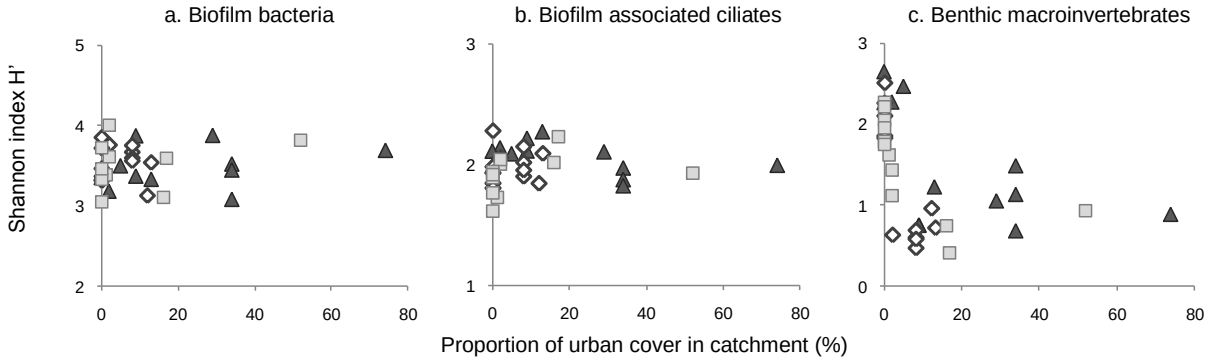

Figure 7.6 Shannon index values (H') along the urbanisation gradient for (a) biofilm bacterial communities, (b) biofilm associated ciliate communities and (c) benthic macroinvertebrates communities. Data are (▲) Oratia stream, (◇) Swanson Stream and (□) Hays Creek. Data has been averaged from 4 replicates of each site for biofilm bacteria and biofilm associated ciliates.

The datasets obtained for bacteria, ciliates and macro-invertebrates were based on very different types of samples and monitoring techniques. To investigate bacterial and ciliate communities, 4 biofilm samples were obtained from each site from a surface of around 100 cm^2 per sample. Biofilm bacterial communities were monitored by ARISA profiles which contained 800 potential variables and biofilm associated ciliates were investigated by T-RFLP profiles providing 1560 potential variables. For all sites of this study, 688 different peaks were present in at least one ARISA profile (compared to 800 potential peaks) and 442 different peaks were present in at least one T-RFLP profile (compared to 1560 potential peaks) (it is to be noted that a single species of bacteria can have multiple intergenic spacer lengths and therefore produce multiple interlinked peaks and that each species of ciliate is expected to produce two different peaks, one for each end of the 18S rRNA gene). Macro-invertebrates were sampled from an area of approximately 1 m^2, more than 20 times the surface sampled for biofilm but provided a total of only 62 taxa for all sites. On average per site, 137 different peaks were obtained on the ARISA profiles, 56 peaks on the T-RFLP profiles and only 14 different taxa of macro-invertebrates.

To investigate the peaks or species most involved in the differentiation between pristine and impacted sites, SIMPER analysis was performed between sites with no urbanisation in their catchment (0 % of urban cover) and sites most impacted by urbanisation (> 20 % of urban cover). The 10 most influential peaks of ARISA or T-RFLP profiles and the 10 most influential macro-invertebrate species are presented in Table 7.2 together with their contribution to the observed differences in community structure. Results reveal that the differentiation between bacterial communities from pristine and impacted sites is based on a

high number of peaks as the 10 most influential peaks contribute to only 17.54 % of the observed differences. The differentiation between ciliate communities includes a moderate number of peaks and the 10 most influential peaks contribute to 34.08 % of the observed differences. Finally, the differentiation between macro-invertebrate communities is based on a much smaller number of species. *Potamopyrgus* explain more than 35 % of the differences between pristine and impacted sites. In total, the 10 most influential species explain more than 83 % of the observed changes.

Table 7.2: Most influential peaks or species in the differentiation between sites with no urbanisation in their upper catchment and sites with > 20 % of urbanisation in their upper catchment. Peaks and species were revealed by SIMPER analysis. Peaks represent ribosomal intergenic spacer lengths (bacterial communities) or terminal 18S rRNA gene restriction fragment lengths labelled with HEX (H) or FAM (F) fluorochromes (ciliate communities).

Bacterial communities		**Ciliate communities**		**Macro-invertebrate communities**	
Peaks (b.p.)	Contribution (%)	Peaks (b.p.)	Contribution (%)	Species	Contribution (%)
484	2.57	H 119	8.12	*Potamopyrgus*	35.32
476	2.01	H 289	5.7	*Paracalliope*	12.41
432	1.92	H 678	3.42	*Paratya*	6.83
483	1.90	F 193	3.03	*Aoteapsyche*	6.35
425	1.73	F 682	2.94	*Physa*	4.66
457	1.65	H 505	2.77	*Deleatidium*	4.3
429	1.57	F 121	2.37	*Hydora*	4.08
421	1.48	F 680	2.03	*Austroclima*	3.9
505	1.36	F 544	1.84	*Austrosimulium*	3.02
430	1.35	F 166	1.84	*Ostracoda*	2.67
Total contribution	17.54	Total contribution	34.08	Total contribution	83.54

7.3.2 Changes in metal concentrations along the urbanisation gradient

At each site, concentrations of As, Cd, Cu, Cr, Pb, Ni and Zn were measured in biofilm, sediments and freshwater snails (*Potamopyrgus*). Concentrations of all 7 metals in every site of the three streams are available in appendix 4. To compare the concentrations of these metals in biofilm, sediments and snails and investigate how urbanisation affected them, the concentrations have been plotted against the proportion of urban cover in the catchment of the sampling sites (Figure 7.7). Graphs reveal that metal concentrations can differ considerably between biofilm, sediments and snails. Most notably, all 7 metals are generally present in higher concentrations in biofilms than in sediments. Freshwater snails can contain similar (Zn and Ni), higher (Cd and Cu) or lower (As, Cr and Pb) concentrations of metals than biofilm, although biofilm are a main source of food of snails (Lamberti, 1996). The

correlation between metal concentration and urbanisation depends highly on the metal considered. Concentrations of As, Pb and Zn appear to increase with urbanisation in biofilm, sediments and snails. Surprisingly, concentrations of Cd and Cu in snails appear to increase with urbanisation whereas no increase in those metals could be revealed in biofilm or sediments. Finally, in the three streams investigated, concentrations of Ni and Cr in biofilm, sediments and snails seem to be minimally correlated with urbanisation.

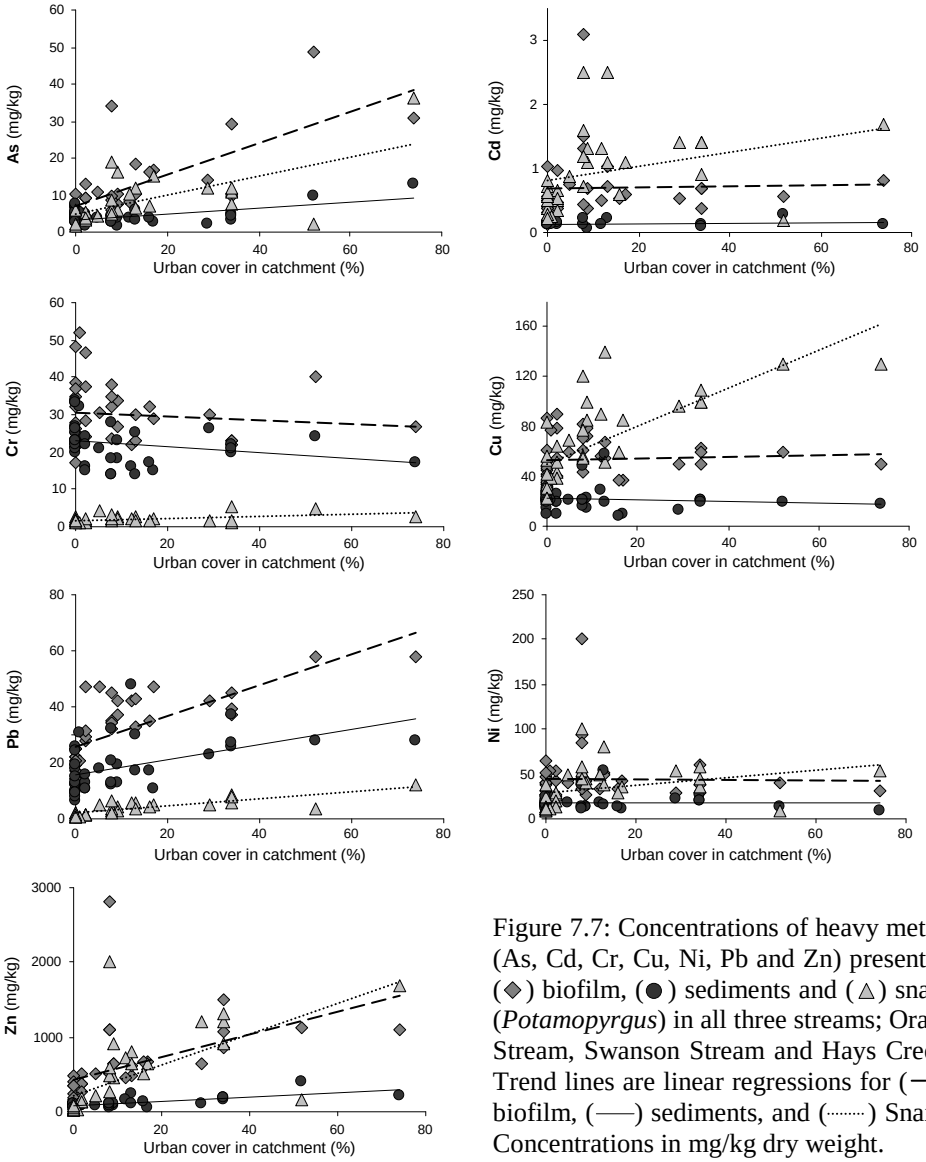

Figure 7.7: Concentrations of heavy metals (As, Cd, Cr, Cu, Ni, Pb and Zn) present in (◆) biofilm, (●) sediments and (△) snails (*Potamopyrgus*) in all three streams; Oratia Stream, Swanson Stream and Hays Creek. Trend lines are linear regressions for (– –) biofilm, (——) sediments, and (·····) Snails. Concentrations in mg/kg dry weight.

7.3.3 Ecological relevance of metal concentrations present in biofilm, sediments and freshwater snails (*Potamopyrgus*)

Heavy metals concentrations are analysed to assess the level of contamination of stream ecosystems and to determine whether metals are present at concentrations that may be

detrimental to aquatic biota. The environmental relevance of metal concentrations measured in biofilm, sediments and freshwater snails can be compared by estimating how well the concentrations are correlated to changes occurring in the community structure of aquatic organisms. In the present experiment, this was achieved by DistLM analysis using biofilm bacteria, ciliates and macro-invertebrates as bio-indicators (Table 7.3). Unexpectedly, concentrations of metals measured in snails generally explained the greatest proportion of the changes occurring in bacterial communities, closely followed by the concentrations of metals in biofilms. The concentrations of all 7 metals measured in sediments explained a smaller proportion of the changes in bacterial communities than concentrations measured in biofilms and snails, suggesting that sediments are the less relevant to assess the detrimental impact of metals on biofilm bacteria. An identical trend appears from the analysis of both ciliate and macro-invertebrate communities. In most cases, concentrations of metals present in biofilm or snails explain a much greater proportion of the changes occurring in the bio-indicator than the concentrations present in sediments. These results suggest that snails and biofilm are the most suitable media to assess the detrimental effect of metals on aquatic biota.

Table 7.3: Relationship between differences in bio-indicator communities and metal concentrations measured in sediments, biofilm and freshwater snails (*Potamopyrgus*). Data were assessed by marginal tests using a distance based linear model (DistLM) and indicate the significance of the relationship (P) and the proportion (Prop) of the variability in the multivariate dataset explained by the concentrations of metals.

	Bacterial communities			Ciliate communities			Macro-invertebrate communities		
	Variable	P	Prop. (%)	Variable	P	Prop. (%)	Variable	P	Prop. (%)
	Sediments	0.341	3.0	Sediments	0.393	3.1	Sediments	0.502	2.6
As	Biofilm	0.006	5.2	**As** Biofilm	0.045	4.8	**As** Biofilm	0.001	13.5
	Snails	0.003	5.3	Snails	0.036	5.0	Snails	0.001	15.9
	Sediments	0.058	3.9	Sediments	0.596	2.7	Sediments	0.072	5.3
Cd	Biofilm	0.001	5.8	**Cd** Biofilm	0.146	4.0	**Cd** Biofilm	0.004	9.2
	Snails	0.001	6.7	Snails	0.07	4.6	Snails	0.001	18.7
	Sediments	0.016	4.4	Sediments	0.183	3.8	Sediments	0.001	13.4
Cr	Biofilm	0.012	4.5	**Cr** Biofilm	0.826	2.2	**Cr** Biofilm	0.553	2.4
	Snails	0.004	5.0	Snails	0.022	5.3	Snails	0.003	9.1
	Sediments	0.463	2.8	Sediments	0.371	3.2	Sediments	0.869	1.5
Cu	Biofilm	0.005	4.9	**Cu** Biofilm	0.164	3.8	**Cu** Biofilm	0.075	4.8
	Snails	0.001	6.0	Snails	0.011	5.9	Snails	0.001	16.4
	Sediments	0.359	3.0	Sediments	0.917	2.0	Sediments	0.518	2.6
Ni	Biofilm	0.004	4.7	**Ni** Biofilm	0.471	2.9	**Ni** Biofilm	0.032	6.6
	Snails	0.001	7.3	Snails	0.09	4.3	Snails	0.001	18.8
	Sediments	0.031	4.1	Sediments	0.038	5.0	Sediments	0.002	9.2
Pb	Biofilm	0.002	6.0	**Pb** Biofilm	0.007	6.7	**Pb** Biofilm	0.001	16.3
	Snails	0.001	5.8	Snails	0.019	5.6	Snails	0.001	17.2
	Sediments	0.013	4.4	Sediments	0.076	4.4	Sediments	0.002	10.1
Zn	Biofilm	0.002	5.6	**Zn** Biofilm	0.005	7.1	**Zn** Biofilm	0.001	14.8
	Snails	0.001	6.0	Snails	0.01	6.2	Snails	0.001	19.0

7.4 Discussion

Biofilm bacterial, ciliate and macro-invertebrate communities changed significantly along the urbanisation gradient (Figure 7.5). The proportion of the urban cover in the upstream catchment of each site was used, in this project, as a general indication of the degree of impact caused by urbanisation on the stream ecosystem. However, the effect of urbanisation results from multiple interacting influences (Allan, 2004) and the intensity of each influence depends not only on the proportion of urban cover but also on many other parameters including the type of urbanisation (typically, industrial areas produce different contaminants than residential areas). This explains the dispersion of data points and the sinuosity of trajectories on MDS plots for all three types of communities. Nevertheless, the statistical analysis revealed the general significant influence of urbanisation on all three types of communities (Table 7.1) and the comparison of the changes occurring in the all three streams on a single MDS plot (Figure 7.4) highlighted that urbanisation affected the communities present in each of the different streams in similar ways. These findings demonstrate that biofilm associated bacteria, ciliates and macro-invertebrates can all provide a reliable indication of the impact of urbanisation on stream ecosystems. Numerous environmental factors, related or not to urbanisation, can influence aquatic communities (Quinn, 2000; Suren, 2000) and environmental parameters such as vegetation type in the stream catchment (Thompson & Townsend, 2004), depth or flow rate (Besemer *et al.*, 2007) can also affect aquatic organisms but have not been considered in this study. This was confirmed by DistLM analysis as urban cover in the upper catchment explained only 4.3, 4.9 and 6.4 % of the changes occurring in bacterial, ciliate and macro-invertebrate communities, respectively (Table 7.1).

Although all three types of communities, bacteria, ciliates and macro-invertebrates, significantly changed along the urbanisation gradient, the response of each community varied greatly. For example biofilm bacteria and macro-invertebrates differed significantly between sites with no urbanisation in their upstream catchment and sites with only a few percent of urban cover in the upstream catchment whereas the response of ciliates was more progressive. The diversity of macro-invertebrate communities also decreased along the urbanisation gradient whereas no decrease in diversity could be observed in either bacterial or ciliate communities. The differences in response to urban impact have significant

implications for the implementation of each type of community as a bio-indicator and highlight the complementary use of bacteria, ciliates and macro-invertebrates as indicators of stream health.

Consistent with previous studies (e.g. Lenat & Crawford, 1994; Stepenuck *et al.*, 2002) the diversity of macro-invertebrate communities decreased in sites with higher proportions of urban cover in their upstream catchment. Runoff from urbanised areas often contains a wide range of contaminants at concentrations that can be toxic to aquatic organisms. Along the three streams investigated in this study, the proportion of urban cover in the catchment progressively increased causing a gradual accumulation of urban contaminants in the stream ecosystem. This was confirmed by the analysis of metals (particularly As, Pb and Zn) in biofilm, sediments and snails. As the contaminant concentrations increase and the stream environment is modified, macro-invertebrate sensitive taxa are excluded causing a decrease in diversity of the community. Although a similar influence on biofilm bacterial and ciliate communities may be expected, no decrease in diversity could be revealed in this experiment. This highlights fundamental differences in adaptability to hostile environments between microbes and macro-organisms. Many bacteria and protozoa have developed resistances against toxic compounds such as heavy metals (Cervantes & Gutierrez-Corona, 1994; Rehman *et al.*, 2008) and hydrocarbons (Lara *et al.*, 2007). Some bacteria can even degrade toxic compound and use them as an energy source (Haritash & Kaushik, 2009). Therefore, sensitive bacteria and ciliate taxa that became excluded from the communities when pollutants reached toxic levels could be replaced by other resilient taxa that are better adapted to the new environment.

The replacement of sensitive bacteria by resilient bacteria also suggests that changes occurred in the presence of genes responsible for the resistance against specific contaminants. Considerable research has been done to investigate the role of particular genes in conferring a resistance against various contaminants, including heavy metals (Abou-Shanab *et al.*, 2007) and organic compounds (Hamann *et al.*, 1999). In addition, recent developments of new sequencing technologies and of gene micro-arrays have enabled the investigation of the presence of a wide range of functional genes in environmental samples (He *et al.*, 2008; Roh *et al.*, 2010) such as those involved in the resistance to, and transformation of, various urban contaminants. A combined analysis of the changes occurring in the community structure of bacterial communities and the presence of genes responsible for the resistance against

specific contaminants could therefore not only reveal where the changes occur but also identify the main drivers of the changes.

Analyses of metals revealed that their concentrations can vary greatly between biofilm, sediments and freshwater snails (*Potamopyrgus*). Consistent with the findings of chapter 4, the concentrations of zinc, copper and lead measured in biofilms were greater than the concentrations measured in sediments. The same trend was also revealed for arsenic, cadmium, chromium and nickel which are other potential contaminants of urban runoff. High concentrations of cadmium and copper measured in freshwater snails compared to biofilms and sediments highlighted a potential accumulation of some metals in snails while very low concentrations of chromium and lead suggested the excretion or exclusion of other metals. The concentrations of several metals also appeared to increase differently in biofilm, sediments and snails along the urbanisation gradient. All these findings highlight the potential bias when assessing contamination with heavy metals in only one substrate. Furthermore, in most cases, metal concentrations present in biofilm and snails explained a much greater proportion of the changes occurring in all three biological indicators used; biofilm bacteria, ciliates and macro-invertebrates. These results highlight that freshwater snails and biofilm are more suitable than sediments to assess the detrimental effect of metals on aquatic biota.

7.5 Conclusions

Biofilm bacterial, ciliate and macro-invertebrate communities were all successfully used to monitor the impact of urbanisation on stream ecosystems. Statistical analysis revealed significant differences in all three types of organisms between the less impacted sites and the most impacted sites although the response of each community varied. Bacteria and macro-invertebrates significantly differ among sites with no urbanisation in their upper catchment and sites with only a few percent of urban cover in the upper catchment while ciliates responded more progressively to the increase of urban cover. The effect on the diversity of the different communities also varied; the diversity of macro-invertebrate communities decreased along the urbanisation gradient whereas no decrease in diversity could be observed for bacterial or ciliate communities. Results confirm the suitability of biofilm bacterial and ciliate communities as indicators of the impact of urbanisation on stream ecosystems. The differences between the responses of the three types of organisms highlight their complementary use as bio-indicators. Analyses of metals revealed that their concentrations can vary considerably between biofilm, sediments and freshwater snails (*Potamopyrgus*). A much greater proportion of the changes occurring in all three types of organisms, bacteria, ciliates and macro-invertebrates was explained by the concentrations of metals present in biofilm and freshwater snails than present in sediments. These results highlight that freshwater snails and biofilm are more suitable than sediments to assess the detrimental effect of metals on aquatic biota.

CHAPTER 8

8 Use of microbial biofilm to monitor the efficacy of a stormwater treatment train

Abstract

The efficacy of a stormwater treatment system and the impact of the discharge on the receiving stream were investigated by monitoring biofilm associated metals and biofilm bacterial community structure within the treatment system and the receiving stream. Located north of Auckland City, this stormwater treatment system has one of the most advanced designs in New Zealand and includes raingardens, grassy swales, a stormwater filter and a wetland. Biofilm was sampled in multiple locations through the treatment system and in the receiving stream above and below the stormwater discharge. Changes in bacterial community structure were assessed by Automated Ribosomal Intergenic Spacer Analysis (ARISA) and concentrations of biofilm associated metals were monitored by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS). Both biofilm bacterial community structure and biofilm associated metals revealed a gradual improvement of water quality through the system and a minimal impact of the discharge on the receiving stream ecosystem, demonstrating the efficacy of the treatment process. This highly original study demonstrates that biofilm composition is sensitive to environmental changes within freshwater ecosystems and an efficient indicator to monitor changes in enclosed stormwater networks where traditional biological indicators are not available.

8.1 Introduction

In this chapter, the new indicators developed along the project were implemented to assess the efficiency of a stormwater treatment train where traditional indicators are not available. This practical application demonstrates actual opportunities for the use of biofilm as indicators of urban impact on freshwater ecosystems.

Urban stormwater often carries high concentrations of contaminants to receiving rivers and streams. These contaminants include suspended sediments, heavy metals, hydrocarbons, nutrients, and a variety of other pollutants that constitute a threat to stream ecosystems (see § 1.1). Because of the complex composition of urban stormwater, a multifaceted 'treatment-train' approach combining several treatment systems is generally desirable to reduce the cocktail of contaminants before discharge into rivers and streams (Davis & McCuen, 2005). In such an approach, stormwater is sequentially directed through various treatment systems linked by an underground pipe network.

As part of a low impact design plan, an integrated stormwater treatment train has been incorporated throughout the site of the Albany Park and Ride Bus Station, New Zealand ($36^{o}43'18$ S, $174^{o}42'45$ E or see Figure 2.7), which currently provides open-air parking for approximately 550 cars. Surface water from the bus station and surrounding area is sequentially channelled through raingardens, grassy swales, a StormFilter™ (for more details refer to www.stormwater360.co.nz) and a wetland before discharge into a nearby creek (Figure 8.1). Enviropod™ catchpit filters that catch trash and debris (for more details refer to www.stormwater360.co.nz) are also fitted to most stormwater drains of the bus station complex. This multifaceted treatment train is highly novel in its design, and while theoretically effective, very little is known about the actual ability to minimise ecological impacts of stormwater on the receiving waters.

Assessing stormwater quality throughout complex, enclosed networks of stormwater pipes is difficult as traditional biological indicators of water quality (e.g. fish and macro-invertebrates) are largely absent. Unlike fish and macro-invertebrates, bacteria are ubiquitous, being detected in virtually all freshwater environments. Therefore, biofilms constitute a highly interesting alternative to assess ecosystem health in confined systems.

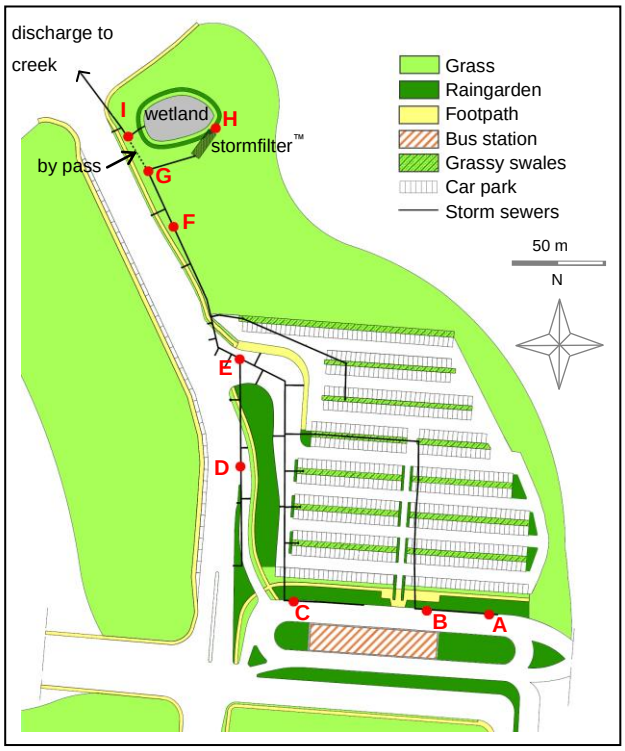

Figure 8.1: Map of the Albany Park and Ride complex showing bus station, car park and components of the stormwater treatment system (36°43'18 S, 174°42'45 E). Letters A to I reveal the location of each sampling site. Sampling sites were stormwater pipes accessed by manholes.

In this study, Automated Ribosomal Intergenic Spacer Analysis (ARISA) was used to evaluate the structure of bacterial communities in microbial biofilm located in different sections of the stormwater treatment network and in the receiving waters of a nearby creek.

Simultaneously, concentrations of various potentially toxic heavy metals associated with sediments, water and biofilm were monitored. The hypotheses of this project were that (i) the concentrations of stormwater associated contaminants would be reduced across the treatment train, causing significant differences in bacterial community structure, and (ii) there would be no significant difference in bacterial community structure or concentrations of biofilm associated metals up and downstream of the stormwater outlet into the receiving waters of a nearby creek.

This project was carried out with help from Dr Gavin Lear and Kelly Roberts (University of Auckland). Auckland Regional Council provided funding and assistance and the North Shore City Council provided help in sample collection. Thanks also to Elizabeth Fassman and Mingyang Liao (University of Auckland) for sharing their expertise in the operation of the Albany Park and Ride treatment train.

8.2 Experimental design

8.2.1 Experiment outline

For the analysis of biofilm bacterial community structure, samples were taken at 9 different locations (A to I, Figure 8.1) along the treatment train on two sampling occasions (29.01.09 and 26.03.09). Stormwater pipes were accessed by manholes and three biofilm samples removed, each by swabbing an area of approximately 100 cm^2 at the bottom of the pipe using a fresh Speci-Sponge™ (Nasco, USA). Three additional samples were removed on the second sampling occasion, in a similar manner, to quantify concentrations of biofilm-associated metals within each site.

Within the receiving waters of Lucas Creek, biofilm samples were obtained from 11 sites; 1 in the stormwater outlet, 5 upstream of the outlet and 5 downstream of the outlet. Sampling sites were located at regular intervals (approximately 10 m apart) and samples taken on three occasions (30.01.09, 26.02.09 and 26.03.09). Six samples were taken from each sampling location and date, three for microbiological analysis and three for biofilm associated metal analysis. Each biofilm sample was obtained by scrubbing an individual rock with a Speci-Sponge™, following the standard protocol (see § 2.2.1). In addition, one 50 ml sample of sediment was taken at each sampling site for metal analysis (see § 2.2.2).

8.2.2 Bacterial community analysis

In the laboratory, biofilm biomass was removed from each sponge and the DNA was extracted following the standard protocol (see § 2.4.1). Changes in bacterial populations were assessed by Automated Ribosomal Intergenic Spacer Analysis (ARISA) as detailed in § 2.4.3. Variations among bacterial population data were assessed by creating a matrix of Manhattan distances and visualised on Multidimensional Scaling (MDS) plot (see § 2.4.3 for more details). The dispersion of data points was assessed using multivariate dispersion (MVDISP) and the statistical significance of any differences observed, assessed by permutational multivariate ANOVA (PERMANOVA) using the software PRIMER© with the add-on PERMANOVA+ (PRIMER-E Ltd., UK).

8.2.3 Metal analysis

Biofilm and sediment samples collected for metal analysis were processed following the standard protocol (see § 3.3.1). Biofilm samples were pooled per site before analysis. Biofilm and sediment samples were sent to Hills Laboratories (Hamilton, New Zealand) for analysis of total recoverable concentrations of Cu, Pb and Zn by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS). A selection of these samples was also analysed for concentrations of As, Cd, Cr and Ni using the same method.

8.3 Results

8.3.1 Bacterial community structure

The drainage system of the bus station and its car park is broadly divided into three parts. Sites A and B are located on the same main pipe that drains the East side of the bus station and the cark park. Site C is located on a pipe collecting water from the West side of the bus station and the car park. Finally, site D is located further West of the complex and mainly drains the side road (see Figure 8.1 for more details about the stormwater network). Biofilm collected from these three separate areas exhibited large differences in community structure (average Manhattan distance between A-C, A-D, B-C and B-D > 0.81, Manhattan distance going from 0 [identical] to 1 [totally different]). Statistical analysis reveals that the differences were significant between each of the sites (P-values, < 0.05). In contrast, biofilm bacterial communities from sites A and B, which are located on the same pipe, did not significantly differ and cluster close to each other on the MDS plot (Manhattan distance = 0.62) (Figure 8.2). The population residing in site E, where the waters from sites A, B, C and D merge, appears to result from the combination of that within the sites located upstream and was significantly different (P-value, all < 0.05) from any of them. Further downstream, the relative differences in bacterial community structure between consecutive sites decreased. For example, the bacterial community structure on both sides of the StormFilter™ (sites G and H) and of the wetland (sites H and I) were not significantly different (P-values G-H, 0.39; H-I, 0.15) and clustered relatively close together (with average Manhattan distances of 0.58 and 0.65, respectively). Overall, the data show that the greatest differences in bacterial community structure occurred upstream of the car park.

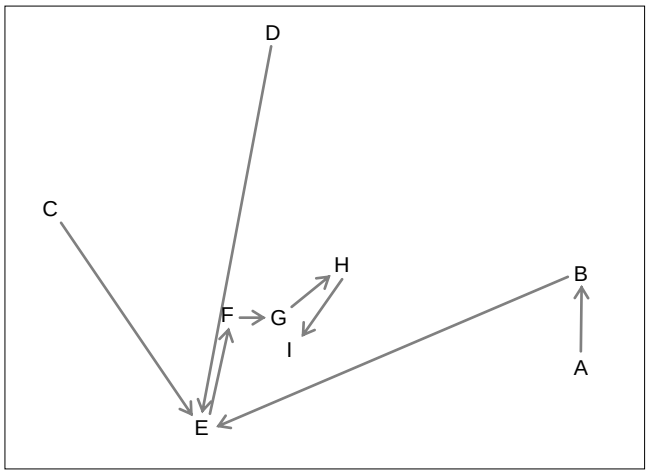

Figure 8.2: Nonmetric Multidimensional Scaling plot of Manhattan distances between bacterial ARISA data along the stormwater treatment system. Capital letters (A to I) are the nine different sites along the treatment train (see Figure 8.1). Data are the average of three replicates from both sampling occasions. 2D stress= 0.07. Arrows show the flow of water through the treatment system.

Within the receiving waters of Lucas Creek, significant differences in bacterial community structure were detected between sampling sites located either upstream, or downstream of the stormwater outlet on each sampling date (PERMANOVA, $P < 0.05$). Figure 8.3 combines the data from all three sampling occasions and shows that changes in bacterial community structure either side of the stormwater outlet are consistent over time.

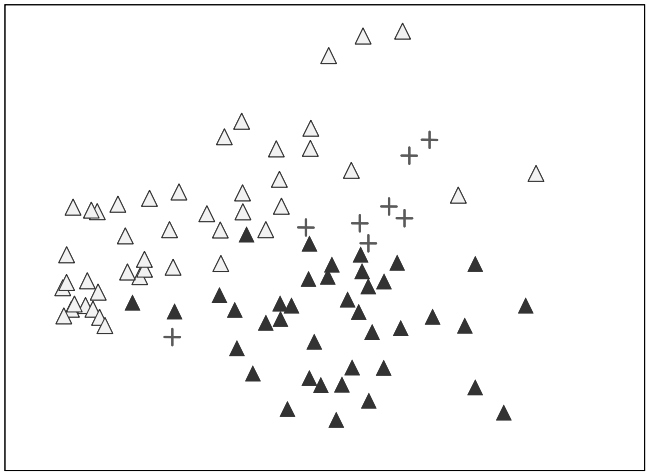

Figure 8.3: Nonmetric Multidimensional Scaling plot of Manhattan distances between bacterial ARISA profiles in the receiving stream (Lucas Creek). Data are: (△) upstream of stormwater outlet, (▲) downstream of stormwater outlet and (+) the stormwater outlet. 2D stress = 0.21

In Figure 8.4, bacterial community data collected in this study is compared to data collected from 18 different Auckland streams, located in a range of both rural and urban catchments (data from chapter 4). Analysis of the ARISA data using multivariate dispersion values

(PRIMER MVDISP) revealed similar variability (dispersion value = 1.020) in bacterial community structure among samples obtained from within the stormwater treatment train, in which the greatest physical distance between sample sites was only 0.3 km, compared to between streams (dispersion value = 0.994) in which the greatest physical distance was up to 100 km between sample sites. Samples abstracted from sections of Lucas Creek in this study varied little in comparison (dispersion value = 0.055) revealing that the stormwater outlet had relatively little effect on the bacterial biofilm community within Lucas Creek. Interestingly, samples from lower sections of the stormwater treatment train (i.e. sites H and I) were the most similar to the communities within Lucas Creek (including sections located upstream of the stormwater outlet).

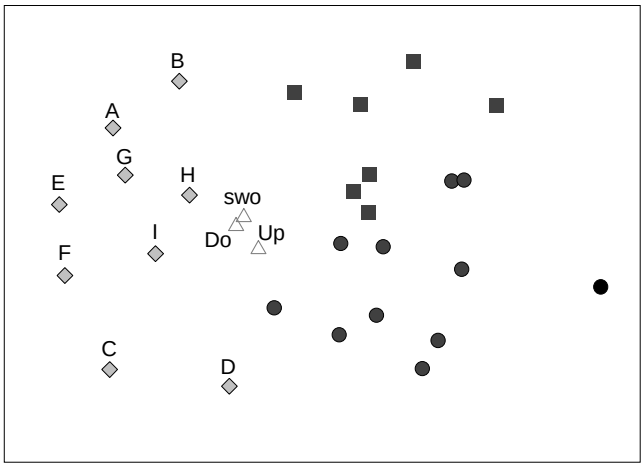

Figure 8.4: Nonmetric Multidimensional Scaling plot of Manhattan distances between bacterial ARISA profiles from this study and a range of Auckland streams. Symbols represent data obtained from bacterial communities: (◇) within the stormwater treatment train, (letters represent sites defined in Fig. 1), (△) within Lucas creek, upstream of the outlet (Up), downstream (Do), outlet (swo) and, different streams within the Auckland Region [data obtained from Lear *et al.* (2009)] located in predominantly (●) urban catchments, (■) rural catchments. All replicate data were averaged. 2D stress = 0.19

8.3.2 Biofilm and sediment associated metals.

Similar to the large differences in bacterial community structure revealed between the three different draining areas, concentrations of biofilm associated metals varied greatly between sites A-B, C and D (Figure 8.5). Concentrations of copper and lead were particularly high in site C while zinc was more concentrated in site D. Further downstream, after the junction of the three main pipes in site E, concentrations of biofilm associated copper, lead and zinc decreased gradually, suggesting the effectiveness of the treatment train in reducing the loads

of these particular contaminants. From site E to site I, concentrations of biofilm associated zinc, copper and lead dropped by 73 %, 46 % and 48 %, respectively. As there are currently no recommended trigger values for biofilm associated metals, concentrations were compared to ANZECC sediment quality guidelines (ANZECC & ARMCANZ, 2000). Concentrations of biofilm-associated copper and lead did not exceed the ANZECC high interim sediment quality guideline (ISQG-High) in any site. In contrast, concentrations of zinc exceeded ANZECC ISQG-High values (0.41 g Zn kg^{-1} dry wt.) in every sampling location, reaching a maximum of 4.8 g kg^{-1} dry wt. at site E, directly downstream of the car park. Although zinc was removed efficiently through the treatment train, concentrations of zinc remained high, only returning to levels similar to sites located upstream of the car park (e.g., 1-2 g kg^{-1} dry wt. in sites A, B and C). As the guidelines have been defined for metal concentrations in sediment and not in biofilm, the concentration of zinc in biofilm samples exceeding the guideline may not suggest a significant ecological risk for the organisms in the receiving waters. Nevertheless, it is worth noting that concentrations of zinc observed within these biofilm samples reached a maximum concentration more than 10 times higher than the current ANZECC ISQG-High trigger value for sediments.

Unexpectedly, concentrations of biofilm-associated arsenic and cadmium increased following passage through the StormFilter™ (located between sites G and H), and the wetland (located between sites H and I). However, concentrations of arsenic, cadmium and chromium remained below the ANZECC ISQG-High values. Conversely, concentrations of nickel exceed the ANZECC ISQG-High values (52 mg Ni kg^{-1} dry wt.) in most sites along the treatment train. Its concentration also increased downstream of the StormFilter™, reaching a maximum of 190 mg kg^{-1} dry wt. at site H.

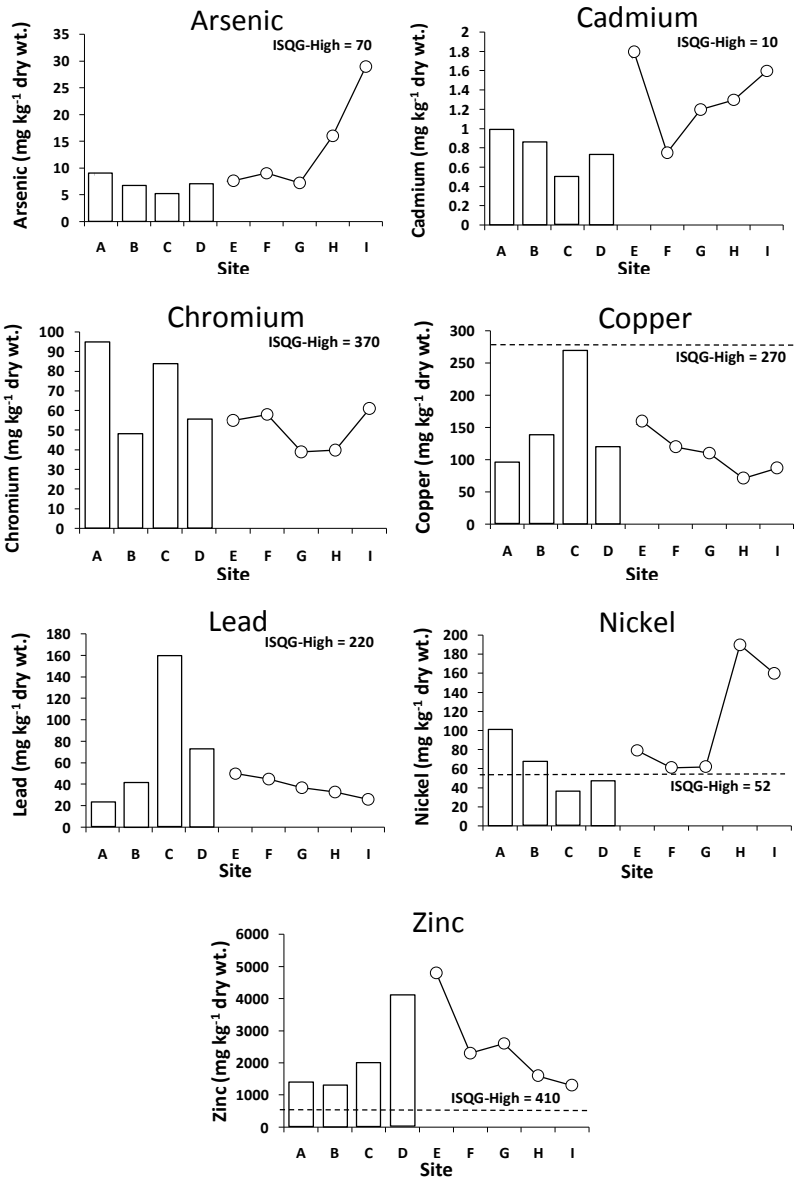

Figure 8.5: Concentrations of As, Cd, Cr, Cu, Pb, Ni and Zn (mg kg^{-1} dry wt. of biofilm) at different sampling locations along the treatment train. Columns represent concentrations in the different draining areas before the confluence of the pipes in site E while lines represent concentrations after the confluence. Data were collected from pooled samples, combining the biofilm biomass of three sponge samples, and are not replicated. The dashed lines show the ISQG-High values (ANZECC & ARMCANZ, 2000) which indicate possible risk to environmental health.

Within Lucas Creek, concentrations of Cu, Pb and Zn were analysed in both sediments and biofilm at all sampling locations (Figure 8.6). Concentrations of biofilm-associated metals were greater than concentrations of sediment-associated metals for all three elements (paired t-test, $P < 0.05$). Concentrations of metals measured in sediments were all below ISQG-high trigger values (ANZECC & ARMCANZ, 2000). Concentrations of metals in either biofilm or sediment did not differ significantly between sampling sites located upstream or downstream

of the stormwater outlet (student t-test, $p > 0.05$), except for concentrations of biofilm associated lead, which were greater in upstream sections (student t-test, $p = 0.012$). Concentrations of all three metals were generally similar for the stormwater outlet as for the surrounding sampling sites within Lucas Creek.

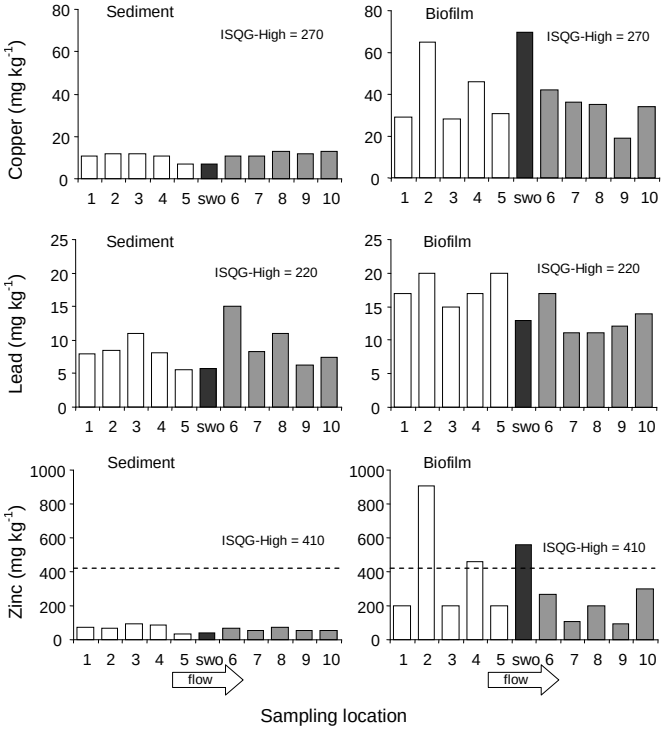

Figure 8.6: Concentrations of Cu, Pb and Zn (mg kg^{-1} dry wt.) in sediment (left) and biofilm (right) within: (□) Lucas Creek sites located upstream of the stormwater outlet; (▩) Lucas Creek sites located downstream of the stormwater outlet; (■) the stormwater outlet. Data for each site were collected from pooled samples, combining either the biofilm biomass of three rocks or three 50 ml sediment samples and not replicated. Labels on the x-axis refer to sampling location within Lucas Creek, SWO is stormwater outlet. The dashed lines show the high interim sediment quality guideline (ISQG-High) values (ANZECC & ARMCANZ, 2000) which indicate possible risk to environmental health

Additional biofilm samples were analysed for concentrations of biofilm associated As, Cd, Cr and Ni in addition to Cu, Pb and Zn at one sampling location upstream and downstream of the stormwater outlet and at the stormwater outlet itself on each sampling date (Figure 8.7). Concentrations of trace metal associated with biofilm in Lucas Creek followed this order: Zn > Ni > Cr > Cu > As > Pb > Cd. Concentrations of copper, nickel and zinc were slightly higher within the stormwater outlet than within the sampling locations upstream. However, there was no significant difference in the concentration of any biofilm-associated metals between the stormwater outlet, the sampling site located upstream and the sampling sites located downstream of the outlet. All elements but Zn and Ni were present in concentrations

below the ISQG-High guidelines. Concentrations of Ni consistently exceeded ISQG-High guidelines at each sampling site and occasion with concentrations up to 190 mg kg^{-1} detected in the stormwater outlet, more than three times the guideline.

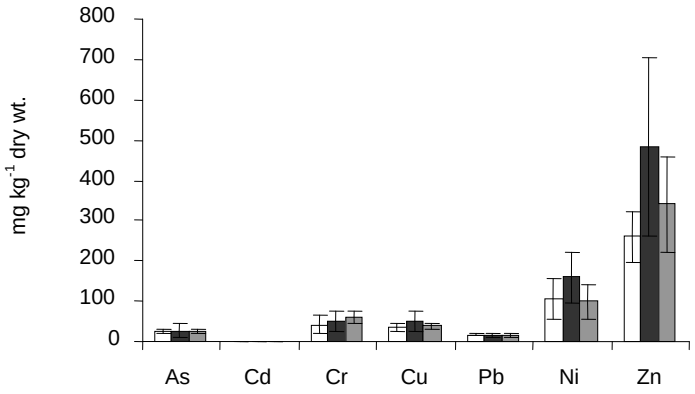

Figure 8.7: Concentrations of As, Cd, Cr, Cu, Pb, Ni and Zn (mg kg^{-1} dry wt. of biofilm) within: (□) Lucas Creek upstream of the stormwater outlet; (■) Lucas Creek downstream of the stormwater outlet; (■) the stormwater outlet. Data for each site were collected from pooled samples, combining the biofilm of three rocks and averaged for the three sampling dates. Error bars are interval of confidence ($\alpha = 0.05$) for the three sampling dates. ISQG-High values are: 70 mg kg^{-1} arsenic, 10 mg kg^{-1} cadmium, 370 mg kg^{-1} chromium, 270 mg kg^{-1} copper, 220 mg kg^{-1} lead, 52 mg kg^{-1} nickel and 410 mg kg^{-1} zinc.

8.4 Discussion

We hypothesised that (i) the concentrations of stormwater associated contaminants would be reduced across the treatment train, causing significant differences in bacterial community structure, and (ii) there would be no significant difference in bacterial community structure or concentrations of biofilm associated metals either side of the stormwater outlet into the receiving waters of a nearby creek.

Both biofilm bacterial community structure and associated metals revealed a gradual improvement in water quality along the treatment train and a minimal impact of the discharge on the receiving freshwater ecosystem. This confirms our first hypothesis of the efficacy of the stormwater treatment train in reducing the contaminant load and in limiting the impact on the receiving waters. This study also supports the use of ARISA as a sensitive and reproducible indicator of the environmental changes within freshwater systems, including enclosed stormwater networks where traditional biological indicators are not present.

Major shifts in biofilm bacterial population structure occurred throughout the treatment train and particularly at the upper reaches. Because sites A-B, C and D have localised and specific catchment areas, it is expected that they received stormwater of different composition. Biofilm associated metals confirm the variability of contaminant loads in those sites with, for instance, high concentrations of both lead and copper in site C and high concentration of zinc in site D. Further downstream, the confluence of different waters in site E generated a mixed stormwater influenced slightly by each of the sources. Analysis of the bacterial community structure reveals the same trend with very different bacterial communities present in each of the sites upstream of the car park, but with populations mixing to generate the combined bacterial community observed at E. Further downstream of site E, changes in bacterial community structure became progressively smaller, but continued to change to become similar to that within the receiving creek, implying an improvement in water quality along the treatment train.

At the discharge into the receiving stream, our study revealed significant differences in bacterial community structure between stream sections located upstream or downstream of the discharge, which lead us to reject our second hypothesis. However, compared to the data collected from a wide range of streams in Auckland area, the observed differences in bacterial community structure were relatively small. This suggests that the stormwater outlet is currently causing minimal disturbance to the ecological health of the receiving waters of Lucas Creek.

Despite the elevated concentrations of metals within the microbial biofilms of the stormwater treatment train, concentrations of copper, lead and zinc within the sediments of Lucas Creek were within the ISQG-High limits and were not significantly greater downstream of the stormwater outlet. Since concentrations of some metals (most notably of copper, lead and zinc) decreased in the downstream sampling sections of the treatment train, this study confirms the effectiveness of the treatment system in reducing the load of contaminants entering Lucas Creek.

In the receiving stream, concentrations of biofilm-associated metals were consistently higher than concentrations of sediment–associated metals. High concentrations of trace elements have previously been observed to accumulate within natural biofilm communities exposed to pollutants (see chapters 4 and 7 and Farag *et al.*, 2007; Holding *et al.*, 2003; Ivorra *et al.*,

1999; Morin *et al.*, 2008). This may have important implications for aquatic systems, since biofilms are the basis of most aquatic food webs (Lamberti, 1996). Concentrations of biofilm associated contaminants could therefore provide a more relevant measure of the effects of human activity on freshwater ecosystem health. This warrants further study to define toxic concentrations of contaminants in biofilm for aquatic biota, since at present, there are no recommended guidelines for levels of pollutants within microbial biofilms.

This study confirms the efficacy of a multifaceted treatment train to minimise the impacts of a stormwater discharge on the receiving stream ecosystem. Major shifts in bacterial population structure observed along the treatment train highlight the changes in environmental conditions and the importance of the stormwater treatment before discharge into the stream. This supports the use of low impact design and integrated stormwater treatment systems to mitigate the impact of urban development on freshwater rivers and streams.

The findings of this study also reveal the successful use of ARISA to monitor differences in bacterial community structure between complex and varied environmental samples. The differences in bacterial community structure within sections of Lucas Creek located upstream or downstream of the stormwater outlet differed very little when compared to the differences in bacterial community structure between different streams. Nevertheless, the ARISA proved its ability to differentiate the bacterial communities located either upstream or downstream of the outlet on all sampling occasions.

8.5 Conclusions

Both biofilm bacterial community structure and biofilm associated metals revealed a gradual improvement of water quality through the treatment train. This confirms the efficacy of a multifaceted treatment train and supports the use of low impact design and integrated stormwater treatment systems to mitigate the impact of urban development on freshwater rivers and streams. This study also demonstrates the successful use of ARISA and biofilm associated metals as efficient indicators to monitor changes in enclosed stormwater networks where traditional biological indicators are not available.

CHAPTER 9

9 General conclusion

This project investigated how urban runoff affects stream biofilm microbial communities and assessed the potential uses of biofilms as indicators of the impact caused by urbanisation on freshwater ecosystems. Initial experiments conducted in flow chamber microcosms indicated that biofilm microbial communities are modified by exposure to urban runoff contaminants and suggested negative implications for stream ecosystems. Investigation in natural streams then revealed differences in the structure of the communities present in unimpacted and impacted streams and in their tolerance to metal contaminants. The rapid and consistent response of biofilm microbial communities to urban runoff contaminants supported their potential use as bio-indicators of the impact caused by urbanisation. Experiments also investigated absorption and desorption rates of metal contaminants in biofilms. Results revealed the rapid accumulation and the retention of metals in biofilms, suggesting that biofilms may play a critical role in the transfer of metal contaminants from abiotic to biotic components of the stream ecosystem. Metal concentrations in biofilms were then tested as indicators of metal contamination in streams and results highlighted their high ecological relevance. Finally, both biofilm bacterial communities and biofilm associated metals were used successfully to investigate the efficacy of a stormwater treatment system. The techniques developed in this project provide innovative tools that facilitate the assessment of urban influences on aquatic environments. Further research is being carried out to develop a Bacterial Community Index that will allow precise assessment of stream ecosystem health based on biofilm bacterial communities.

9.1 Impact of urban runoff contaminants on biofilm microbial communities and implications for freshwater ecosystems

Urban runoff carries a wide range of contaminants, frequently in concentrations that can alter aquatic biota. The detrimental effects of urban runoff and its potential contaminants on fish and macro-invertebrates has been well described, however, little is yet known about their impact on aquatic microbial communities. Step by step, this project provides an insight into how urban runoff affects biofilm microbial communities, from the effect of specific metal contaminants investigated in flow chamber microcosms to the general impact of urbanisation on natural stream biofilms.

To start with a simplified model, initial experiments were conducted in flow chamber microcosms and investigated the effect of specific metal contaminants of urban runoff (Zn, Cu and Pb) on biofilm microbial communities (chapter 3). Results revealed that significant differences in bacterial community structure could be detected within only 1 to 3 days of exposure to the different treatments. A significant decrease in the number of biofilm associated protozoa could also be observed after only 1 day of exposure to metals. The toxicity of metals on freshwater biofilm has generally focused on photosynthetic organisms (e.g. Gold *et al.*, 2002; Soldo & Behra, 2000). A few studies revealed a decrease in biofilm bacterial activity under zinc or cadmium exposure (Admiraal *et al.*, 1999; Lehmann *et al.*, 1999) suggesting that metals affect bacterial communities but only Massieux *et al.* (2004) showed, using Denaturing Gradient Gel Electrophoresis (DGGE), changes occurring in bacterial community structure following exposure to copper. In the present study, the changes in biofilm bacterial community structure revealed by ARISA confirm the structural sensitivity of the communities and highlight their rapid response to metal exposure.

When the biofilm was exposed to metal contaminants and then returned to uncontaminated water, differences in bacterial community structure remained detectable after 14 days of recovery in clean water (chapter 3). In stormwater receiving environments, both metal concentrations in the runoff and the frequency of rain events would determine the impacts on aquatic ecosystems. Considering that more than 14 days were required for the recovery of bacterial communities after exposure to metals, if rain events happen more frequently, a permanent shift in bacterial communities towards metal tolerant populations can be expected in urban streams. As stream biofilms play key roles in stream ecosystems (Fischer, 2003) and provide a major source of nutrients for organisms grazing on them (Hall & Meyer, 1998; Lamberti, 1996), changes in bacterial and protozoan community structure triggered by urban runoff metal contaminants are expected to alter the functions of biofilms (Boivin *et al.*, 2006; Leland & Carter, 1985) and affect the whole stream ecosystem.

In chapter 5, biofilms originating from different streams were exposed to metal contaminants and the response of bacterial communities was investigated. The increased tolerance to metal exposure of bacterial communities originating from the streams most impacted by urbanisation confirmed the alteration of bacterial communities frequently exposed to urban runoff. These results complete the findings of Admiraal *et al.* (1999) and Lehmann *et al.* (1999) that revealed the decrease in metabolic response to metal exposure of biofilm bacterial

communities originating from environments contaminated by metals. Using a molecular fingerprinting technique (ARISA), we demonstrate that the tolerance to metals is also induced in the structure of biofilm bacterial communities frequently exposed to metal contaminants.

In chapter 6, the effect of different types of stormwater on biofilm bacterial communities was investigated. Although urban runoff are known to affect freshwater algae, invertebrates and fish (Kennedy, 1999; Suren, 2000), very little research has focused on their effect on aquatic microorganisms (Kayhanian *et al.*, 2008) and to our knowledge this is the first study to investigate the effect of urban runoff on biofilm bacterial communities. Results revealed the rapid response of bacterial communities to exposure to all different treatments. In addition, bacterial communities were more affected by exposure to most types of stormwater than by exposure to Zn, Cu and Pb at concentrations known to be toxic to aquatic organisms. This highlights the great impact of the complex association of very diverse contaminants present in urban runoff. Investigation of the main drivers causing the shift in bacterial communities (chapters 4 and 6) revealed that no single contaminant could explain a major proportion of the changes observed and suggested the complex co-influence of all the different contaminants present in the water. Again, as biofilms play key roles in the stream ecosystems, the changes caused by urban runoff exposure are expected to alter the functions of biofilms (e.g. modification in their nutritional value) and affect the whole stream ecosystem. Furthermore, as chapter 3 revealed that more than 14 days were necessary for the bacterial communities exposed to metals to recover, it is expected that in urban runoff receiving streams, the changes in biofilm microbial communities generated during storm events will influence the ecosystem long after the rain stopps and the concentrations of contaminants return to their normal levels.

In chapter 7, biofilm bacterial and ciliate communities in streams progressively impacted by urbanisation were investigated. Results revealed significant differences between the less impacted sites and the most impacted sites and confirmed the influence of urbanisation on microbial communities in streams. Consistent with Lear & Lewis (2009), analysis of bacterial community structure could differentiate communities originating from sites with a rural dominated catchment and communities originating from sites with an urban dominated catchment. This strongly supports the use of bacterial communities as bio-indicators of freshwater ecosystem health.

9.2 Use of biofilm microbial communities as an indicator of freshwater ecosystem health

Communities of macro-invertebrates are traditionally used as biological indicators of stream health (Boothroyd & Stark, 2000; Carter *et al.*, 2006) and their response to environmental changes has been well described (Rosenberg & Resh, 1993; Roy *et al.*, 2003). In contrast, because investigations of microbial communities were limited in the past by the lack of methodological tools, few studies have looked at the use of microorganisms as indicators of aquatic ecosystem health (Cordova-Kreylos *et al.*, 2006; Lear *et al.*, 2009). Nevertheless, recent advances in molecular microbiology now provide a rapid and detailed description of microbial communities (Dorigo *et al.*, 2005) and enable their use as alternative bio-indicators. In this study, two fingerprinting techniques were used successfully to screen a large number of samples and to monitor the differences in community structure. ARISA was used to monitor bacteria embedded in biofilm and T-RFLP was used to monitor ciliate protozoa associated with biofilms.

The use of organisms as bio-indicators relies on their sensitivity to the environmental perturbation investigated (Jamil, 2001). The rapid changes in bacterial and protozoan community structure following exposure to urban contaminants (chapters 3 and 6) demonstrate the sensitivity of biofilm microbial communities and their potential use as bio-indicators of the impact of urban runoff on freshwater ecosystems. In chapter 7, biofilm communities present in different streams progressively impacted by urbanisation were investigated. Similar changes in bacterial and ciliate community structure occurred in the separate streams suggesting that healthy biofilm can be differentiated from an unhealthy biofilm based on the bacterial or ciliate community structure. This confirms a study in which Lear & Lewis (2009) identified differences in bacterial communities from streams impacted by different land uses (rural vs. urban). These findings also demonstrate that biofilm microbial communities can provide a reliable indication of stream health.

In chapter 6, stormwater collected from different urban areas affected biofilm bacterial communities in very different ways revealing that each treatment influenced a different combination of bacteria. This suggested that members of the bacterial community are sensitive or resistant to specific contaminants. Therefore, monitoring the abundance of certain

bacteria in biofilm could reveal the presence of specific contaminants in streams, as recently suggested for hyporheic microbial communities (Feris *et al.*, 2009). This would significantly improve stream health bio-monitoring techniques as the use of macro-invertebrate communities generally relies on an index value representing the general stream quality which gives poor insight into the presence of specific contaminants (Maher & Norris, 1990). The reorganisation of bacterial communities under exposure to contaminants and the replacement of sensitive bacteria by resilient bacteria also suggest that changes occur in the presence of genes responsible for the resistance against specific contaminants. Considerable research has been done to identify particular genes conferring a resistance against various contaminants including heavy metals (Abou-Shanab *et al.*, 2007) and organic compounds (Hamann *et al.*, 1999). In addition, recent developments of new sequencing technologies and of gene micro-arrays have enabled the investigation of the presence of a wide range of functional genes in environmental samples (He *et al.*, 2008; Roh *et al.*, 2010). A combined analysis of the changes occurring in the structure of bacterial communities and of the presence of genes responsible for resistances against specific contaminants could therefore reveal not only where the changes occur but also the main drivers of the changes.

9.3 Accumulation of metals in biofilms and implication for organisms grazing on biofilms

Initial experiments in flow chamber microcosms revealed the fast accumulation of metals in the biofilm matrix. Enrichment factors up to 500:1 for zinc, 1500:1 for copper and 6000:1 for lead measured between the biofilm wet weight and the water after 21 days of exposure confirmed the high absorptive capacity of biofilms (Ivorra *et al.*, 2002; Mages *et al.*, 2004; Xie *et al.*, 2010). Results also highlighted that accumulated metals were retained in the biofilm for weeks after transfer to uncontaminated water. After 14 days of recovery in clean water, 10 to 16 % of zinc and copper accumulated during exposure still remained in the biofilm. The release of lead was even slower and more than 35 % of accumulated lead remained in the biofilm after 14 days of recovery. Further investigation in natural streams (chapters 4 and 7) revealed that the concentrations of As, Cd, Cr, Cu, Pb, Ni and Zn were frequently higher in biofilm than in sediments, corroborating previous studies (Farag *et al.*, 1998; Farag *et al.*, 2007; Holding *et al.*, 2003). Analyses of sediment and biofilm composition suggested that both the organic content and the size of inorganic particle size were responsible for the greater concentrations of metals in biofilms than in sediments.

The toxicity of trace metals depends not only on the exposure of freshwater organisms to dissolved metal ions but also on the transfer of precipitated metals throughout the food chain (Clearwater *et al.*, 2002; De Schamphelaere *et al.*, 2004). As biofilms are intensively grazed by many organisms, the accumulation of metals in the biofilm matrix is likely to form a critical link in the movement of urban contaminants from abiotic to biotic components of the stream as suggested in Farag *et al.* (2007) and Rhea *et al.* (2006). The quick sorption of metals in biofilms, as well as the relatively slow release following a return to uncontaminated water also suggest that during storm events biofilms can quickly accumulate urban runoff contaminants and retain them for several days after the event, increasing the impact of contaminants on aquatic biota.

9.4 Use of biofilm associated metals as an indicator of their detrimental effect of on aquatic biota

Metals associated with sediments are commonly used to assess whether heavy metals are present in stream ecosystems at concentrations that are detrimental to aquatic biota (ANZECC & ARMCANZ, 2000; USEPA, 2005). Although biofilms are constantly growing to regenerate parts that have been grazed or stripped off by the stream flow, they are relatively sessile structures and have been suggested to be an alternative indicator of metal contamination (Holding *et al.*, 2003; Mages *et al.*, 2004). The fast accumulation of metals and their retention in biofilms observed in this project support the use of biofilm associated metals as indicators of stream contamination. The greater concentrations of most trace metals found in biofilms compared to sediments in chapters 4 and 7 also suggest that monitoring biofilm associated metals could improve the detection of contaminants present at low concentrations in aquatic environments.

As biofilms are a main source of nutrients for many aquatic organisms, their content in contaminants may be a highly relevant indicator of the potential threat for stream ecosystems. Therefore, in chapters 4 and 7, the ecological relevance of biofilm associated metals was investigated using biofilm bacterial communities, biofilm associated ciliates and macro-invertebrates as bio-indicators. Metals associated with sediments were simultaneously investigated to provide a comparative measurement. Farag *et al.* (2007) compared metal concentrations in water, biofilms, sediments and macro-invertebrates and suggested that

biofilm may be an important link in the movement of metals through the food chain. From a toxicity perspective, Rhea *et al.* (2006) revealed the correlation between macro-invertebrates communities and concentrations of metals in biofilm, suggesting that biofilm associated metals are relevant indicators of the impact on aquatic organisms. However, the present study is the first to provide a detailed comparative assessment of the ecological relevance of metal associated with biofilms and sediments based on three different types of bio-indicators. Results revealed that a much greater proportion of the changes occurring in all three types of organisms, bacteria, ciliates and macro-invertebrates, were explained by the concentrations of metals present in biofilm than in sediments. This confirms the greater environmental relevance of biofilm associated metals, not only for bacteria embedded in biofilms and therefore directly exposed to accumulated metals but also on motile, ciliated protozoa and macro-invertebrates that feed on biofilms. These findings highlight the ecological importance of biofilm associated contaminants and warrants further study to define toxic concentrations of contaminants in biofilm for aquatic biota, since at present, there are no recommended guidelines for levels of pollutants within microbial biofilms.

9.5 Applications and further research

Throughout this project, numerous potential applications of the new monitoring techniques developed have been highlighted.

A wide range of pollutants can contaminate freshwater ecosystems. Assessing the threshold of toxicity for each of the contaminants is a complex process and often requires the use of invertebrates as bio-indicators (e.g. Girling *et al.*, 2000; Grapentine *et al.*, 2008; Roman *et al.*, 2007). Macro-invertebrates are difficult to manipulate and maintain in laboratory microcosms, which complicates the assessment as the effects of contaminants are often not seen before adulthood (Mason, 1996). The use of biofilm microbial communities in flow chamber microcosms to assess the toxicity of specific contaminants (as demonstrated in chapter 3) greatly simplifies the process. The rapid response of microbial communities to environmental changes is expected because of their short lifespan (Paerl & Pinckney, 1996) and molecular methods enable a precise monitoring of the response. Using a similar protocol, biofilm microbial communities can also be used to assess the ecological toxicity of urban runoff. Due to the great diversity of potential contaminants present in stormwater, it is difficult to assess

the ecological toxicity of stormwater based on chemical analyses. The relatively simple protocol used in chapter 6 constitutes a highly interesting way of assessing quickly and efficiently the ecological impact of different types of stormwater on aquatic ecosystems.

Biofilm microbial communities and associated metals can also be used in situ to provide an indication of their natural freshwater environment. In chapter 8, biofilm was collected from different parts of a stormwater treatment system and in the receiving stream to assess the efficacy of the treatment process and the impact of the discharge on the stream ecosystem. Assessing the ecological improvement of stormwater along a treatment system generally requires laboratory based eco-toxicological tests (Grapentine *et al.*, 2008). The use of biofilm bacterial communities extracted from the stormwater pipes as a bio-indicator simplified considerably the assessment. The assessment of the impact of a stormwater discharge on the receiving streams generally relies on the analysis of macro-invertebrate communities (Gómez *et al.*, 2008; Neumann & Dudgeon, 2002). However, the impact of urban runoff is cumulative from numerous discharges and the impact of a single discharge is often too small to be detected by analysis of macro-invertebrate communities (Davis & George, 1987; Perdikaki & Mason, 1999). In chapter 8, although the discharge had little effect on the receiving creek, significant differences were revealed between bacterial community structure within sections of the creek located upstream or downstream of the stormwater outlet. This highlights the sensitivity of the ARISA technique to detect small perturbations. The use of biofilm as an indicator also has the advantage of being widely applicable because of the ubiquity of biofilms in freshwater environments.

Throughout this project, biofilm microbial communities were used as bio-indicators of the changes occurring in their surrounding environment. Alterations of the microbial community structure and/or composition were assessed by comparison with a control treatment (chapters 3 and 5) or with unimpacted sites (chapter 7). The need for a comparative reference system increases the number of samples to analyse and makes the experiment more complex and laborious. In addition, when the efficacy of a stormwater treatment system was investigated (chapter 8), although the most influential steps in the treatment system could be pinpointed, it remained difficult to assess whether the influences were beneficial or detrimental. Therefore, a microbial biotic index is needed to enable a direct deduction of freshwater ecosystem health based on microbial community structure. Biotic indices combine the abundance of individual species with their pollution tolerance to provide an estimation of the stream ecosystem health

(Pinto *et al.*, 2009). Such an approach is commonly used for the analysis of macro-invertebrate communities (Boothroyd & Stark, 2000) for which pollution tolerance scores have been developed based on the extensive research existing about individual macro-invertebrate species. The macro-invertebrate community index provides a value of stream ecological health calculated from the relative abundance of all the species of the community and their relative tolerance to pollutants (Boothroyd & Stark, 2000). The development of a biotic index requires that pollution tolerance scores can be assigned to individual species of the targeted community (Pinto *et al.*, 2009). In chapter 7, similar changes in bacterial and ciliate community structure occurring along the urbanisation gradient in different streams suggested that specific species or peaks could reveal the level of urban impact of the stream sites. Lear & Lewis (2009) also identified differences in bacterial communities from streams impacted by different land uses (rural vs. urban), confirming the possible development of a biotic index based on bacterial community structure. By analysing the presence and abundance of numerous bacterial species and their relative tolerance score, a microbial index could assess the general quality of the ecosystem. As chapter 6 revealed that different contaminants influenced different bacteria, a bacterial community index may also enable the identification of specific stress factors. This would constitute a major advantage compared to macro-invertebrate community indices which provide little information regarding the exact cause of the pollution (Maher & Norris, 1990). Following the results of this thesis, the development of a bacterial community index for New Zealand has been undertaken by the 'Stream Biofilm Group' based at the University of Auckland. Investigations are being carried out in more than 300 streams all around New Zealand. By comparing the presence and abundance of the different peaks and various physico-chemical parameters of the streams, the different peaks are being assigned ecological values. The resulting index should provide an easy and reliable assessment of stream health that will facilitate the identification of anthropogenic pollution and help water resource managers to succeed in their stream restoration attempts.

APPENDICES

10 Appendices

List of appendices :

Appendix 1

Table App-1: Comparison of biofilm bacterial populations from the long term experiment, based on abundances of cloned 16S rRNA gene sequences, identified at the genus level. Columns are: (st0) the population at the beginning of the long term experiment and after 7 days of exposure to (ct7) the control treatment with no metal added, (mr7) synthetic urban runoff, moderately contaminated and (hr7) synthetic urban runoff, highly contaminated.

Genus \ Samples	st0	ct7	mr7	hr7
	%population			
Aquabacterium	-	2.3	1.1	-
Azospirillum	-	1.2	-	-
Brevundimonas	-	1.2	-	-
Caulobacter	3.6	-	2.2	2.4
Chitinophaga	1.2	-	2.2	-
Clostridium	1.2	-	-	-
Cupriavidus	-	-	1.1	-
Cytophaga	-	-	2.2	-
Desulfosporosinus	-	-	-	1.2
Devosia	2.4	1.2	-	-
Gemmata	-	5.8	-	-
Gemmatimonas	-	1.2	-	-
Hyphomicrobium	-	1.2	-	-
Hyphomonas	1.2	-	-	-
Ideonella	1.2	-	2.2	1.2
Isosphaera	-	1.2	-	-
Lactobacillus	-	-	1.1	-
Legionella	8.4	2.3	-	2.4
Leptospira	-	1.2	1.1	1.2
Leptothrix	4.8	-	2.2	-
Limnothrix	-	3.5	1.1	1.2
Lysobacter	1.2	-	1.1	-
Methylobacterium	-	-	1.1	-
Methylocapsa	-	1.2	-	-
Methylophilus	-	2.3	9.7	4.8
Methylosinus	1.2	-	-	-
Niastella	-	-	1.1	-
Nostocoida	-	-	-	1.2
Novosphingobium	-	3.5	1.1	14.3
Paucibacter	-	-	1.1	-
Pedomicrobium	-	-	-	2.4
Phenylobacterium	-	1.2	1.1	1.2
Porphyrobacter	-	4.7	5.4	1.2
Prosthecobacter	-	-	1.1	-
Pseudomonas	7.2	1.2	3.2	14.3
Ralstonia	-	-	-	1.2
Reyranella	2.4	2.3	2.2	1.2
Rhodobacter	3.6	1.2	1.1	-
Rickettsieae	-	1.2	-	-
Roseateles	1.2	-	-	2.4
Rubrivivax	-	1.2	-	-
Runella	-	-	1.1	-
Sandarakinorhabdus	1.2	-	2.2	4.8
Sphingobacterium	-	-	-	1.2
Sphingomonas	8.4	7.0	3.2	1.2
Steroidobacter	-	1.2	-	-
Tistrella	-	1.2	-	-
Variovorax	-	2.3	-	-
unknown	49.4	46.5	48.4	39.3

Appendix 2

Table App-2: Inorganic particulate matter and inorganic particles < 63 µm remaining after acid digestion (see § 2.3.2 for more details about the digestion) in % of biofilm and sediment samples dry weight for each of the 23 stream sampling sites. Data are averages of replicates (3) except for the biofilm from Ngakoroa Stream for which the data was not replicated.

	Sediments		Biofilm	
	Inorganic particulate matter (% sample dry wt.)	Inorganic particles<63 µm (% sample dry wt.)	Inorganic particulate matter (% sample dry wt.)	Inorganic particles<63 µm (% sample dry wt.)
Cascades stream	61.8	23.1	42.2	30.8
Matakana River	87.1	42.2	42.6	38.7
Mahurangi, site 2	86.6	23.9	72.9	51.0
Ngakoroa Stream	72.0	34.5	26.1	24.0
Opanuku Stream	80.8	11.9	42.8	32.9
Wairoa River	85.3	19.1	57.7	41.8
Waiwera River	88.7	43.4	78.3	52.1
Hoteo River	89.4	26.6	73.6	44.6
Rangitopuni River	72.2	60.8	60.1	57.3
Kumeu River	78.6	22.8	57.7	55.5
Mahurangi, site 1	87.6	11.1	52.9	50.3
Papakura Stream	72.3	15.4	65.4	63.9
Otara Creek, site 1	84.1	41.0	68.5	58.2
Vaughans Stream	90.9	21.6	25.3	22.5
Puhinui Stream	79.6	21.0	11.3	10.7
Lucas creek	81.6	50.9	70.5	61.2
Oteha Stream	65.0	12.5	14.6	11.5
Pakuranga Creek, site 2	84.6	19.5	37.3	35.0
Otara Creek site 2	72.4	15.7	20.9	18.0
Pakuranga Creek, site 1	72.6	32.5	46.2	40.0
Oakley Creek	88.0	19.8	62.6	52.9
Otaki Creek	75.2	11.2	43.6	37.1
Omeru Creek	63.1	41.9	23.7	22.9
Average	79.1	27.1	47.7	39.7

Appendix 3

Table App-3: Most influential peaks in the differentiation between biofilm bacterial communities in the control treatment and in the stream or storm water treatments. The peaks were identified by SIMPER analysis based on the data from both sampling occasions (3 and 6 days of exposure). The five most induced peaks are listed together with their presence (x) in the original biofilm and/or in the treatment water.

	Cascades stream					Sylvia Park stormwater					Oakley Creek					Wairau Creek				
Peak position (b.p.)	767	812	768	513	645	578	579	588	478	618	512	588	397	513	445	512	589	505	517	506
Present in initial biofilm	x	x	x	x	x	x		x			x	x	x	x	x	x	x	x		x
Present in treatment water							x							x					x	x

	Albany Busway stormwater					Tamaki stormwater					Lucas Creek					Mission Bay stormwater				
Peak position (b.p.)	397	396	398	470	477	488	513	620	512	444	813	812	768	397	590	588	396	761	760	606
Present in initial biofilm	x	x		x		x	x		x	x	x	x	x	x		x	x	x	x	x
Present in treatment water	x		x	x		x									x	x				

Appendix 4

Oratia Stream

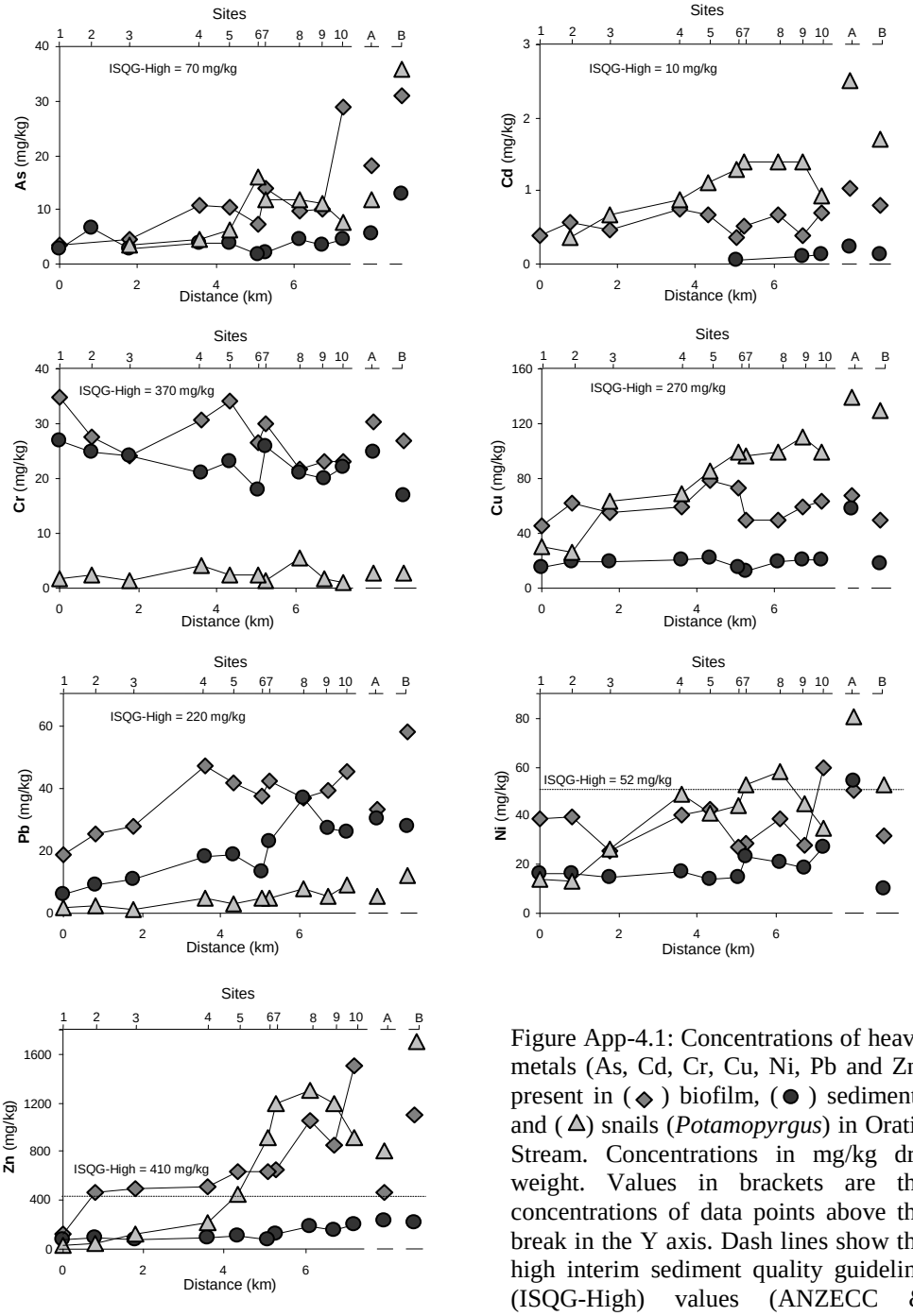

Figure App-4.1: Concentrations of heavy metals (As, Cd, Cr, Cu, Ni, Pb and Zn) present in (◆) biofilm, (●) sediments and (△) snails (*Potamopyrgus*) in Oratia Stream. Concentrations in mg/kg dry weight. Values in brackets are the concentrations of data points above the break in the Y axis. Dash lines show the high interim sediment quality guideline (ISQG-High) values (ANZECC & ARMCANZ, 2000).

Swanson Stream

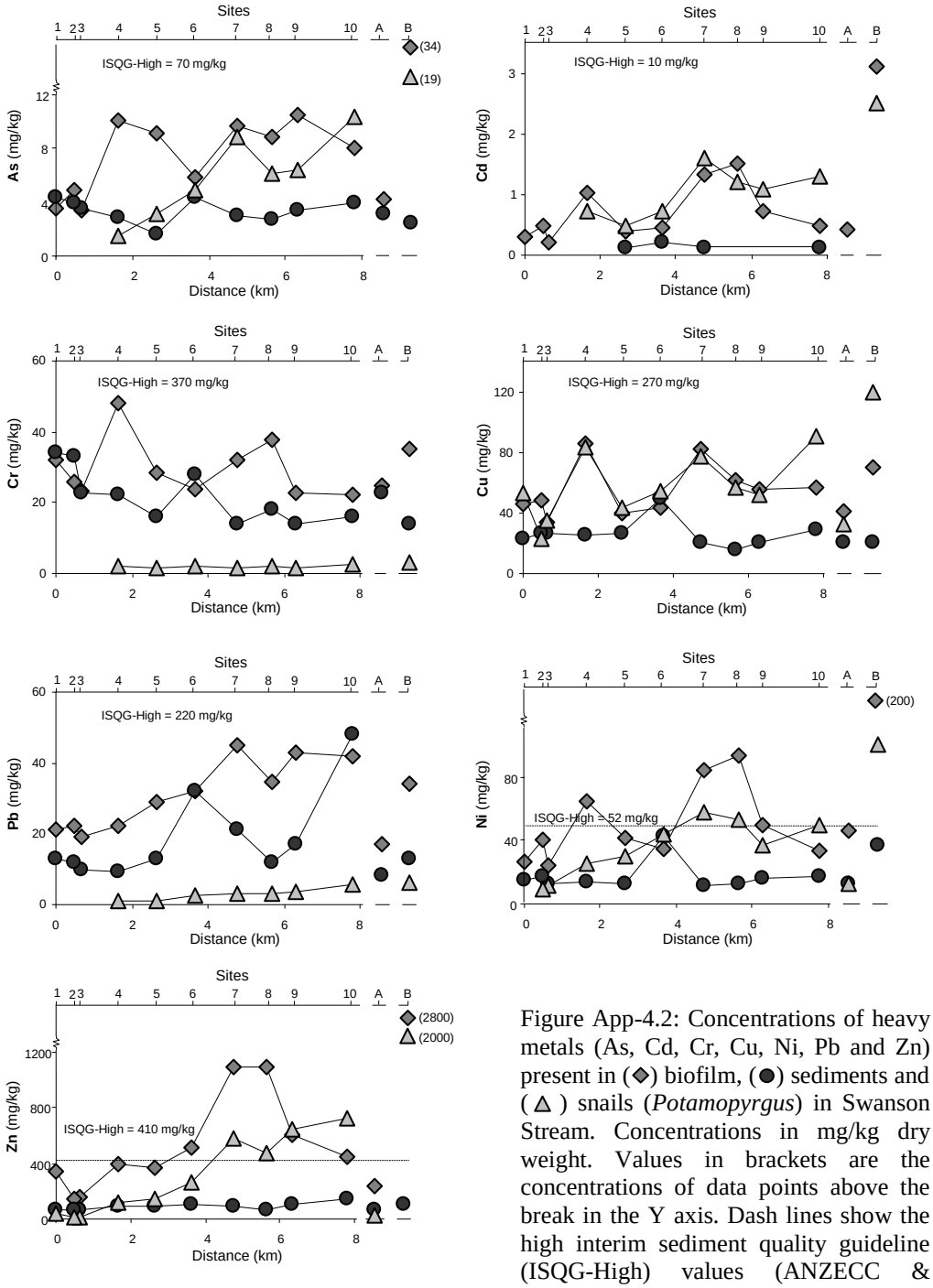

Figure App-4.2: Concentrations of heavy metals (As, Cd, Cr, Cu, Ni, Pb and Zn) present in (◆) biofilm, (●) sediments and (△) snails (*Potamopyrgus*) in Swanson Stream. Concentrations in mg/kg dry weight. Values in brackets are the concentrations of data points above the break in the Y axis. Dash lines show the high interim sediment quality guideline (ISQG-High) values (ANZECC & ARMCANZ, 2000).

Hays Creek

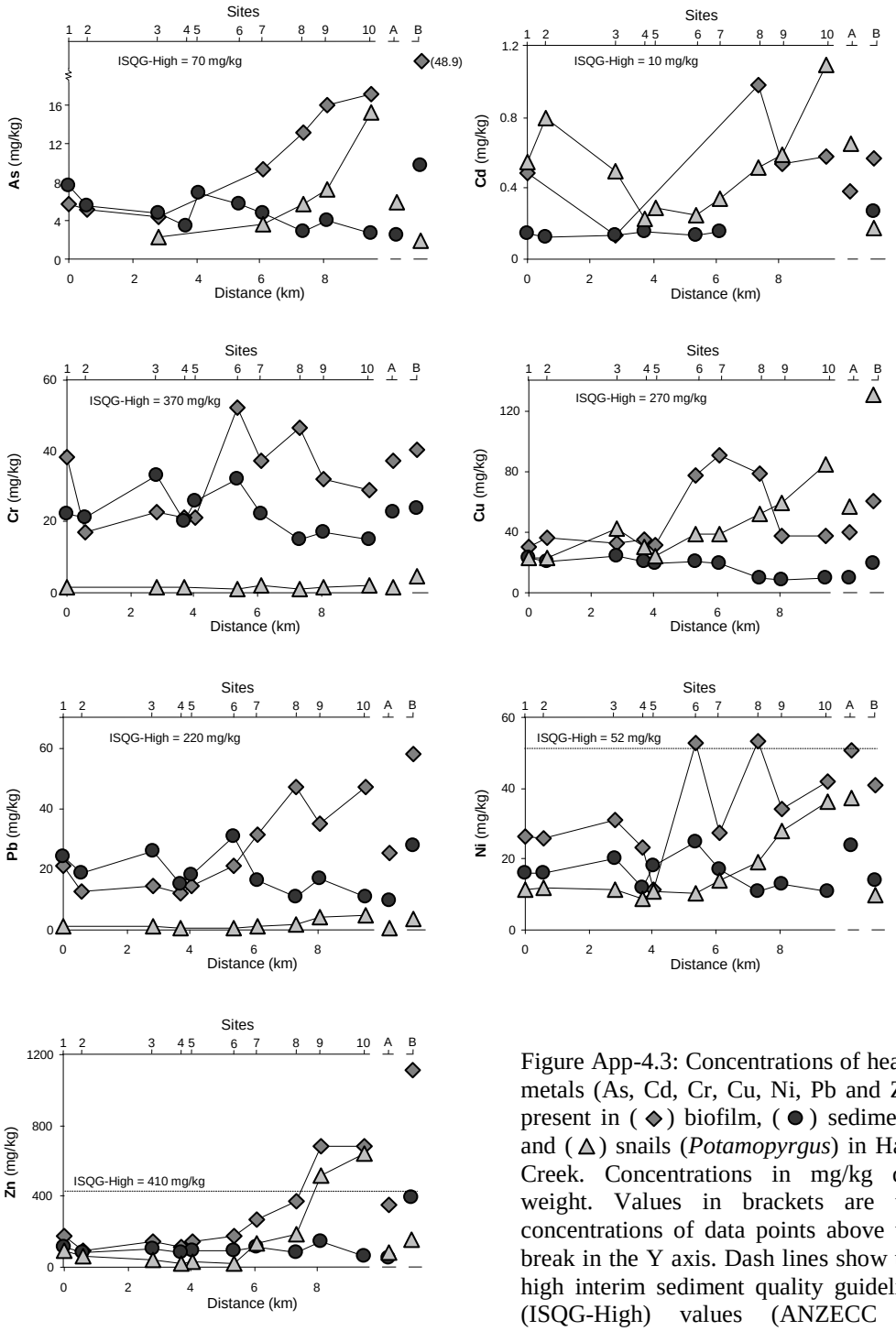

Figure App-4.3: Concentrations of heavy metals (As, Cd, Cr, Cu, Ni, Pb and Zn) present in (◆) biofilm, (●) sediments and (△) snails (*Potamopyrgus*) in Hays Creek. Concentrations in mg/kg dry weight. Values in brackets are the concentrations of data points above the break in the Y axis. Dash lines show the high interim sediment quality guideline (ISQG-High) values (ANZECC & ARMCANZ, 2000).

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