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Factors Affecting the Response of Marine and Estuarine Bacterial Communities to Trace Metal Enrichment

by

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ABSTRACT

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FACTORS AFFECTING THE RESPONSE OF MARINE AND ESTUARINE BACTERIAL COMMUNITIES TO TRACE METAL ENRICHMENT

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Studies of the effects of trace metal perturbation on microbial communities have, to date, concentrated on contaminated environments, single phylotypes or single metals. The effects of trace metals on bacterial communities in estuarine and marine environments have been largely overlooked. The aim of this research was to advance the scientific knowledge in this area by the implementation of incubation experiments. Experiments were designed to determine the effects of trace metal enrichment and aggregate formation on the structure and function of bacterial communities. Environmental samples included bacterioplankton from a pristine estuary (Erme, Devon), a moderately contaminated estuary (Tamar, Devon), a contaminated estuary (Fal, Cornwall), a coastal station (M1) and a truly marine station off the continental shelf (M2).

Key factors influencing the response of bacterial communities to trace metal enrichment were identified. In marine communities the most influential factors were; environmental parameters, such as type and concentration of organic matter; initial community composition and ambient concentration of zinc. The addition of trace metals resulted in a decrease in diversity in the bacterial community from the Tamar Estuary, however, bacterial association with aggregates appeared to reduce this effect. Community dynamics of bacteria from a pristine estuary (Erme) demonstrated remarkable bacterial resilience under trace metal stress, particularly in samples dominated by bacteria from the *Rhodobacteraceae*. Some metals were shown to have a more profound effect on community dynamics than others, resulting in the division of trace metals into Type 1 and Type 2 categories as a function of bacterial response. RNA derived community fingerprints were more different between incubation conditions than DNA derived fingerprints, and were thus a more sensitive indication of response to trace metal enrichment. The wider implications of the effects of trace metals on bacterial communities in estuarine and marine environments are discussed, along with possible future research directions. Recommendations are made for future investigations of the effects of metal contamination in light of the results presented here.

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DECLARATION OF AUTHORSHIP

I, RACHEL MARY JONES declare that the thesis entitled

Factors Affecting the Response of Marine and Estuarine Bacterial Communities to Trace Metal Enrichment

and the work presented in it are my own. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
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- I have acknowledged all main sources of help;
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- none of this work has been published before submission.

Signed: ……..

Date:…….

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Abbreviations

µL	microlitres
µm	micrometres
AMD	acid mine drainage
AMP	ampicillin
AONB	area of outstanding natural beauty
APS	ammonium persulphate
ARDRA	amplified ribosomal DNA restriction analysis
ARISA	automated intergenic spacer analysis
bp	base pair
cDNA	complementary DNA
CDOM	chromophoric dissolved organic matter
CFU	colony forming unit
CT	controlled temperature
CTAB	cetyltrimethylammonium bromide
D/TGGE	denaturing / temperature gradient gel electrophoresis
DAPI	diamidino-2-phenyindole
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DPM	disintegrations per minute
EDTA	ethylenediaminetetraacetic acid
FAA	0.2µm filtered, aged and autoclaved
FASW	0.2µm filtered, autoclaved sample water
HGMP	Human Genome Mapping Project
IMS	industrial methylated spirits
IPTG	isopropyl β-D-1-thiogalactopyranoside
KOAc	potassium acetate
L	litre
lacZ	beta-D-galactosidase gene
LB	Luria Bertani
MDS	Multi-Dimensional Scaling

MilliRO	high purity, reverse osmosed water created using the Millipore Milli-RO system
mL	millilitres
mm	millimetres
MQ/ Milli-Q	18.2Ω, high purity, reverse osmosed, UV treated, 0.2µm filtered water created using the Millipore Milli-Q system.
NaCl	sodium chloride
O.T.U	operational taxanomic unit
OD600	optical density at 600nm
PCAA	particulate combined amino acid
PCR	polymerase chain reaction
PEG	polyethylene glycol
pers. comm.	personal communication
PML	Plymouth Marine Laboratory
pmoles	picomoles
POC	particulate organic carbon
PON	particulate organic nitrogen
R.R.S	Royal Research Ship
RCF	relative centrifugal force
rDNA	Ribosomal DNA
RNA	ribonucleic acid
rRNA	Ribosomal RNA
rpm	revolutions per minute
SAC	special area of conservation
SAPs	successive alkalinity producing system
SCDOM	slope of CDOM absorbance curve
SOB	buffered growth medium for high efficiency competent E.coli cells
SOC	growth medium designed to ensure maximum transformation efficiency (*E. coli*)
SPA	special protection area
SPM	suspended particulate matter
spp.	species
SRB	sulphate reducing bacteria

SSCP	single strand conformation polymorphism
SSSI	site of special scientific interest
Taq	DNA polymerase from *Thermus aquaticus*
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TFBI	transformation buffer I
TFBII	transformation buffer 2
TMZ	Turbidity Maximum Zone
T-RFLP	terminal restriction fragment length polymorphism
TSR	template suppressing reagent
UV	Ultra Violet
X-Gal	5-bromo-4-chloro-3-indoyl β-D-galactopyranoside

1 Introduction

This thesis will present multi-disciplinary research encompassing molecular microbial ecology and the effects of aggregate formation and trace metal enrichment on bacterial communities. This chapter gives an overview of why the study of bacterial communities is necessary and summarises methodologies and research areas of direct relevance to the research performed.

1.1 Bacteria

1.1.1 Why study the effects of trace metals on marine bacterial community structure?

The study of bacteria in aquatic environments is essential to determine how impacts on their numbers and diversity, particularly through anthropogenic activities, will affect the cycling of organic matter and nutrients. This study focuses on the effects observed with trace metal contamination and spans aquatic environments from estuaries to coast and the open sea.

1.1.1.1 Importance of bacteria in aquatic environments

The importance of bacteria in aquatic environments has been recognised since the early eighties when the elegant paradigm 'the microbial loop' was proposed by Azam *et al.,* (1983). The microbial loop described the role played by bacteria in recycling and repackaging of organic matter in terms of the phytoplankton based marine food web and is updated progressively as new links and pathways are identified. The role of bacteria in the flux and cycling of carbon and nutrients in aquatic environments is still a subject of intense scientific activity (Cho and Azam, 1988; Turley and Mackie 1994; Azam 1998; Ploug and Grossart, 1999; Ducklow 2000; Zubkov *et al.,* 2001; Sherr and Sherr 2003; Zehr *et al.,* 2003; van Mooy *et al.,* 2004) and as such has prompted the development of improved methodologies. As a result of improved methodologies for measurement of parameters such as microbial biomass, metabolic activity, growth rate and production, microbial ecology remains one of the most exciting and important fields in modern science.

Bacteria are amongst the most abundant and diverse organisms in aquatic environments. They vary in numbers from 10^5 mL^{-1} in oligotrophic regions of the open ocean, to 10^6 mL^{-1} in coastal regions, to 10^7 mL^{-1} in eutrophic and estuarine regions (Li,

1998). This variation in bacterial numbers, controlled predominantly by nutrient levels, is also apparent in phytoplankton (Li *et al.,* 2004). However, the decrease in abundance observed in low nutrient environments is not as extensive in bacteria, which are able to utilise lower levels of nutrients than phytoplankton. This means that, in extreme cases, production by bacteria can contribute more significantly to marine food webs than that of phytoplankton (Del Giorgio *et al.,* 1997; Cotner and Biddanda, 2002).

1.1.1.2 Bacteria play an essential role in biogeochemical cycles

Carbon, hydrogen, oxygen, nitrogen, phosphorus and sulphur are the major elements of life and bacteria play an important role in the cycling of all of these elements (Cho and Azam, 1988; Turley and Mackie, 1994; Azam 1998; Ploug *et al.,* 1999; Ducklow 2000; Zubkov *et al.,* 2001; Sherr and Sherr 2003; Zehr *et al.,* 2003; van Mooy *et al.,* 2004). Bacterial metabolism can take many forms e.g. aerobic respiration, anaerobic respiration, oxygenic photosynthesis, anoxygenic photosynthesis, fermentation, lithotrophy and photoheterotrophy. As a result of this metabolic diversity bacteria are able to use a wide variety of dissolved organic matter as a substrate. The metabolic processes of bacteria always require an energy source and an electron acceptor and different types of bacteria are frequently grouped together based on the energy source or electron acceptor they use. Examples of aerobic respiration include nitrifying bacteria which gain energy (electrons) by oxidising NH_3^+ to NO_2^- or NO_2^- to NO_3^{2-}, sulphur oxidisers via oxidation of H_2S or S to SO_4^{2-} and iron bacteria from the oxidation of Fe^{2+} to Fe^{3+}. These bacteria are also all lithotrophic i.e. they use inorganic compounds as a source of energy. Anaerobic respiration is more common in sediments, although it has been detected in the water column usually associated with regions of intense productivity. Anaerobic respiration includes the metabolism of sulphate reducing bacteria which reduce SO_4^{2-} to H_2S or S and denitrifying bacteria which reduce NO_3^{2-} to NO_2^- or NH_3^+. Photoheterotrophs use energy provided by sunlight to reduce organic carbon. Photosynthesis in bacteria is either oxygenic, which uses electrons derived from light harvesting to reduce carbon dioxide to oxygen, or anoxygenic, which uses an external reducing agent such as H_2S to reduce carbon dioxide (or an alternative carbon source).

Bacteria are of crucial importance in the carbon and nitrogen cycles where they perform unique functions through these diverse metabolic activities. Particularly in the nitrogen cycle they alone can transform both excretory products and atmospheric

nitrogen to NO_3^{2-}, a form of nitrogen which is available to phytoplankton (Capone, 2001; DeLorenzo *et al.,* 2001).

1.1.2 Methods used to study microbial ecology

1.1.2.1 Traditional

Bacterial ecology as a discipline was confounded in the early days by the high degree of visual similarity of bacteria with extremely different phenotypic characteristics. Accordingly bacteria of similar appearance were commonly grouped together. To discover more about the roles played by bacteria in ecosystems, conventional approaches relied heavily on cultivation techniques. Enrichment and isolation techniques were typically used for bacterial identification and characterisation. However it has been estimated that less than 1% of marine bacteria have been cultured using these methods (Amann *et al.,* 1995), making assemblage dynamics and composition difficult to assess (Murray *et al.,* 1998). The low proportion of species successfully cultivated could be because traditional culture techniques do not successfully mimic real environmental conditions, under which natural populations flourish (Ward *et al.,* 1990). Alternatively, the physiological state of the cell, as few as 10% of cells may be active at any one time (Bernard *et al.,* 2000), may affect their culturability. It has been estimated that only half of the known major bacterial phylogenetic groups have members in cultivation (Rappe & Giovannoni 2003). Nold *et al.,* (1995; 1996) contributed, in part, to reducing the discrepancies between cultivated and molecular retrieval via the use of more relevant incubation temperatures, substrate types and substrate concentrations. Comparison of bacterial populations detected by traditional and molecular approaches have described the communities present as completely different (Santegoeds *et al.,* 1996). As recently as five years ago isolation procedures were still failing to adequately investigate microbial diversity (Nübel *et al.,* 1999).

More recent approaches, based on reflecting environmental conditions and targeting bacteria which grow more slowly, have been more successful. Connon and Giovannoni (2002) achieved the culture of 14% of cells from coastal seawater via dilution to extinction in very low nutrient medium (this is an improvement of between 14 and 14 000% depending on the initial estimate i.e. 0.01 or 1%). They also pioneered the use of microtiter plates as incubation vessels (enabling the analysis of nearly 2 500 extinction cultures over a three year period) to create a high throughput methodology to

speed up the laborious process of dilution to extinction. Zengler *et al.,* (2002) have derived a gel microdroplet system which encapsulates single cells from environmental samples. These droplets can be incubated together in a variety of flowing media for a minimum of 5 weeks after which time analysis by flow cytometry allows the droplets containing colonies to be selected for further study. The beauty of this technique is that nutrient levels are controlled by diffusion through the gel matrix and growth factors and other signalling molecules are present. Thus, environmental conditions which are impossible to mimic under any other conditions are reflected, whilst still allowing isolation in pure culture. High throughput techniques which limit nutrient availability have also been used to isolate and cultivate bacteria from the Oligotrophic Marine Gammaproteobacteria, a group only known to exist because of clone library construction (Cho and Giovannoni 2004).

1.1.3.2 Molecular techniques

Molecular phylogenetic approaches, based on cloning, the polymerase chain reaction (PCR) and comparative analysis, have alleviated many of the limitations placed on bacterial ecology. They have been shown to detect microbial species consistent with microscopic detection in the same environments, and have also brought to light many novel species. New insights on phylogenetic diversity and characterisation of naturally occurring non-cultured organisms via 16S rRNA cloning, amplification and sequencing (Pace *et al.,* 1986; Wise *et al.,* 1997; Nübel *et al.,* 1999) have proved invaluable. A further advantage of molecular techniques is that isolation of rRNA genes for phylogenetic analysis is less selective and thus provides a more representative view of microbial community structure than classical techniques (Reysenbach *et al.,* 1992) . These advantages over conventional methodologies have been demonstrated in the analysis of natural samples from fresh, coastal and marine waters, (Ward *et al.,* 1990; Britschgi and Giovannoni 1991; Øvreas *et al.,* 1997; Bernard *et al.,* 2000). Indeed, to demonstrate the importance of molecular techniques, it is worth noting that it was the application of DNA sequencing which brought to light the basic phylogenetic split between the Archaea and Bacteria and resulted in the introduction of the domains Archaea, Bacteria and Eucarya (Woese 1990). It was also these techniques that allowed the further classification of the bacteria into 11 major divisions (Woese *et al*., 1990). However, to gain greater understanding of the physiology and ecology of bacterial species their isolation in pure culture remains an essential part of microbial ecology.

Indeed it has been observed (Ellis *et al.,* 2003) that the cultivable portion of the community may give greater clues to the effects of contaminants than analyses of diversity.

Variability in the efficiency of PCRs is a common observation (Suzuki and Giovannoni 1996), and can arise for a number of reasons. One reason is the phenomenon known as PCR drift. PCR drift can result in different PCR products from the same template and is attributed to stochastic variations in the early stages of PCR. Mutations introduced in the early stages of a PCR reaction can become established during the exponential phase, this phenomenon is thought to occur more frequently in samples containing low concentrations of template. Drift may also occur due to variations in the thermal profile and variable ramping characteristics in different areas of the thermocycler used. A second consideration when using PCR is the possibility of bias (Reysenbach *et al.,* 1992; Wagner *et al.,* 1994). Bias, in this case, is where certain parts of the starting template are amplified preferentially and so make up a greater proportion of the resulting product than of the original template. Bias can arise for a number of reasons; firstly, the proportional guanine and cytosine content of template DNA. Secondly, differential binding energies derived from primer degeneracy (Suzuki and Giovannoni 1996) and thirdly, the influence conferred by template folding, i.e., secondary structure formation of templates in the annealing stage of the process. These factors combined mean that it is likely that the bias could be against certain groups of bacteria.

It is possible to minimise the effects of PCR bias by using the minimum number of cycles and appropriate template concentrations. Another consideration is the formation and subsequent analysis of hybrid or heteroduplex molecules, a problem common to all conserved phylogenetic markers. Critical analysis of derived sequences is essential for detection of any chimeric sequences. This analysis can be done in two ways, by checking the secondary structure of highly conserved helices or by calculation of separate phylogenetic trees for individual domains of the sequences concerned. The RDP-II website (http://rdp.cme.msu.edu/) provides an interface designed to aid with the latter. A simple experimental methodology applied to detect heteroduplex formation is the subsequent excision from a denaturing gradient gel electrophoresis (DGGE) gel and re-amplification of the relevant bands, followed by a repeat DGGE analysis and identification of the component phylogenies (Ferris and Ward 1997).

Despite these limitations molecular techniques are currently the best tool available for studies of bacterial diversity. Exciting and versatile methods are constantly being

developed for this purpose and have been successfully applied to the questions facing microbial ecologists, one of the most important being how bacterial communities change in response to perturbation.

1.1.3 Detection of community change in response to perturbation

Bacteria play an essential role in the majority of biogeochemical cycles (Section 1.1.1). They are ubiquitous, inhabiting even the most extreme environments and furthermore, as a result of short generation times, they respond rapidly to environmental perturbations. In light of these characteristics it seems logical that they should be included in ecological risk assessment. Whilst bacteria are regularly utilised in this manner it is generally as pure cultures (e.g. Microtox®) which clearly do not reflect the diversity of tolerance levels and substrate use observed in environmental bacterial communities. Whilst such toxicity tests are undoubtedly useful indicators of environmental effects of pollutants they do not necessarily encompass the full range of effects of such pollutants on biogeochemical cycling. Traditional methods of assessing bacterial community diversity also share this fundamental flaw as a result of inherent selection for cultivable bacterial phylotypes. In contrast molecular methods can detect subtle changes in environmental bacterial populations and as a result have been suggested as more relevant measures of the ecological harm caused by perturbation (Ellis *et al.,* 2001).

1.1.3.1 Molecular methods used to track changes in bacterial communities as a result of perturbation

The most useful methodologies appear to be those which can be used to compare multiple samples and which give a rapid profile or fingerprint of the community under investigation. High throughput molecular methods which fit these criteria include: Terminal restriction fragment length polymorphism (T-RFLP), a technique which exploits sequence differences to provide distinct patterns of terminal (identified by fluorescent marker) fragment lengths following treatment of the DNA with restriction enzymes; Automated ribosomal intergenic spacer analysis (ARISA), an automated method for comparing the ribosomal intergenic spacer region between the 16S-23S rRNA genes; Amplified ribosomal restriction analysis (ARDRA), analysis of amplified ribosomal DNA restriction sites (analogous with RFLP); Denaturing / Temperature gradient gel electrophoresis (D/TGGE), separation of DNA fragments according to

denaturing / melting characteristics conferred by sequence of bases; Single-strand conformation polymorphism (SSCP), analysis of secondary structure and migration of single stranded DNA. The advantages and disadvantages of each method and environments in which these techniques have been applied are given in Appendix A (Table A.1). Despite a number of caveats regarding the use of DGGE it is the most widely used and accepted methodology for the rapid fingerprinting of dominant phylotypes from complex microbial assemblages, particularly in aquatic systems, and as such is utilised in this study.

1.1.4 Molecular methods used in this study

1.1.4.1 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) utilises two opposing oligonucleotides to exponentially amplify fragments of DNA. PCR is particularly suited to the analysis of evolutionarily conserved genes, e.g. highly conserved regions of the genes encoding ribosomal RNA (rRNA). Oligonucleotides (primers) are available which are complementary to the rRNA genes of a broad range of even distantly related organisms (Britschgi and Giovannoni 1991). These allow the amplification of a broad spectrum of rDNA types (Giovannoni *et al.,* 1990) and can be applied to a mixed microbial community (Suzuki and Giovannoni 1996). This technique is especially powerful for those organisms which cannot be grown as pure cultures and for the detection of molecular diversity of microbial populations (Liesack *et al.,* 1991).

1.1.4.2 Denaturing gradient gel electrophoresis (DGGE)

To reduce the analysis time required to characterise complex microbial communities fingerprinting techniques have been developed which separate amplified genomic DNA fragments based on sequence characteristics (section 1.1.3.1, Appendix A.1). One such example is denaturing gradient gel electrophoresis (DGGE) of PCR amplified 16S rRNA genes, a molecular technique used to study the dynamic behaviour of complex microbial assemblages (Muyzer *et al.,* 1993), and to determine genetic diversity of natural microbial communities (Teske *et al.,* 1996[a,b] ; Moeseneder *et al.,* 1999; Casamayor *et al*., 2002; Gillan 2004; Ovreas *et al.,* 2003[a,b]; Cummings *et al.,* 2003; Gillan 2004; Massieux *et al.,* 2004). DGGE has been used to resolve PCR-amplified 16S rDNA based solely on differences in nucleotide sequence (Øvreas *et al.,* 1997). As the name suggests DGGE is an electrophoretic technique where DNA molecules migrate into

a gradient of ascending concentration of denaturants, in this case formamide and urea, forming distinctly different bands based on the specific sequence of base pairs. The denaturant gradient is equivalent to a corresponding gradual increase in temperature at which the lower-melting temperature domain of the double stranded DNA helix melts. The melting occurs in 'discrete melting domains' (Muyzer *et al.,* 1993) and results in branching, i.e. transition of helical to partially melted strands, thus decreasing fragment mobility (Sheffield *et al.,* 1989). The DNA molecules undergo this abrupt decrease in mobility (compared to completely helical molecules) at a characteristic depth which corresponds with their melting temperature. This results in positions and patterns that change little if application of the electrical field is continued (Fischer and Lerman 1983). The retardation depth in the gradient is determined by the least stable part of the molecule, i.e. the part of the molecule with the lowest 'melting' temperature, and is relatively insensitive to other parts of the sequence or to the overall fragment length. This method is extremely sensitive within denaturant concentrations corresponding to the lowest melting domains and can distinguish between fragments with a single base substitution. The melting temperature is altered even at this high resolution due to differences in stacking interactions between adjacent bases in each DNA strand (Sheffield *et al.,* 1989). However, higher melting domains cannot be separated due to complete strand separation at higher denaturant concentrations. To increase the resolution of DGGE to include these higher melting domains (i.e. expand the least stable part of the molecule to encompass regions with greater stability) a GC Clamp can be applied, or the lowest melting domain excised.

The GC clamp was introduced by Myers *et al.,* (1985) working with mouse DNA, they found that, if a 300bp sequence rich in Guanine and Cytosine was added to the DNA fragments during PCR amplification, resolution increased from detection of roughly 40% of all mutations to a minimum of 95%. Later work combined with theoretical considerations indicated that a GC Clamp 40-45bp long would be adequate to increase the resolution a comparable amount (Sheffield *et al.,* 1989). Use of a GC Clamp is now routinely applied in an increasing number of studies. Most of these studies are qualitative, aimed at determining species richness for calculations of diversity, or elucidating community composition of natural assemblages of microorganisms in both aquatic (Moeseneder *et al.,* 1999; Gillan 2004; Ovreas *et al.,* 2003[a,b] ; Cummings *et al.,* 2003, Massieux *et al.,* 2004) and terrestrial (Nakatsu *et al.,* 2000; Sandaa *et al.,* 2001; Nicol *et al.,* 2003) environments.

1.1.4.3 Does DGGE of rRNA derived products give us more information about the active portion of the population?

The presence of large numbers of ribosomes within cells and the regulation of their bio-synthesis in proportion to cell growth make these molecules ideally suited to ecological studies (Britschgi and Giovannoni 1991). DGGE of 16S rRNA derived PCR product is semi-quantitative in that the production of rRNA is directly related to the cellular growth rate of the microbial population (Britschgi and Giovannoni 1991). The other important aspect of this relationship to consider is that metabolically active cells contain more rRNA than those which are resting or dormant and thus DGGE of reverse transcribed, PCR amplified 16S rRNA 'conceptually represents the metabolically active, rRNA rich, bacterial populations' (Teske *et al*., 1996[a]). These variations in abundance / activity can be assessed by scanning and integrating staining intensity of bands, a function of the relative abundance of a phylotype (Teske *et al.,* 1996; Øvreas *et al.,* 1997; Zhongtang and Mohn 2001; Casamayor *et al.,* 2000). However, recent work has shown that band intensity may not be directly representative of relative abundance between populations. This can be attributed primarily to variations in PCR efficiency, possible PCR bias (Wagner *et al.,* 1994; Suzuki and Giovannoni 1996; Konopka *et al.,* 1999; Zhongtang and Mohn 2001), and also to variations in proportion of rRNA to cell numbers. This factor is dependant on both species and growth rate (Giovannoni *et al.,* 1994; Kemp 1994). Band intensity indicates instead the relative differences between populations, thus giving an indication of an organism's ecological importance (Giovannoni *et al.,* 1994). Other examples argue that the status of organisms within an environment has little or no effect on the appearance of DGGE gel patterns, with comparison between active portion, as determined by CTC reduction and flow cytometry (Sherr *et al.,* 1999), and whole assemblage rRNA gels showing high levels of similarity. This similarity suggests that at least some populations include both active and inactive cells (Bernard *et al.,* 2000) a possibility strongly supported by recent work regarding nucleic acid content (Lebaron *et al.,* 2001). The application of RNA- derived DGGE fingerprinting has been demonstrated in a number of environments including; rhizospheres (Duineveld *et al.,* 2001); an aquatic mesocosm (Schafer *et al.,* 2001); marine waters (Teske *et al*., 1996[a]); soil (Griffiths *et al*., 2000; Norris *et al*., 2002; Girvan *et al.,* 2003) and wastewater (Ebie *et al*., 2004). All these studies compared results obtained using DNA and RNA derived fingerprints and in most cases considered the RNA derived

fingerprint to represent the active portion of the microbial community (Teske *et al*., 1996[a]; Griffiths *et al*., 2000; Duineveld 2001; Ebie *et al.,* 2004). In the majority of studies the diversity, i.e. the complexity of the fingerprint, was lower in the RNA derived DGGE. Furthermore the bands present in the RNA derived profiles were invariably also apparent in the DNA derived profile. Girvan *et al.,* (2003) found that the RNA derived profile was more indicative of short-term management change effects i.e. they were able to distinguish between samples from different farms *c.f.* different soil types using DNA derived fingerprints.

It is important to note possible pitfalls of the DGGE technique concerned with estimation of diversity. It is possible that the formation of heteroduplex or chimeric sequences during PCR, which are not detected by the methods described above, may lead to overestimation of species richness, i.e. the number of species present. It is also a possibility that if sequences are not amplified equitably, i.e. undergo PCR selection or drift, or share melting temperatures then species richness may be under-estimated (Wagner *et al.,* 1994).

1.1.4.4 Sequencing

In the past fifteen years the extensive use of 16S rRNA gene sequences in studies of microbial systematics, evolution and ecology has lead to the creation of large computer databases, e.g. RNA Data Base Project (Maidak *et al.,* 2001), which encompass the phylogenetic diversity found both within culture collections and environmental samples. Following sequence analysis sequences can be compared with known sequences from such databases. This gives an indication of what kind of prokaryotes are present within any given sample whether they are held in culture or not. If they are novel species or strains, comparison will also give a distance matrix and their relative position to known individuals on a phylogenetic tree, thus providing information about likely growth conditions and substrate requirements.

Up until ten years ago it was common to use cloning and sequencing of PCR products to facilitate diversity assessment. This approach is time consuming in that extensive analysis of large numbers of clones is required, followed by sequencing of selected individual populations. Also, organisms which make up only a small percentage of the population are not adequately or readily detectable by this method. Sequencing of selected components of the community can however give insight into the dominant phylogenies (as determined by DGGE) in any given sample.

1.1.4.5 The importance of a multifaceted approach when investigating response of bacterial communities to perturbation

The use of molecular techniques provides useful data regarding the organisms which are present in a particular environment and change in diversity shows which bacteria are impacted most strongly by perturbations. However, it is important to also understand what affect such perturbations have on the metabolic capability of the bacterial community. A number of techniques are available which can be used to determine changes in metabolism of bacterial communities. In this study the ability of bacteria to incorporate ^{3}H-Leucine into cellular protein (Kirchman, 1993) has been used as a measure of the production of the bacterial community and will be referred to as bacterial production in the remainder of the text.

In the early stages of the 'molecular revolution' in bacterial ecology large numbers of researchers used molecular approaches in isolation to document bacteria present in a wide variety of environments and/or how a bacterial community changed in response to perturbation (e.g. Moffett *et al.,* 2003; Sandaa *et al.,* 2001; Toms-Petersen *et al.,* 2003; Nakatsu *et al.,* 2000; Beaulieu *et al.,* 2000; Santegoeds *et al.,* 1996; Crump *et al.,* 1999). More recently the desire to link structure of bacterial communities with their function has become a key part of microbial ecology. The measurement of metabolic activity indicators such as growth, protein production and enzyme production in conjunction with measurements of bacterial community diversity allow inferences regarding the bacterial types responsible for such metabolic activities to be made. It also allows the researcher to better assess the implications of and reasons for changes in community composition. Simek *et al.,* (2001) attributed changes in community composition to a shift in balance between population specific growth and mortality rates. A second study showed that a change in bacterial community resulted in higher protein content per cell (Beardsley *et al.,* 2003).

1.2 Trace Metals

1.2.1 Presence and sources of trace metals in aquatic environments

Trace metals can be defined as those metals occuring in minute quantities, often at concentrations less than picomoles per litre (Chester 1990). Essential metals such as iron (Fe), copper (Cu), zinc (Zn), nickel (Ni) and molybdenum (Mo) are required in minute quantities to support many biochemical processes involved in the metabolic functioning of the cell (Nies 1992). They act as micronutrients, are utilised in redox processes, stabilise molecules through electrostatic interactions, regulate osmotic pressure and are essential co-factors for a number of enzymes (Bruins *et al.,* 2000). Trace metals are found naturally occurring in rocks, soils and sediment and are released gradually through natural phenomena such as weathering and erosion. Typical concentrations of metals found in relevant environments are given in Table 1.1.

Metal	Estimated dissolved oceanic concentrations[A]	Range of dissolved oceanic concentrations[B]	Concentration of dissolved metals in the Tamar Estuary[C]
Cadmium	620 $pmol.kg^{-1}$	0.1-1 $nmol.kg^{-1}$	4.3 $ng.L^{-1}$
Copper	2.4 $nmol.kg^{-1}$	1-10 $nmol.kg^{-1}$	2.5 $\mu g.L^{-1}$
Lead	13 $pmol.kg^{-1}$	0.01-0.1 $nmol.kg^{-1}$	0.2 $\mu g.L^{-1}$
Nickel	8.2 $nmol.kg^{-1}$	1-10 $nmol.kg^{-1}$	1.1 $\mu g.L^{-1}$
Zinc	5.4 $nmol.kg^{-1}$	1-10 $nmol.kg^{-1}$	7.4 $\mu g.L^{-1}$

Table 1.1 Concentration of relevant metals in the ocean ([A]Bruland 1980; [B]Libes 1992) and Tamar Estuary (Devon, UK; [C]DEFRA 2003).

Anthropogenic activity introduces trace metals into aquatic environments at comparable concentrations to natural inputs. However, anthropogenic sources are likely to be more localised and thus result in elevated concentrations *c.f.* naturally derived background levels. Dominant sources of metals to aquatic environments vary

with distance from land. Anthropogenic sources of metals are discussed in further detail below.

1.2.1.1 Sources of trace metals in estuarine and coastal environments

The majority of trace metal inputs to estuarine and coastal environments are of anthropogenic origin (e.g. Cobelo-Garcia *et al.,* 2004). Point-source origins include storm drains, sewage work discharge, industrial effluent from activities such as mining; smelting and other metal purification methods and the manufacture and use of metallic products. More diffuse sources stem from agricultural use of pesticides and fertilizers in the catchment area of the estuary in question. Metals in these environments are found predominantly associated with suspended particulate matter or sediment and concentrations vary according to other physico-chemical factors in the immediate environment (Section 1.3.2.2). For example the amount of suspended particulate matter can be directly linked with removal of trace metals as a result of abiotic and biotically enhanced adsorption onto surfaces (Section 1.3.2.2). This process is enhanced further if conditions are favourable for the formation of iron or manganese hydroxides which act as attractants and speed up precipitation of other metals (Millward and Moore, 1982).

1.2.1.2 Sources of trace metals in open ocean environments

Inputs to open ocean environments are usually more natural in origin with dominant sources being atmospheric deposition of dust and desert sands (depending on location). Other atmospheric sources include volcanic products and airborne contamination resulting from the burning of fossil fuels. Non-atmospheric sources of trace metals in oceanic regions may originate from hydrothermal vents, coastal regions as a result of currents and diffusion, and dissolution of ship associated metal and wastes. In oceanic environments the majority of metals are present as metal-ligand complexes (Sunda and Huntsman 1991; Ellwood and Van den Berg 2000; Ellwood and van den Berg 2001; Ellwood 2004).

1.2.2 Toxicity of trace metals

Metals which are required for metabolic functioning of the cell (e.g. zinc; Blindauer *et al*., 2002) can become toxic at high concentrations and those for which no biological use has yet been determined (such as lead) are likely to be toxic at lower

concentrations. The range of minimum concentrations at which an effect on microbial metabolism is seen (Sandrin 2003) is strongly influenced by factors such as contents of experimental media, pH and the form in which the metal is added i.e. its bioavailabilty (see section 1.2.3.2). To understand the affects of trace metals in different environments it is first necessary to understand how trace metal toxicity occurs at the cellular level and how physicochemical factors influence toxicity.

1.2.2.1 Modes of action of trace metal toxicity.

The first mode of toxicity is an effect on the redox state of the cell which results in oxidative stress. One example of this is the production of hydroxyl (OH^-) ions within the cell. Solioz and Stoyonov (2003) demonstrated that an influx of cupric (Cu^{2+}) ions can create OH^- excess via redox reactions with H_2O_2. Redox inactive metals can also induce oxidative stress by displacing redox active metals from their binding sites. Reactive oxygen species can damage phospholipids and reduce the fluidity of the cell membrane via peroxidation. They also target DNA base and sugar moieties resulting in single and double-stranded breakage. Furthermore proteins (including enzymes) can be damaged via oxidation of sulfhydryl groups and reduction of disulphides. Effects of oxidative stress and protein damage by reactive oxygen species are examined in Cabiscol *et al.,* (2000) along with bacterial defence mechanisms.

The second mode of toxicity is via substitution of metabolically active metal anions with non-required anions in enzymes. For example if Zn^{2+} is replaced by Cd^{2+} the enzyme function is compromised. This action is frequently seen associated with sulfhydryl (-SH) containing enzymes which bind strongly to metals and are essential for a number of metabolic activities. A similar effect is observed when metal oxyanions such as arsenate replace structurally similar non-metal oxyanions such as phosphate compromising nutrient transport systems (Nies 1999). In summary the overall effect of metal toxicity is damage of cell membranes, DNA structure, alteration of enzyme specificity and general disruption of cellular function (Bruins *et al.* 2000).

1.2.3.2 Influence of physicochemical parameters on metal toxicity

Speciation of toxic metals plays a crucial role in their impact on the organisms being studied. Metal species can be broadly divided into two categories; the solution phase which approximates the bioavailable fraction, and the solid phase which may be

present as sediment-adsorbed, particulate, colloidal or complexed states, all of which are less available to and are therefore less toxic to the cell. The distribution of metals between these phases is affected by a number of physicochemical parameters.

1) Ionic strength: The availability of H^+ ions has been shown to influence the toxicity of metals, particularly within aquatic systems. Variation in pH can result in increased precipitation of trace metals as particulate oxides or phosphates thus decreasing availability. For example, minimum solubility of metal hydroxides ranges from pH 8.1 for copper to pH 11 for cadmium. Thus, increased pH frequently results in the removal of metal ions from solution, a phenomenon traditionally utilised in the remediation of acid mine drainage. The effect of ionic strength is particularly apparent in artificial microbiological media where phosphate is routinely used as a buffer, because the use of such media reduces the free metal ion concentration. Also related to media for bacterial growth is the addition of adsorbing surfaces such as those observed in yeast extract which again reduce bioavailability. Villaescusa *et al.,* (2000) observed a decrease in toxicity of trace metals with the addition of NaCl to incubation medium, measurements showed that this decrease in toxicity corresponded to a decrease in the proportion of zinc present as 2^+ ions. A similar phenomenon is observed in the presence of substances associated with water hardness.

2) Organic matter: The production of biological polymers such as those released by bacteria in sewage sludge systems (Rudd *et al.*, 1984) can be a response to increased metal concentrations. Complexation of metals with such polymers prevents them from binding with the active sites on cell membranes or enzymes within the cell, thus decreasing toxicity. Chen *et al.* (1995) championed the use of bacterial polymers as agents of metal complexation / removal for bioremediation of contaminated sites. A second biological derivative known to influence metal toxicity is humic acid (Pandey *et al*., 2000). Humic acids tend to be terrestrially derived in aquatic systems and highly refractory. Koukal *et al.* (2003) demonstrated that the presence of humic acids decreased metal toxicity by forming complexes which were stable with regard to metal exchange and hence reducing bioavailability. A second effect was adsorption of humic acids onto cell surfaces forming a barrier which reduced influx of free metal (Cd^{2+} and Zn^{2+}) ions. It should be noted that the stability and strength of complexation is a key factor when determining the availability of metal ions to biota. Tubbing *et al.* (1994)

have shown that concentrations of intermediately labile copper (retained on Chelex-100 columns) correlated significantly with inhibition of bacterial growth and algal photosynthesis.

1.2.3 Mechanisms of trace metal tolerance in bacteria

Metal resistance can be a by-product of cellular metabolism and result from redox processes such as oxidation of metals to obtain energy or conversely from reduction of metals which act as terminal electron acceptors (dissimilatory reduction). In addition, a number of bacteria have evolved specific metal resistance strategies (e.g. Choudbury and Srivastava 2001) which can be divided into two categories, avoidance and sequestration (Nies 1999).

1) **Avoidance**: Metal influx can be prevented or decreased by a permeability barrier external to the cell, e.g. extracellular capsular polymers (Chen *et al*., 1995), or via plasmid encoded changes to cell wall structure. Structural changes which decrease permeability of metal (Cd^{2+}) ions have been observed in *Staphylococcus aureus* (McEntee *et al*., 1986). A second mechanism of avoidance is active transport of excess metal ions out of the cell by cation transporting ATPases. Some of which can switch between influx and efflux depending on the intracellular metal concentration (Table 1.2). Characterised metal transporting ATPases belong to five main groups: ABC (ATP-Binding Cassette), P-type, A-type, RND (Resistance-Nodulation-cell Division), and CDF (Cation Diffusion Facilitator). A third mechanism involves transformation (reduction) by enzymes which convert metals to a less bioavailable form, however, this mechanism is only well characterised for enzymes which detoxify mercury and arsenate (which becomes more toxic prior to removal by an efflux pump (Bruins *et al*., 2000).

2) **Sequestration:** Sequestration by exopolysaccharides or other biopolymers (e.g. proteins and enzymes) prevents or reduces metal toxicity by forming stable complexes either inside or outside the cell. For example, metallothioneins, whilst more often associated with eukaryotic cells, have been induced in cells of the cyanobacterium *Synechococcus* (Ybarra and Webb 1999) following exposure to divalent metal ions. Genes encoding metallothioneins have also been identified in bacteria (Blindauer *et al.*, 2002) Post-efflux binding has been suggested as a mechanism to prevent expelled ions returning to the cell (Bridge *et al*., 1999), thus reducing the energetic cost of

detoxification. Citrobacter have been shown to utilise phosphate to complex cadmium, presumably for this purpose (McEntee *et al*., 1986).

Family	**Direction of transport**	**Energy source**	**Metal ions transported**
ABC	Uptake / efflux	ATP	Mn^{2+}, Zn^{2+}, Ni^{2+}, Fe^{2+}
P-type	Uptake / efflux	ATP	Mg^{2+}, Mn^{2+}, Ca^{2+}, K^{+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+}, Ag^{+}
A-type	Efflux	ATP	Arsenite
RND	Efflux	Proton gradient	$Co^{2+,}$ Zn^{2+}, Cd^{2+}, Ni^{2+}
CDF	Efflux	Chemiosmotic	Zn^{2+}, Cd^{2+}, Co^{2+}

Table 1.2 Protein families involved in metal transport (efflux) across the bacterial cell membrane. Table adapted from Nies (2003).

1.2.4 Bacterial diversity in metal contaminated environments

1.2.4.1 Experimentally contaminated environments

Most experimental studies of the response of microbial communities to metal contamination focus on effects of single metals. One exception is a study performed by Stephen *et al.* (1999) who observed an increase in the relative abundance of sequences belonging to the C cluster of the AmoA gene in soil bacteria, (a structural gene involved in the production of the ammonia monooxygenase gene) thus metal contamination may have implications for the cycling of nitrogen in soil environments. Sandaa *et al.* (2001) detected a change in the proportion of the community DNA binding with primers specific to different bacterial groups (community change) as a result of metal rich sludge amendment. Although these effects will not translate directly to the environments in which this study was performed (i.e. estuarine and marine), due to differences in organic matter, ionic strength and salinity of the media, they do give an indication of the spectrum of effects which may be observed in such

systems. Recent work investigating the effects of acute exposure to copper (a 2.5 fold increase in bioavailable copper in the sediment phase) in North Sea sediment microcosms, indicated no significant affect on genetic diversity or cell density (CFUs) of the bacterial community (Gillan 2004). However, a significant increase in chitin metabolism was observed after 12 days. Laboratory experiments investigating the effects of metals on *Desulfovibrio desulfuricans* (a representative strain of sulphate reducing bacteria) showed a reduction of 25% in maximum specific growth rate at a concentration of 6 μM Cu^{2+}, reduction in cellular protein was also observed at this concentration. The same study found that addition of 16 μM Zn^{2+} and 5 μM Pb^{2+} increased lag time by 48-72 hours but had no significant affect on the amount of cell protein produced when compared to the control (Sani *et al.,* 2001). Few experiments of this kind have looked at marine or estuarine microbial communities. Possibly because in other environments contamination is easier to control, one such example, which is well-researched for obvious reasons, is agricultural soil.

Moffett *et al*., (2003) examined the effect of zinc-enriched sewage solids on the bacterial diversity of agricultural soil communities. They observed a decrease in operational taxonomic units (OTUs), which correspond with species richness, of approximately 25% with the addition of zinc-enriched sludge when compared with addition of sludge alone. This finding was accompanied by a decrease in evenness indicating an overall decrease in diversity, probably a result of selection processes for phylotypes which were more able to cope with the additional zinc. The authors noted that the dominant phylotypes emerging as a result of treatment with zinc enriched sludge were also detected in the sludge-only treatment. This indicates that the ability of these bacteria to cope with zinc addition was also beneficial in the presence of sludge. A second experimental study used soil microcosms to investigate how the microbial community was affected by a one time zinc addition. After 15 days of incubation a decrease of 85% in viable bacteria was observed (Kelly 1999). This incubation was continued for a total of 420 days after which time viable counts in zinc enriched and control microcosm populations were not significantly different. This information alone would suggest recovery of the bacterial population, however, development of colour on BIOLOG plates was slower in zinc amended samples and dehydrogenase activity was significantly reduced. Furthermore a shift in community, presumably to favour zinc tolerant bacteria, was detected using both phospholipid-fatty acid (PLFA) profiles and BIOLOG data. The observed shift suggests that the community had adapted. This

study illustrates the importance of incubation length and the use of a multifaceted approach. A shift in community from sensitive to less sensitive was also detected by Baath *et al.* (1998) following the addition of copper (8 g.kg of soil^{-1}), nickel (4 g.kg of soil^{-1}) and zinc (16 g.kg soil^{-1}), again working in agricultural soil. Conversely Hemida *et al.* (1997), also working with copper and zinc in soil (200µg and 2mg kg^{-1} in both cases), only detected reduced cell density (as determined by the number of CFUs) in the copper enriched conditions, possibly as a result of the much lower concentrations added. Notably the authors also detected a decrease in urease activity (Hemida *et al.,* 1997), again indicating that the effects of metals on bacterial communities may have implications for the cycling of nitrogen by bacteria in soil. The addition of copper (0-1 mg.g^{-1}) to soil (Ekelund *et al.,* 2003) resulted in a shift in bacterial community from Gram -ve to Gram +ve as determined by analysis of phospholipids fatty acids. Despite these notable changes in community the authors observed only slight changes in overall bacterial abundance and activity, again demonstrating the importance of measurement of metabolic or functional potential of a community.

In addition to studies of how bacterial communities change with metal perturbation the ability of bacterial communities to adapt (adaptive response) to elevated metal concentrations has been investigated. Ganguly and Jana (2002) observed a more profound negative effect of cadmium perturbation on ammonia oxidising bacteria and denitrifying bacteria than on cellulose decomposing bacteria in an aquatic environment. Measurement of tolerance using thymidine incorporation of acclimated bacterial communities (long-term incubations over 28 months) showed two mechanisms of tolerance development of bacterial communities. Firstly, the initial mortality of metal sensitive species (first 48 hours of exposure) left behind a community consisting of bacteria more able to tolerate metals. Secondly, a more long-term response attributed to different adaptation and competitive abilities of the remaining bacteria was observed (DiazRavina and Baath 1996). The authors also observed increased tolerance as a result of glucose addition.

1.2.4.2 Historically contaminated environments

Studies carried out on effects of multiple metals tend to be opportunistic and focus on environments which are historically impacted. Studies of native microbial populations in metal-rich or impacted environments are few, with the exception of acid mine or rock drainage (AMD/ARD) which has been researched by a number of workers

(e.g. Gray 1997; Johnson *et al.,* 2001; Johnson and Hallberg 2003; Edenborn 2004 and references therein) as a result of its relevance to bioremediation (Antsuki *et al*., 2003). Such studies rarely have a control site or sample for comparative purposes.

Stein *et al*., (2002) investigated diversity of microbial communities associated with metal rich particles in a fresh-water reservoir. Construction and analyses of a clone library utilising 16S DNA from the extractable portion of the community showed dominance of 16S genes closely related to those from a single cluster within the candidate division OP10 (Geobacteraceae). Previous detection of this OP10 cluster in question was derived from genes cloned from sludge systems. The dominance of Geobacteraceae in this environment provided a reference point for work carried out by Cummings *et al.* (2003). The authors found evidence that Geobacteraceae inhabited metal-polluted fresh-water lake sediments using group specific 16S gene targeting primers suggesting an important role of this group of bacteria in metal polluted anaerobic sediment environments. This is one of the few studies which made reference to a control site and although some overlaps were identified only two phylotypes were found exclusively in the pristine sediments whereas six of the phylotypes identified from the polluted sites were absent from the pristine communities, suggesting they had a competitive advantage under metal-stressed conditions.

Labrenz (2004) used molecular methods to determine diversity of bacterial biofilms which precipitate ZnS in a mine drainage system (approx. neutral pH). Microbial clusters belonging to the *β-, γ- and ε-proteobacteria*, CFB, *Planctomycetales, Spirochaetales*, *Clostridia* and green non-sulphur bacteria were detected. *Desulfobacteriaceae* (SRB) were discovered to be a significant proportion of the established biofilms.

The geothermal fluid released in hydrothermal vent systems creates an area of highly concentrated metals when contrasting with surrounding seawater. Holden and Adams (2003) give an overview of micro-organisms found in such environments and describe the ways in which they detoxify the metals therein. They divide the micro-organisms into four groups based on habitat i.e. those found in pore spaces and cracks below the surface; those who gain their nutrition from sulphide deposits and form mats; those existing in symbiosis with invertebrates; and those within the plume itself.

With the exception of the first group most of the microbes isolated from these environments are mesophillic aerobic bacteria including members of the

Thermatogales, *Bacillus, Pseudoaltermonas, Shewanella, Geobacter, Thermococcus, Thermotoga,* and *Thermanaerobacter.* Micro-organisms isolated from the first habitat are more likely to belong to the anaerobic hyperthermophillic archea as a result of temperatures typically exceeding 100°C.

1.3 Aggregates

1.3.1 Contrasting nature of estuarine and marine aggregates

Aggregates can be defined as rapidly sinking particles > 0.5 mm in diameter and can be responsible for 7-45g C m^{-2} yr^{-1} of total organic material flux in aquatic environments (Walsh and Gardner 1992). Aggregation of such substances takes place via coagulation which comes about through processes such as Brownian motion, fluid shear and particle settling. The nature and formation of aggregates is highly dependant on the materials available and the types of physical forcing within specific environments.

1.3.1.1 Marine snow formation

Marine snow is a term used to describe oceanic aggregates >0.5 mm in diameter (Alldredge and Silver 1988). Marine snow has been extensively researched and is of critical importance for the determination of flux of organic matter between the upper mixed layer and the deep sea (Silver and Alldredge 1981; Lochte and Turley 1988). Formation of marine snow tends to be biologically mediated (reviewed by Turley 1992), one such example is particle formation as a result of phytoplankton cell lysis. This leads to release of extracellular enzymes by colonising bacteria and subsequent release of polysaccharides, proteins and other exopolymers which act as flocculating agents (Busch and Stumm 1968; Biddanda 1988; Vandevivere and Kirchman 1993). Furthermore, production of bacterial and diatom fibrils (Heissenberger *et al.,* 1996), capsular material (Heissenberger *et al.,* 1996), and transparent exopolymer particles (TEP) (Logan *et al.,* 1994) also contribute. These processes affect particle size, composition and surface characteristics. In addition, sticky carbohydrates / exopolymers originating from biological sources such as diatom blooms (Passow *et al.*1994; Passow and Wassmann 1994) significantly increase the likelihood of aggregation. Free DNA can also serve as a flocculation agent (Alldredge and Silver 1988). Increase in particle size as a result of enhanced stickiness (Biddanda 1988; Jenkinson *et al.*1991) results in increased probability of collision (Stolzenbach 1993) and also contributes to increased sinking velocities.

Larger scale organisms, which hold a place higher in the food chain, also contribute to formation of marine snow via the excretion of faecal pellets (Pomeroy *et*

al., 1984; Turner 2002). Faecal pellets provide large amounts of relatively labile material and are rapidly colonised as they move through the water column. These pellets also introduce an additional dimension to the food chain by concentrating bacteria in substrate large enough to be consumed by grazing organisms (Lampitt *et al*., 1993). Another biological process important for aggregate formation is the production of mucus by various organisms including foraminifera and coral, coelenterates and phytoplankton (Alldredge and Silver, 1988) and the creation of aggregates through abandoned larvaecean houses (Alldredge 1972; Hansen *et al.*, 1996).

1.3.1.2 Turbidity maxima formation

Whilst oceanic aggregate (marine snow) formation is dominated by biological mechanisms estuarine aggregate formation is strongly influenced by abiotic factors. For aggregation to take place smaller, component particles must first come into contact. Once the component particles have collided they must be sticky enough to adhere to one another. Elevated concentrations of suspended particulate matter are frequently encountered at the fresh-water/salt-water interface (FSI) of estuaries, which are termed turbidity maxima zones (TMZs). One major factor contributing to the formation of TMZs is surface charge. For example, colloidal clay particles are coated by negatively charged organic matter resulting in repellant activity which needs to be overcome at the point of collision to result in cohesion. This is not possible in freshwater, however, upon reaching water with a salinity of 1-3 these forces are weakened and overcome, usually by Van der Waal forces, and aggregates are formed (the processes involved are described in Gregory and Duan (2001)). Thus a TMZ is generally associated with the FSI. Similarly, negatively charged particles can be linked by bridging divalent cations which enhance attraction and cohesion (Pers comm. P. Statham, Southampton Oceanography Centre; Gregory and Duan 2001). The boundary layer between the sediment and the overlying estuarine water is rich in mucopolysaccharides produced by benthic diatoms which enhance the stickiness of particles which, in turn, increases the likelihood of the formation of aggregates. These factors combined with the biotic factors responsible for aggregate formation in marine systems result in enhanced aggregate formation in estuaries. The TMZ is formed and maintained by predominantly physical factors such as reduced turbulence due to stratification (induced by saline intrusion, Geyer 1993), topography of the estuary (decreasing depth can result in residual current in the landward direction, Jay and Musaik, 1994), tidal forcings

(Weir and McManus 1987) and shear forces which are responsible for the resuspension of the 'fluffy' layer at the boundary between sediments and estuarine water. These shear forces change over the tidal cycle and result in gravitational circulation: 'settling during slack tide, resuspension during the early ebb and early flood, flocculation of fine particles into large ones during most of the tide, and deflocculation of large flocs into smaller particles during or after settling to the bottom' (Eisma and Li 1993).

1.3.2 Importance of aggregates in aquatic environments

1.3.2.1 Bacterial cycling of organic matter

Affect of aggregate association on bacterial abundance and production

The micro-environment associated with aquatic particles has long been acknowledged to provide an area of increased nutrient availability when compared to the surrounding water (Shanks and Trent 1979, Table 5 in Simon *et al.,* 2002). Therefore it is no surprise that the abundance of bacteria associated with aggregates tends to be enriched *c.f.* surrounding water column of equal volume (e.g. Caron *et al*., 1986). The extent of this phenomenon depends primarily on the size and composition (Turley and Mackie 1994) of the aggregates and enrichment factors range from 0.6 to 5,700 (Turley and Mackie 1994; Simon *et al.,* 2002). The number of bacteria per aggregate shows positive correlation with aggregate size, however, enrichment factors relative to surrounding water decrease with increasing aggregate size (Simon *et al*., 2002). Bacteria associated with aggregates can be as little as 5% of the total bacterial numbers in oligotrophic marine environments and as much as 90% of total bacterial numbers in estuarine environments with high SPM concentrations (Crump *et al.,* 1998). The contribution of aggregate association to bacterial biomass is clearly highly variable depending on aggregate number, size and composition.

Bacterial production rates on aggregates have been correlated with aggregate size (Alldredge and Gotschalk 1990; Grossart and Ploug 2000) and, when calculated on a per cell basis, values tend to be equal to or slightly exceeding those of their free-living counterparts, possibly as a result of increased substrate availability (Turley and Stutt 2000). Aggregate associated bacterial production has been estimated to range from 0.39% of total bacterial productivion in the subtropical Atlantic Ocean (Alldredge and Youngbluth 1985), 3-12% of production in the surface water (70m) of the

Mediterranean (Turley and Stutt 2000) to 53% of total bacterial production in the Columbia River Estuary (Crump and Baross 1996). The discrepancy between values is a result of both different sampling environments and methodology used. As a rule, aggregate associated bacterial production in pelagic marine and lake environments comprises <14% of the total while in eutrophic and estuarine environments production can exceed 30% of the total (Simon *et al.,* 2002). It has been shown that production on settled aggregates is lower than that on suspended aggregates hence the development of rolling bottle and upflow incubation techniques. It should be noted that upflow type incubations can provide more favourable oxygen conditions than would be encountered in the field and thus may overestimate in situ production values. These techniques are nevertheless more representative of field measurements than incubations in which aggregates are allowed to settle.

Affect of aggregate association on respiration and hydrolysis

Respiration by aggregate associated bacteria has been shown to increase with aggregate size. However, respiration determined on a per cell basis is lower on larger aggregates (Ploug 2001), a phenomenon attributed to reduced lability of substrate (Grossart and Ploug 2000). Respiration has also been found to correlate positively with particulate organic carbon (POC) in riverine environments (Grossart and Ploug 2000; Ploug and Grossart 2000). The age of aggregates also has implications for bacterial respiration and indeed growth efficiency (Grossart and Ploug 2000). In the first three days of incubation high growth efficiencies were noted in samples taken from the Weser Estuary. The authors concluded that in the early stages of aggregate formation transfer of organic matter is both rapid and efficient. From day 3 onwards ectoenzyme production exceeded the direct requirement of aggregate associated bacteria for substrate and a net export of hydrolysed organic matter was observed which is believed to contribute to substrate demand of free-living bacteria.

Bacteria associated with aggregates have 'higher potential ectoenzymatic hydrolysis rates per cell' (Simon *et al.,* 2002), as a result, labile aggregate associated biopolymers are rapidly hydrolysed by enzymes including aminopeptidase, phosphatase and glucosidase produced by such bacteria (Smith *et al.,* 1992; Unanue *et al.,* 1998; Grossart and Ploug 2001; Lehman and O' Connell 2002). By measuring enzymatic activity and incorporation of radiolabelled amino acids Grossart and Ploug (2001) showed that 87% of the decrease in particulate organic nitrogen (PON) could be explained by turnover of particulate combined amino acids (PCAA) by the aggregate

associated food web and that PCAA were preferentially decomposed when compared to particulate organic carbon (POC). They concluded that significant transformation and remineralization was performed by the aggregate associated food web. In an earlier study (Grossart and Ploug 2000) variability in bacterial production and respiration was attributed to a changing bacterial community. This change comprised a shift in community from α- and β-proteobacteria dominance to cytophaga and γ-bacteria dominance. This suggests a shift in food source from labile to more refractory as the aggregate ages.

The determination of the differences between aggregate associated and free-living bacterial communities in terms of these metabolic activities highlights the important role aggregate associated bacteria play in the recycling and remineralization of organic matter in aquatic environments (Fowler and Knauer 1986; Alldredge and Silver 1988; Turley and Mackie 1994; 1995; Grossart and Simon 1998). Coexistence of bacteria, phytoplankton and microzooplankton (Patterson *et al.,* 1993) associated with aggregates creates a micro-environment with enhanced nutrient cycling and thus growth and biomass production capacity. In addition to these functions, the colonisation of aggregates by bacteria and other micro-organisms provides a short-cut in the food chain in a similar manner to faecal pellets (Baylor and Sutcliffe 1963; Lampitt *et al.,* 1993). As a result detritus and/or grazing organisms can obtain nutrition from bacterial biomass which would otherwise be unavailable due to their small size.

1.3.2.2 Trace metals removal / transport

Particles are known to play a dominant role in controlling the trace element distribution in riverine environments (Trefry *et al.,* 1985; Ellwood 2004), the open ocean (Morris 1986), and they have also been shown to influence the behaviour of trace elements in estuaries (Luoma and Davis 1983; Valenta *et al*., 1986; Turner 1996) and coastal seas (Balls 1989). However, these studies do not account for biological influence on trace metal dynamics. Further details of the affects of sediment and biology on trace metal dynamics in aquatic systems are discussed below.

Adsorption to iron oxyhydroxides / co-variance with salinity

One theory suggests that the uptake of trace metals by suspended particulate matter (SPM) is a result of adsorption to iron oxyhydroxide, a highly efficient trace metal scavenger (Millward and Moore 1982). This theory indicates that uptake of

manganese and zinc is directly related to salinity i.e. 'adsorption onto fresh iron (oxyhydroxide) precipitates will (also) be favoured for manganese and zinc in the freshwater above the salt-wedge' and that the relative partitioning of these metals between the solid and dissolved phases is dependant mainly on salinity. The relationship is not apparent for copper which will adsorb onto such precipitates at all pH and salinity ranges expected within a brackish water region such as an estuary (Millward and Moore 1982). Removal of copper has been observed in the low salinity regions of both the Tamar (Morris *et al.,* 1986) and the Rhine (Duinker *et al.,* 1978). Interactions between iron oxyhydroxides and trace metals are well documented, however, it is important to note that the low salinity/ fresh water above the salt-wedge where such interactions are frequently observed may also be an area of high biological activity. An area of low dissolved oxygen was located downstream from the TMZ in the Tamar estuary (Morris *et al*., 1986) indicating a high degree of biological activity the effects of which on trace metal distribution are unaccounted for within this theory.

Influence of biology on trace metal removal / transport

Work carried out in the Dover Strait supports the direct relationship between metal removal and salinity (discussed above), although it states that no clear relationship is evident for manganese in channel waters (James *et al.,* 1993). In this study biological activity is accounted for, the authors suggest that partitioning of a metal between the dissolved and particulate forms may vary according to both biological activity and particle exchange processes (James *et al.,* 1993). This theory also concludes that copper is one of the most conservative trace metals, with a pronounced affinity for biogenic matter during estuarine mixing and states that nickel shows 'distinct similarities' (James *et al.,* 1993). Cadmium has also been shown to have an affinity for biogenic material (Valenta *et al.,* 1986).

Influence of suspended particulate matter (SPM) on trace metal removal / transport

James *et al.,* (1993) considered the size of SPM present as another factor that can influence trace metal removal/transport. They suggest that there is proportionally more metal (specifically nickel and copper) associated with the finer fractions of the SPM and that particle size is probably an important variable in determining trace metal concentration in the SPM. Cobalt and manganese showed a linear relationship to SPM on a mass/volume of seawater basis. It should be noted that these conclusions are drawn from studies on channel waters which, despite varying salinity, were not specifically 'estuarine' in nature. In addition, extensive laboratory work has shown that

for each metal the partition co-efficient decreases with increased SPM loading (Zhou *et al*., 2003), it was also shown that scavenging under high particle concentrations was more efficient (Zhou *et al*., 2003). Again these studies do not satisfactorily include biological aspects. Another important factor requiring consideration is the fact that maintenance of a non saturated state with regards to trace metals is essential to allow prolonged removal of those metals by sorption to particulate matter. This requirement is to a degree fulfilled by the constant turnover of resuspendable particles within the estuary and subsequent removal of both metals and SPM to the marine environment or by longer-term settling processes. It should be noted however that these relationships have been shown to break down in the presence of decreased pH, a condition common to many metal impacted environments (Achterberg *et al.,* 2003)

1.3.2.3 Artificial aggregates for laboratory studies

Artificial substrates

Artificial substrates used for laboratory experiments have the advantage of being clearly defined. For example Kiorboe *et al.,* (2002) designed experiments to determine mechanisms and rates of bacterial colonisation of aggregates. The use of agar droplets approximately 0.2cm in diameter with varied enrichments allowed the authors to determine that colonisation of enriched (Marine broth and DMSP) spheres was increased by a factor of 5 to 10 in 'tumbling' bacterial strains and not affected in non-tumbling strains. This indicated that the tumbling behaviour was effective for location of food source. Numerous other conclusions were drawn regarding the nature of attachment and detachment demonstrating the usefulness of such studies. These included; the discovery of rapid exchange between attached and unattached bacterial assemblages; the observation that motility plays a crucial role in attachment to particles and that bacterial may be able to survive with aggregates as a sole food source.

Natural substrates

Artificial aggregates can be formed using either inorganic or organic particles. The first laboratory produced aggregates were created by Shanks and Edmondson (1989). Since then the use of rolling bottles to simulate differential settling and shear forces has been adopted by a number of researchers. For example a study carried out by Grossart and Ploug (2000) created aggregates by rolling water taken from the Weser

River (W. Germany) for assessment of bacterial growth efficiency and metabolism in relation to bacterial community structure. This study showed that bacterial production and respiration was dependant on community composition rather than availability of nutrients. A second relevant study by the same authors (Grossart and Ploug 2001) utilised three diatom species, *Thalassiosira weissflogii, Skeletonema costatum* and *Chaetocerous debilis* to form aggregates in the laboratory. These aggregates were used to investigate the impact of aggregation on cycling of organic matter by bacteria, 87% of the total decrease in particulate organic nitrogen over the time course of the experiment was attributed to transformation and remineralization of particulate combined amino acids by the aggregate associated food web. Unanue *et al.* (1998) working with autoclaved diatom cultures investigated aminopeptidase production and leucine incorporation by aggregate associated bacteria. They found that attached bacteria support growth of free-living bacteria via production of aminopeptidase enzymes, they determined that this wasn't a result of excessive enzyme production, rather a result of lower uptake rates in attached bacteria *c.f.* free-living counterparts. Similar results were obtained using aggregates formed by rolling lake water, a study in which identity of aggregate colonisers was also determined using fluorescent *in situ* hydridisation (FISH)(Ploug 2001; Schweitzer *et al.,* 2001). Diatom cultures were also utilised in an investigation of the effects of flow and diffusion of oxygen on remineralization by aggregate associated bacteria. *Chaetocerous debilis* and *Skeletonema costatum* cultures and faecal pellets originating from cultures of *Acartia tonsa* were aggregated by incubation in aged or artificial seawater (1.5 L) on roller tables. Oxygen micro-electrode measurements through artificial aggregates showed that remineralization was determined by substrate quality and quantity rather than oxygen transport limitation.

1.4 Aims and objectives

The overall aim of this study was to investigate, using molecular and cell specific methods, the interactions between bacteria, aggregates and trace metals in aquatic systems. Specific research objectives were as follows:

Objective I) To determine the effects of trace metal enrichment on bacterial community structure and function (Chapters 3, 5 and 6).

Objective II) To compare the ability of bacterial consortia from different marine environments to tolerate elevated concentrations of zinc (Chapter 3)

Objective III) To assess the affect of incubation strategy including artificial aggregate formation on bacterial community structure and function under trace metal impacted conditions (Chapter 4).

Objective IV) To determine the capacity of bacterial consortia derived from a pristine environment to tolerate trace metals (Chapter 5).

Objective V) To investigate bacterial communities associated with different sizes classes of aggregates originating from estuarine systems (Chapter 6).

Objective VI) To assess the suitability of different nucleic acids for the detection of changes in bacterial communities as a result of trace metal enrichment (Chapter 6).

2 Materials and Methods

2.1 Materials

Material	**Supplier**
Microbiological materials	
Ampicillin	Sigma-Aldrich UK
Bactoagar	Difco Laboratories
Bactopeptone	Difco Laboratories
L-[4,5-^{3}H]-leucine	Amersham Pharmacia Biotech
Optiphase Hisafe III	Perkin Elmer
SYBR Gold II	Molecular probes Inc.
Tryptone	Difco Laboratories
Yeast Extract	Difco Laboratories
Strains and plasmids	
E. coli DH5α	Invitrogen, UK
E.coli XL1-Blue MRF'	Stratagene, UK
pGEM-T	Promega, UK
Nucleic acid materials	
100bp markers	Promega UK Ltd
ABI Big-dye reagents	Applied Biosystems
Acrylamide:bis-acrylamide (40%)	Sigma-Aldrich UK
Bromophenol Blue	Sigma-Aldrich UK
Custom oligonucleotides (Primers)	MWG-biotech UK
dNTPs	Promega UK Ltd
Formamide	Fluka (Sigma-Aldrich UK)
Hi-Dye Formamide	Applied Biosystems
*Hin*d III	Promega UK Ltd
Magnesil Green	Promega UK Ltd
pGem-T	Promega UK Ltd
Rain-X	Halfords (UK)

SYBR Green I	Molecular probes Inc.
Taq DNA polymerase	Promega UK Ltd
TEMED	Promega UK Ltd
TSR	Amersham Biosciences UK Ltd
Wizard SV Miniprep kit	Promega UK Ltd
X-Gal	Promega UK Ltd
Xylene Cyanol	Promega UK Ltd
Λ DNA	Promega UK Ltd

All reagents were of an appropriate grade (i.e. Analar, microbiological or molecular biology grade) and obtained from VWR unless listed above.

2.2 Water collection and manipulation

2.2.1 Water collection and precautions for preventing contamination

At each site the salinity, pH and temperature were determined prior to water collection. Marine water was collected from RRS Discovery (cruise D261) using trace-metal clean externally closing Niskin bottles on Kevlar® hydroline. Estuarine water was collected in trace metal clean containers at a salinity of approximately 15 as determined using the YSI salinometer. Water was transferred to low density acid washed, sterile polycarbonate bottles (2 L, 4 L, 10 L or 20 L capacity) and incubated in the dark for up to 41 days. All collection and manipulation containers were made trace metal clean by acid washing and sterilised by autoclaving (121°C, 20 minutes) or rinsing with ethanol (100%). Prior to sample collection containers were thoroughly rinsed with water from the target environment. Sampling was routinely performed upstream of the sampler and collection vessels were completely and rapidly submerged to minimise air-borne and surface micro-layer metal contamination. Non-talc disposable gloves were worn throughout initial sampling and subsequent sub-sampling. All sample manipulation was performed using metal-free apparatus (e.g. pipettes and aggregate sampler).

2.2.2 Incubation strategies

2.2.2.1 Marine stations 1 and 2

Water was collected from the marine stations 1 (M1) and 2 (M2) on the 2nd and 4th of April 2002 from the RRS Discovery (for details see Chapter 3). Externally closing Niskin bottles were used to collect water (>6 L) which was then decanted into and incubated in 2 L polycarbonate bottles. One bottle from each environment was enriched with 100nM zinc, a second bottle with 1µM zinc and the third bottle was left untreated as a control. Bottles were incubated at ambient sea temperature (Table 2.1) in the dark for a total of 41 days. Samples were stirred continually for the duration of the experiment on magnetic stirrers with a sterile magnetic bar. Sub-samples were taken regularly throughout the incubation period (Table 2.1).

Station	Date	Location	Temperature (°C)	Treatments Applied	Control	Incubation Length (days)	Sub-samples collected (days)	Parameters assessed
Marine 1 (M1)	02/04/2002	50° 02'N, 04° 22'W	10.7	100nM and 1µM zinc	Unenriched	41	0, 1, 4, 8, 20, 27,36,41	Numbers, production, diversity (DNA)
Marine 2 (M2)	04/04/2002	48° 41'N, 11° 12'W	12.1	100nM and 1µM zinc	Unenriched	41	0, 1, 4, 8, 20, 27,36,41	Numbers, production, diversity (DNA)
Fal estuary	24/03/2003	50° 09'N, 05° 02'W	12±1	Rolled, Stirred, Still	N/A	21	0, 0.25, 1, 2, 4, 7, 14, 21	Numbers, production, diversity (DNA)
Erme estuary	09/06/2003	50° 26'N, 04° 11'W	16±1	1µM and15µM zinc, 18nM cadmium, 950nM copper, 200nM nickel, 300nM lead, cocktail of zinc15µM, cadmium, copper, nickel and lead (concentrations as above).	Unenriched	21	0, 0.25, 1, 3, 7, 11, 14, 21	Numbers, production, diversity (DNA)
Tamar estuary	16/03/2004	50° 18'N, 03° 57'W	10±1	1µM zinc, and cocktail as per Erme	Unenriched	15	0,6,10	Size fractionated diversity (RNA and DNA derived)

Table 2.1 Sample sites information, treatments and sampling regime.

2.2.2.2 The Fal Estuary

The Fal Estuary was sampled at high tide on the 24th of March 2003. Bulk water (60 L) with a salinity of 15 PSU was collected in six 10 L polycarbonate bottles. Samples were transferred to a controlled temperature (CT) laboratory at Plymouth Marine Laboratory (PML) for manipulation. Bottles were incubated on an aggregate roller (Figure 2.1) to enhance the formation of coherent aggregates. A second bottle was incubated with stirring (as per the marine samples) and a third bottle was incubated without agitation. Samples were incubated in the dark at ambient estuarine temperature (Table 2.1) in a CT laboratory and sub-sampled (Table 2.1) over a total duration of 21 days.

2.2.2.3 The Erme Estuary

The Erme estuary was sampled at high tide on the 9th of June 2003. Water was bulk sampled at a salinity of 15 in a large volume container (40 L) and 4 litres decanted into eight bottles. Samples were transferred to a CT laboratory at PML for manipulation. Metal additions to sample bottles were made within 2 hours of sample collection (details of metal concentrations and addition protocols are given in Table 2.1 and Chapter 5 respectively). All samples were incubated in the dark at ambient estuarine temperature (Table 2.1) for a total of 21 days. Sub-samples were taken at regular intervals (Table 2.1).

2.2.2.4 The Tamar Estuary

The Tamar estuary was sampled from the PML research vessel *Tamaris* on the 16th of March 2004. Water was pumped directly into four 20 L low density, acid washed, sterile polycarbonate bottles at a salinity of 15. Samples were transferred to a CT laboratory at PML for manipulation. Metal additions were made to two sample bottles within 2 hours of sample collection, details of metal concentrations and addition protocols are given in Table 2.1 and Chapter 6 respectively. The third bottle was treated with antibiotics and the fourth bottle was unamended as a control. All samples were incubated in the dark at ambient estuarine temperature (Table 2.1) for a total of 21 days. Sub-samples were taken at regular intervals (Table 2.1).

2.2.3 Sub-sampling

The parameters measured for each experiment are given in Table 2.1. Briefly, for each experimental condition and each time point sub-samples were taken for the analysis of bacterial diversity, bacterial numbers and bacterial production. Samples were either fixed or processed immediately using a combination of microbiological and molecular methods for analysis of the natural bacterioplankton communities (Sections 2.5 and 2.6 respectively).

Throughout sub-sampling and incubations all manipulations were performed using trace metal clean techniques. Powder free gloves and lab-coat were worn when collecting sub-samples which were decanted from the bottles to prevent contamination from metal components of pipettes, filter apparatus or pumps. Aggregates, when collected, were retrieved using plastic 10mL pipette tips connected to a syringe with silicon tubing.

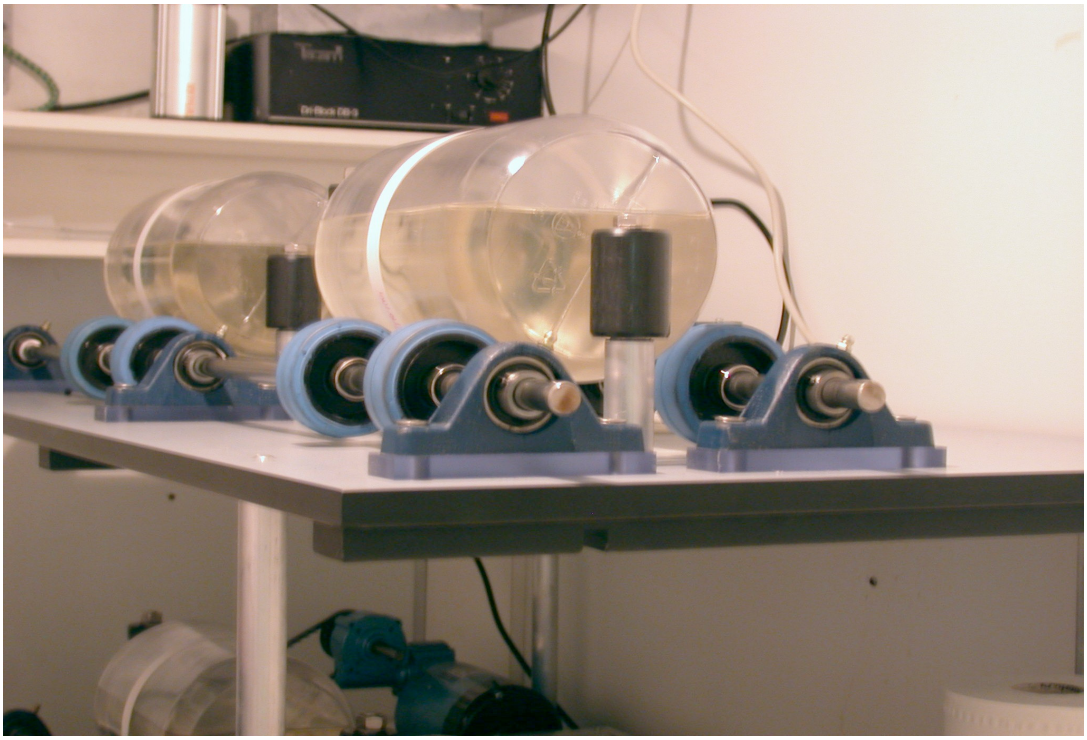


Figure 2.1 'Aggregate roller' system showing two 10 l polycarbonate bottles containing water from Restronguet Creek (Cornwall, UK).

2.3 Established microbiological methods

2.3.1 Media for microbiology

2.3.1.1 Media for cell culture

Luria-Bertani (LB) medium

Tryptone	10.0 g
Yeast Extract	5.0 g
NaCl	10.0 g
MilliQ water	to 1 L

LB/ Ampicillin (LB/Amp)

LB medium was prepared as above. Following sterilisation and cooling to approximately 50°C 250 µL of filter sterilised, 100 $mg.mL^{-1}$ ampicillin stock was added per 250 mL LB medium (final concentration of 100 $\mu g.mL^{-1}$). [Ampicillin stock solution was prepared by dissolving ampicillin in MilliQ water to a final concentration of 50 $mg.mL^{-1}$ followed by filter (0.22 µm) sterilisation. Stocks were stored in 200 µL aliquots at -20°C].

210 Sea water yeast peptone medium (210)

Yeast extract	3.0 g
Bactopeptone	5.0 g
FAA water	750 mL
Milli RO water	to 1 L
pH adjusted to 7.3 using concentrated NaOH	

Solid media

Plates were prepared by adding 1.5 g Bactoagar to 100 mL media (final concentration 1.5%) prior to autoclaving. Following sterilisation, plates were poured using standard aseptic techniques in a laminar flow hood, 20 mL medium was used per plate (90mm diameter). Plates for lac selection were treated with 2 µl isopropyl β-D-1-thiogalactopyranoside (IPTG) and / or 20 µl of 5-bromo-4-chloro-3indoyl β-D-galactopyranoside (X-Gal). IPTG and X-Gal were spread onto the surface of the plate and dried in a laminar flow hood. IPTG is not required for selection of DH5α cells.

2.3.1.2 Media for preparation and transformation of chemically competent cells

SOB		SOC	
Bacto-tryptone	20 $g.L^{-1}$	SOB	4.825 mL
Yeast extract	5 $g.L^{-1}$	2M $MgCl_2$	25 µL
NaCl	0.5 $g.L^{-1}$	1M Glucose	100 µL
KCl	0.186 $g.L^{-1}$	1M $MgSO_4$	50 µL

TFBI		TFBII	
0.1 M RbCl	12.9 $g.L^{-1}$	10 mM MOPS pH 8	0.418 $g.200mL^{-1}$
50 mM $MnCl_2$	9.9 $g.L^{-1}$	75 mM $CaCl_2$	2.2 $g.200mL^{-1}$
35 mM KOAc	3.43 $g.L^{-1}$	10 mM RbCl	0.24 $g.200mL^{-1}$
10 mM $CaCl_2$	1.47 $g.L^{-1}$	15% Glycerol	30 $ml.200mL^{-1}$
15% Glycerol	150 mL	Filter sterilised (0.22 µm pore size)	

pH adjusted to 5.8 using dilute acetic acid.
Filter sterilised (0.22µm pore size).

All the above solutions and media were sterilised by autoclaving at 121°C for 20 minutes unless stated otherwise.

2.3.2 Preparation of chemically competent cells (E. coli XL1-BLUE MRF')

E. coli cells were taken from -70°C glycerol stocks, streaked onto LB plates and incubated overnight at 37°C. A single colony from this plate was used to inoculate 5 mL of LB medium and incubated overnight at 37°C with shaking (225 rpm). This pre-culture was added to 50 mL of pre-warmed (37°C) LB medium and incubated with shaking (225 rpm) until early exponential growth phase was reached i.e. when an OD_{600} of between 0.6 and 0.8 was recorded. The 50 mL culture was added to 250 mL pre-warmed (37°C) LB medium and incubated with shaking (225 rpm) until exponential growth phase (OD_{600} 0.6-1.0) was achieved. The culture was transferred to 4 chilled, sterile Oakridge tubes and incubated on ice for 15 minutes. Aliquots were centrifuged (RCF = 2 500) for 4 minutes at 0°C (Eppendorf benchtop centrifuge), the culture medium removed and cells resuspended in 25 ml of ice cold TFBI (Section 2.3.1.2). Resuspended cells were pooled in two Oakridge tubes and centrifuged (RCF = 2 500, 4 minutes, 0°C). The supernatant was discarded and cells resuspended in 50 mL TFBI (total 100 ml). The cell suspension was incubated on ice for 30 minutes prior to

centrifugation (RCF = 2 500, 4 minutes, 0°C). The supernatant was discarded, cells resuspended in 6 mL ice cold TFBII (Section 2.3.1.2) and 500 µL aliquots placed into chilled microcentrifuge tubes using chilled pipette tips. Aliquots were immediately snap frozen in liquid nitrogen and stored at -70°C.

On occasions competent cells (DH5α) were purchased from a commercial supplier (Invitrogen). These were stored at -70°C in 50µL aliquots according to the manufacturer's instructions.

2.3.3 Transformation of chemically competent cells

2.3.3.1 Laboratory stock E. coli XL1-BLUE MRF'

Aliquots (500 µL) of competent *E. coli* were thawed on ice and 100µL aliquots added to individual pre-chilled Falcon 2059 tubes on ice. Ligation mixture (1-5 µL) was added to a 100 µL aliquot of cells and mixed gently using the pipette tip. As a positive control 1-5 µL of supercoiled plasmid was added to a second aliquot. A negative control using the same volume of MilliQ water was added to a third aliquot. The cell suspensions were incubated on ice for 30 minutes, followed by heat shock in a 42°C water bath for 45 seconds. Cells were returned to ice for 2 minutes. SOC medium (900 µL) (Section 2.3.1.2) was added to each tube and the cell suspensions incubated (37°C) with shaking (225rpm) for 1 hour. A 250µL aliquot of the cell suspension was spread onto an LB/Amp plate (Section 2.3.1.1) treated with IPTG and X-Gal. Plates were incubated at 37°C overnight.

2.3.3.2 Invitrogen sub-cloning efficiency™ E. coli DH5α™ competent cells

A 50 µL aliquot of chemically competent DH5α cells was thawed on ice for approximately 5 minutes. Ligation mixture (2 µL) was added and stirred using a pipette tip. Positive and negative controls were prepared as above. The resulting mixture remained on ice for 30 minutes prior to heat shock treatment at 37°C for 20 seconds. Following heat shock the cell mixture was returned to ice for 2 minutes. LB medium (450 µL) (Section 2.3.1.1) was added to the mixture and incubated at 37°C with shaking at 225 rpm for 30 minutes. The incubated mixture (50 µL) was spread onto an LB/Amp plate surface treated with X-gal and incubated at 37°C overnight.

2.3.3.3 Selection and storage of successfully transformed cells.

Following overnight incubation single colonies containing recombinant plasmid were selected based on the X-Gal/IPTG/LacZ blue/white selection system (i.e white colonies selected). Colonies were first resuspended in 10 µL of MilliQ water, streaked onto LB/Amp plates and inoculated into 5 mL LB/Amp. The liquid culture was incubated overnight at 37°C with shaking (225 rpm). An aliquot (500 µL) was combined with 30% sterile glycerol in a sterile cryovial to give a 15% glycerol stock, snap frozen in liquid nitrogen and placed at -70°C for long term storage. The LB/Amp plates were incubated at 37°C overnight and stored at 4°C for short term storage.

2.4 Established molecular methods

2.4.1 Buffers and solutions for DNA extraction and manipulation

2.4.1.1 Lysis buffer

Proteinase K (10 µL of 20 mg.mL^{-1} filter sterilised stock) was added to 10 mL pre-warmed (55°C) 0.5% sodium dodecyl-sulphate (SDS) in a sterile 20 mL universal container and mixed by gentle inversion. Lysis buffer was always prepared immediately before use.

2.4.1.2 TAE buffer (50x stock solution)

Tris base	242 g
Acetic acid (glacial)	57.1 mL
0.5M EDTA (pH 8.0)	100 mL
MilliQ water	to 1 L

The buffer was sterilised by autoclaving and stored at 4°C.

2.4.1.3 Loading dyes

DGGE (2×) Loading dye

Bromophenol blue (2%)	0.25 mL
Xylene cyanol (2%)	0.25 mL
Glycerol (100%)	7.0 mL
MilliQ water	2.5 mL

Agarose (6×) Loading dye

Bromophenol blue (2%)	1.25 mL
Xylene cyanol (2%)	1.25 mL
Glycerol (100%)	3.0 mL
MQ water	4.5 mL

Loading dyes were sterilised by filtration (0.2 µm), aliquotted (1 mL) and stored at -20°C. Dyes in use were stored at 4°C. Stock solutions of xylene cyanol (2%) and bromophenol blue (2%) were stored at room temperature.

2.4.1.4 DGGE solutions

0% denaturant solution

40% Acrylamide:bisacrylamide (37.5:1)	15 mL
50× TAE	2 mL
MilliQ water	to 100 mL

All components were combined in a measuring cylinder and decanted into a light protected bottle.

80% denaturant solution

40% Acrylamide:bisacrylamide (37.5:1)	15 mL
50x TAE	2 mL
Urea	33.6 g
Deionised formamide	32 mL
MilliQ water	to 100 mL

All components except for MilliQ water were combined in a glass beaker. The mixture was placed on a magnetic stirrer/heater and stirred with a magnetic bar with gentle heating to dissolve the urea. When the urea had dissolved the solution was transferred to a measuring cylinder, MilliQ water added to a final volume of 100 mL and the solution transferred to a light protected bottle. Both the 0% and 80% solutions were degassed under vacuum for approximately one hour to prevent inhibition of setting due to oxidation of the gel components. Solutions were stored at 4°C for up to 1 month.

2.4.2 Extraction and amplification of DNA

2.4.2.1 CTAB extraction of bacterial DNA

Bacterial cells were lysed in 500 µL Lysis buffer (Section 2.4.1.1) and incubated for 30 minutes at 55°C with gentle mixing at 10 minute intervals. CTAB (100 µl of 10% stock solution) and 80µL of 5 M NaCl were added and inverted to mix. A second incubation of 10 minutes at 65°C was performed. The lysate was then extracted once with chloroform: isoamyl alcohol (Section 2.4.2.2). For each environment, the efficiency of alternative precipitation methods was tested and the aqueous phase precipitated with either 2.5 volumes of ethanol (Section 2.4.2.3) or 1 volume of isopropanol (Section 2.4.2.4) accordingly (see individual chapters for

details). The pellet was resuspended by the addition of 25 µL MilliQ water and incubation at 4°C overnight.

2.4.2.2 Chloroform extraction of DNA

An equal volume of chloroform:iso-amyl alcohol (24:1) was added to the DNA solution and the mixture vortexed briefly until the phases combined. This mixture was then centrifuged (RCF =16 000, 5 minutes) to separate the two phases. The upper aqueous phase was transferred to a clean 1.5 mL microfuge tube for precipitation.

2.4.2.3 Ethanol precipitation of DNA

Ethanol (2.5 volumes) was added to the aqueous phase and mixed by gentle inversion. Following incubation for 2 hours at -70°C the DNA was collected by centrifugation (RCF = 16 000, 10 minutes). The supernatant was removed and the pellet washed using 70% ethanol. Following a second centrifugation (RCF = 16 000, 8 minutes) the pellet was dried in a laminar flow hood. When all traces of ethanol had been removed the pellet was resuspended in 25 µL of MilliQ water.

2.4.2.4 Isopropanol precipitation of DNA

Isopropanol (1 volume) was added to the aqueous phase and mixed by gentle inversion. Following incubation for 20-30 minutes at room temperature the DNA was collected by centrifugation (RCF = 16 000, 10 minutes). The pellet was washed, dried and resuspended as per ethanol precipitation (Section 2.4.2.3).

2.4.2.5 PCR amplification of DNA

Master mix:

dNTPs (2 mM)	5 µL
Forward primer (100 pmol.mL^{-1})	1 µL
Reverse primer (100 pmol.mL^{-1})	1 µL
Taq polymerase 10 x buffer	5 µL
Taq DNA polymerase enzyme	0.5 µL
Template	≈10 ng
MilliQ water	to 50 µL

General cycling parameters:

An initial denaturation step of 94°C for 4 minutes was followed by an annealing step (x°C depending on primer set used see Table 2.2) for 30 seconds, an extension step of 72°C for 1 minute and a denaturation step of 94°C for 1 minute. These three steps were repeated a further 34 times, followed by a subsequent 2 minute annealing step and final 5 minute extension (72°C) step.

2.4.3 Electrophoresis of DNA

2.4.3.1 Agarose gel electrophoresis

To determine presence and quality samples of extracted DNA and PCR products were electrophoresed on agarose gels (1% or 1.4% agarose respectively). Agarose was dissolved in an appropriate volume of 1x TAE by heating and allowed to cool to approximately 50°C. A final concentration of 0.5 $\mu g.mL^{-1}$ of ethidium bromide was added and mixed gently by swirling. The solution was then poured into a gel former with comb and allowed to set. The comb was removed and the gel submerged in an electrophoresis tank containing 0.5$\mu g.mL^{-1}$ ethidium bromide in 1× TAE. Samples were mixed with an appropriate volume of 6 × loading dye (Section 2.4.1.3) and 6 µL of sample mixture loaded per well. Samples were electrophoresed with either a λ Hind III for DNA (markers at sizes: 23.1kb, 9.4kb, 6.6kb, 4.4kb, 2.3kb, 2.0kb, 0.5kb, 0.125kb) or 100bp for PCR product (100-1000bp in 100bp increments) marker. Electrophoresis was performed at a constant voltage of 10 $V.cm^{-1}$. The gel was visualised by ethidium bromide fluorescence on a UV transilluminator and photographed using the SynGene GeneGenius gel documentation system.

2.4.3.2 Denaturing Gradient Gel Electrophoresis

Glass plates were de-greased using a detergent wash followed by an acetone wash. To enhance gel removal plates were cleaned using IMS and treated with Rain-X according to the manufacturer's instructions. Plates were clamped together separated by 1 mm spacers. High and low concentration denaturant solutions (15 mL) were made up (see Table 2.3) and 18 µL of TEMED and 120 µL of APS added. Solutions were inverted four times to mix and transferred to individual 30 mL syringes which were then attached to a gradient pourer (Bio-Rad DCode DGGE system). Gels were poured and a 16 or 25 well comb placed in the top. Gels were allowed to solidify for a minimum of 1 hour. The gels were clamped into place and lowered into pre-heated buffer (1 × TAE, 60°C) to pre-heat. After 15-30 minutes well-forming combs were

removed and the wells rinsed with warm buffer to remove excess denaturants and unpolymerised acrylamide. PCR products at equal concentrations (determined using a spectrophotometer) were mixed with equal volumes of 2 × loading dye (Section 2.4.1.3) prior to loading. Electrophoresis was performed for 5 minutes at 200V (to allow DNA to migrate into the gels) after which the buffer circulation pump was activated and electrophoresis continued at 60V for 18-20 hours.

2.4.4 Cloning of amplified bacterial DNA

2.4.4.1 Vector

The pGEM-T vector (Promega™) was routinely used for the cloning of PCR products. This vector is supplied pre-cut with *Eco*R V and has an additional terminal 3' thymidine on both strands (Figure 2.2). This improves the efficiency of ligations of PCR product into the vector by preventing recircularisation. Also, the use of *Taq* DNA polymerase for PCR results in the addition of a terminal adenosine on the 3' end of the PCR product, enhancing compatibility with the vector.

Figure 2.2 Promoter and multiple cloning sequence of the pGEM-T Easy Vector. Primer regions are highlighted in pink, multiple cloning sequence highlighted in pale blue.

2.4.4.2 DNA Ligations

Ligations were performed using the pGEM-T easy vector system. The mix was made up on ice in 0.5 mL microfuge tubes and mixed using a pipette tip.

Reaction mix:

2 × Rapid ligation buffer	5 µL
pGEM-T (50 ng)	1 µL
PCR product (30 ng) / control insert DNA	x µL
T4 DNA Ligase (3 $U.\mu L^{-1}$)	1 µL
MilliQ water	to 10 µL

Reactions were incubated at room temperature for 1 hour or overnight at 4°C. Vectors containing inserts were transformed into *E. coli* strains DH5α or XL1-Blue MRF' (Section 2.3.3) by heat shock at 42°C for 45 seconds or 37°C for 20 seconds respectively. Successful transformations were identified by blue/white lac selection (Section 2.3.3.3), confirmed by colony PCR (Section 2.4.4.3) and insert containing plasmids prepared for sequence analysis as described below (Section 2.4.4.4).

2.4.4.3 Colony PCR

To confirm presence and size of insert, white colonies were picked using a 1 µL disposable loop, suspended in 10 µL MilliQ water and subjected to PCR analysis. Colonies were also streaked onto LB/Amp plates and incubated at 37°C overnight in preparation for plasmid purification.

Reaction mix:

dNTPs (2mM)	2 µL
10 × *Taq* DNA Polymerase buffer	2 µL
Primer M13F	0.5 µL
Primer M13R	0.5 µL
Taq DNA polymerase	0.2 µL
Cell suspension	10 µL
MQ water	4.8 µL

Cycling parameters:

Reactions were subjected to 94°C for approximately 10 minutes and amplified over 25 cycles at a denaturing step of 94°C for 1 minute, an annealing temperature of

50°C for 1 minute and subsequent 1 minute extension at 72°C. Products were electrophoresed on 1.4% agarose gels and visualised to confirm presence of an insert. Successfully transformed colonies were selected for plasmid preparation and sequencing.

2.4.4.4 Plasmid preparation (Promega Wizard® Plus SV miniprep DNA purification system)

Following overnight incubation of streaked colonies (2.4.4.3), a single colony was used to inoculate 5 mL of LB/Amp medium and incubated overnight at 37°C. The resulting culture (3 mL) was centrifuged (RCF = 13 200, 5 minutes) to pellet the cells, and the pellet resuspended in 250 µL of cell suspension solution (50 mM Tris-HCl, 10 mM EDTA, 100 $\mu g.ml^{-1}$ RNase A). Cell lysis solution (250 µL, 0.2 M NaOH, 1% SDS) was added and the mixture incubated at room temperature for a maximum of 5 minutes. Alkaline protease solution was then added and the mixture incubated at room temperature for 5 minutes. Cell neutralisation solution (350 µL, 4.09 M guanidine hydrochloride, 0.759 M potassium acetate, 2.12 M glacial acetic acid) was added and inverted repeatedly until the solution became clear. The clear lysate was transferred to a spin column and centrifuged (RCF = 16 000, 1 minute) to separate plasmids from eluate, and eluate discarded. Column wash solution (500 µL, 162.8 mM potassium acetate, 22.6 mM Tris-HCl (pH 7.5), 0.109 mM EDTA (pH 8.0)) was added and centrifuged as above (RCF = 16 000, 1 minute). The wash solution was discarded and the wash repeated with 250 µL of column wash solution (RCF = 16 000, 1 minute). The column was then transferred to a sterile microfuge tube and MilliQ water (100 µL) added. The column was centrifuged (RCF = 16 000, 2 minutes) and discarded. The eluted DNA in the microfuge tube was stored at -20°C until cycle-sequencing.

2.4.5 DNA Sequencing

2.4.5.1 Cycle sequencing reaction

Reaction mix:

ABI BigDye 3.1 RR mix	2 µL
Dilution Buffer	3 µL
M13f / 341f (3.2 $pmol.\mu L^{-1}$)	2 µL
Plasmid prep / PCR Fragments	~200 ng
MilliQ water	to 20 µL

Cycling parameters:

The reaction mix was denatured at 96°C for 3 minutes followed by 25 cycles of 96°C for 15 seconds, 50°C annealing for 10 seconds and 60°C extension for 2 minutes. The reaction was stored at -20°C until clean-up prior to sequencing (see below).

2.4.5.2 Cycle sequencing clean-up 1 (single capillary sequencer)

This method was used when a small number (<10) of samples were being processed. Removal of unincorporated dye terminators was achieved using Wizard® MagneSil™ GREEN according to the manufacturer's instructions. Briefly, 20 µL of sequencing reaction was added to 180 µL of Wizard® MagneSil™ GREEN particles and the mixture incubated at room temperature for 5 minutes. The solution was vortex mixed at the beginning and half way through the incubation. Particles were trapped using a magnetic stand and the overlying liquid removed. The particles were resuspended in 100 µL of 90% ethanol, incubated at room temperature with vortexing as before (ethanol wash 1). The liquid was then removed and the ethanol wash step repeated (ethanol wash 2). Pellets were air dried for five minutes at room temperature in a laminar flow hood and the dry DNA eluted in 20 µl of Template Suppressing Reagent (TSR). Following incubation at room temperature for two minutes the particles were again trapped and the DNA-containing TSR transferred to clean tubes.

2.4.5.3 Cycle sequencing clean-up 2 (multi-capillary sequencer)

Unincorporated dye terminators must be removed prior to sequencing using the ABI multi capillary automated sequencer. This was achieved using precipitation of labelled DNA using ethanol and EDTA using a modification of the BigDye® Cycle Sequencing Kit protocol. Briefly, 5 µL of EDTA (125 mM) and 60 µL of 100% ethanol were added to 20 µL sequencing reactions in 0.2 mL PCR tubes (or 96 well plate). The tubes / plate were sealed and inverted 4 times to ensure thorough mixing and incubated at room temperature for 15 minutes. Reactions were centrifuged (RCF = 3 000, 30 minutes) at 4°C in a pre-cooled bench-top centrifuge fitted with a swing out rotor. The supernatant containing the dissolved dye terminators was decanted, removing as much supernatant as possible. Ethanol (60 µL, 70%) was added and the reaction centrifuged (RCF = 1 650, 15 minutes). The supernatant was removed and the pellet dried in the dark. Once dry, the pellet was resuspended in 20 µL of Hi-dye™

formamide and stored at -20°C until sequencing. This method was preferable for higher throughput sequencing efforts.

2.4.5.4 Automated sequencing and sequence analysis

Sequence detection and analysis was performed using an ABI 310 (or ABI 3100) capillary sequencer and accompanying DNA Sequencing Analysis Software. Sequences were aligned using the Seqman (Lasergene) software package. Plasmid and primer sequences (Figure 2.2) were removed and the remaining sequence manually checked for misreads. The edited sequences were submitted to the HGMP BLAST interface using the prokaryote search option. Sequences were manually checked for ambiguous base calls, and where misreads occurred sequencing was repeated using the reverse complement to confirm base identity.

2.5 Microbiological methods for natural community analysis

2.5.1 Bacterial enumeration

2.5.1.1 Fixation

For each station and time-point triplicate 900 µL sub-samples were fixed with 100 µL of 0.2 µm filtered 25% gluteraldehyde (2.5% final concentration). Samples from the marine stations were stored at 4°C prior to analysis. Estuarine samples were incubated at 4°C for 24 hours and stored at -20°C prior to analysis.

2.5.1.2 Direct counts

Aliquots (5 mL) of filtered, autoclaved sample water (FASW) were placed in the filtration manifold and 1 mL fixed sub-samples added. Staining of bacterial cells was performed by adding 0.6 ml 50 µg.mL^{-1} diamidino-2-phenyindole (DAPI) (5 µg.mL^{-1} final concentration) and incubating for 7 minutes at room temperature. Cells were then filtered under low vacuum onto 25 mm diameter, 0.2 µm pore size, black Nuclepore track-etched polycarbonate membranes and rinsed with 2-3 mL FASW. The membranes were air-dried and mounted onto microscope slides using non-fluorescing immersion oil. Filters were stored in the dark until examination. Slides were examined within one week.

Observation was carried out under 1 250 × magnification (10 × ocular, 100 × objective) with an epifluorescence microscope (OLYMPUS BH-2) fitted with a UV (340-380 nm) excitation filter. Using an ocular grid the number of DAPI stained bacteria in 10-15 random fields were counted per slide (approximately 300 cells per slide in total). Cell density per ml was calculated using the following formula:

$$\text{Bacteria.mL}^{-1} = (\text{Membrane conversion factor}^* \times N) / D$$

When N = average no. of bacteria per field and D = volume (mL) of sample filtered.
*conversion factor = number of large quadrats per effective filter area = 56533.717
i.e for 25 mm filters on 25 mm sartorius filter units with 100 × objective lens the large quadrat area is 82 µm × 82 µm (6724 µm^2), and the effective filter area = 380 133 mm^2 (effective filter area = (πd^2) / 4 when d = effective diameter. The effective diameter for the filtration scenario described above is 22 mm).

2.5.1.3 Flow cytometer counts

Triplicate sub-samples (900 µL) were stained for 1 hour at room temperature using 10 µL 100 × SYBR Green I stain (Marie *et al.*, 1997) (9×10^{-5} final dilution of commercial stock), 100 µL potassium citrate (24.5 mM final concentration) was added as per Zubkov *et al*. (2000).

Prior to sample counts Beckman-Coulter™ Flow-Set™ fluorosphere solution containing a known concentration (1.214×10^5.mL^{-1}) of (singlet, doublet and triplet) 3.6 µm polystyrene beads stained with a wide emission fluorochrome was run through three sample cycles on the flow cytometer. The resulting counts for all sets of beads were used in the following formula to calculate flow rate:

Total count = (1 × singlet) + (2 × doublet) + (3 × triplet)

Flow rate = (Total count / 1.214×10^5.mL^{-1}) × 1000) / number of minutes analysed

Cells were counted on the basis of SYBR Green fluorescence (measured at 530 +/- 15 nm) forward and side scatter parameters on a Perkin Elmer FACSort flow cytometer. The original sub-sample was subject to dilution by both fixation and staining procedures and thus counts were corrected based on the calculation below:

Fixed sample = 1000 µL original sample + 100 µL fixative ∴90.9% is original sample. The analysed sample = 900 µL fixed sample + 110 µL stain etc ∴89.1% is fixed sample thus 80.99% ((89.1/ 100) x 90.9) is original sample.

2.5.2 Determination of bacterial protein synthesis (Smith and Azam 1992)

Four replicate 1.7 mL live and two 1.7 mL TCA (Trichloro-acetic acid) killed control sub-samples were spiked with 20 nM final concentration L-[4,5-^{3}H]-Leucine (Amersham Pharmacia Biotech) and incubated at ambient water temperature in the dark for 1 hour. Activity in the samples was terminated by addition of 89 µL 100% TCA (5% final concentration) and samples were stored at 4°C until extraction.

Protein extractions were carried out after 1 hour precipitation of TCA insoluble material at 20°C. TCA insoluble material including protein was concentrated using 20 minute centrifugation (RCF = 9 200) and the supernatant was discarded. The pellet was resuspended in 500 µL of 5% TCA and the washing processes (i.e. centrifugation 20 minute, RCF = 9 200, supernatant removal and re-suspension) was repeated. The

remaining material was resuspended in 1 mL scintillation cocktail (Optiphase HiSafe III). Radioactivity, in the form of counts per minute (CPM), incorporated into bacterial protein was counted at Plymouth Marine Laboratory by Dr. Joanna Dixon. Counts were obtained using a Perkin-Elmer Winspectral 1414 advanced liquid scintillation counter system.

Counting efficiency was determined using the external standards method:

Efficiency = ((sample and spike CPM) - sample CPM) / known spike DPMs

Counts per minute were corrected to disintegrations per minute (DPMs):

Total DPM = Sample CPM / Efficiency

Sample DPM = Total DPM - control DPM

DPMs were then converted into the rate of leucine incorporation into cellular protein.

pmol uptake = (Sample DPM × (4.5×10^{-13})) / specific activity

pmol. hr^{-1} = pmol uptake / incubation time (hr)

pmol.hr^{-1}.mL^{-1} = pmol.hr^{-1} / sample volume in mL

pmol.hr^{-1}.L^{-1} = pmol.hr^{-1}.mL^{-1} × 1000

Leucine incorporation was converted into µgC.L^{-1}.day^{-1} using a conservative estimate of 1×10^{-14} gC per cell and 1×10^{18} cells per mole of leucine incorporated:

µgC.L^{-1}.day^{-1} = pmol leu $\times10^{-12}$ (pmoles/mole) ×24 (hours/day) × 2 (dilution factor) × 10^{18} (cells per mole of leucine) $\times10^{-14}$ (grams carbon per cell) × 10^{9} (cells per mL*)

2.5.3 Bacterial diversity

Bacterial diversity was assessed using variation in the gene encoding the small sub-unit ribosomal RNA (16S rRNA). Biomass was collected from a range of environments and incubated in polycarbonate bottles (Table 2.1). Throughout the incubations sub-samples were filtered through 0.2 µm, 25 mm diameter Nuclepore polycarbonate filters and stored at -20°C prior to CTAB extractions (as per Section 2.4.2.1). Simultaneously 6 mL sub-samples were concentrated by centrifugation and stored in 100 µL MilliQ water at -20°C for freeze/thaw extractions (Section 2.6.1). Extracted DNA was amplified (Section 2.6.2) and the product subjected to DGGE (Section 2.6.3) and sequencing where appropriate.

* mL used in place of L to allow use of µg units for carbon.

2.6 Molecular methods for natural community analysis

2.6.1 Collection of biomass and extraction of DNA from natural samples

2.6.1.1 Collection of biomass from natural samples

Bacteria not strongly associated with temporarily suspended particles (TSP, i.e. those particles not in suspension after a 30 minute settling period) were sampled by two methods. Firstly, TSP were allowed to settle out prior to removal of 250 mL of the remaining water which was then filtered under vacuum through a 0.2 µm pore size, 47 mm diameter, track-etched Nuclepore polycarbonate filter. Secondly, 6 mL of sample water was centrifuged (Eppendorf microcentrifuge) (RCF = 16 000, 20 minutes). The supernatant was removed and the pellet resuspended in 100 µL of MilliQ water. Filters and concentrates were stored at -20°C until DNA extraction. Bacteria incorporated into or attached to the surface of TSP were harvested following gravity induced settlement. A slurry of TSP and surrounding water was sampled to a final volume of 10 mL and filtered and stored as above.

2.6.1.2 Extraction of DNA from natural samples

Bacterial cells were lysed in 500 µL Lysis buffer (2.4.1.1) and incubated for 30 minutes at 55°C with gentle mixing at 10 minute intervals. The lysate was extracted with CTAB (Section 2.4.2.1) and extracted once with chloroform: isoamyl alcohol. The aqueous phase was precipitated with either 2.5 volumes of ethanol or 1 volume isopropanol as appropriate (see Section 2.4.2.3 and 2.4.2.4 and individual chapters for details). The pellet was resuspended by addition of 25 µL MilliQ water and incubated overnight at 4°C. Alternatively, bacterial cells concentrated by centrifugation were subjected to freeze-thaw extraction. Cells were removed from storage and thawed at room temperature. Cell suspensions were then subjected to three cycles of -70°C for 2 hours followed by 100°C for 5 minutes. Presence of high molecular weight genomic DNA was established by agarose gel electrophoresis of samples with a λ *Hin*d III marker (Section 2.4.3.1).

2.6.2 Primers used for amplification of bacterial DNA

A number of PCR primer pairs were utilised to amplify bacterial DNA extracted from natural samples. The most commonly used primer pair was 341f (GC) and 907r.

The forward primer 341f is specific to bacterial 16S rDNA and has a GC-rich 'clamp' attached at the 5' end for use in DGGE analysis (Muyzer 1993). The reverse primer 907r is a degenerate universal primer which corresponds to positions 907-926 of the *Escherichia coli* 16S rRNA gene. These primers are designed to amplify a DNA fragment of 566 base pairs. Nested PCR was used to obtain amplification product from challenging environments or low DNA content samples. External primers for nested PCR (indicated by * Table 2.2) were designed to amplify a fragment of 1338 base pairs. Internal amplifications were performed using 341f (GC) and 907r as described above. DGGE bands were re-amplified prior to sequencing using stringent PCR parameters (57°C annealing temperature, 25 cycles) to increase specificity. The internal primer pair for re-amplification of DGGE bands was designed to fall inside 341f (GC) and 907r. For sequencing primers compatible with the pGEM-T vector were used. M13f and M13r are designed to target the cloning vector just outside the T7 promoter region (Figure 2.2). Primer sequences are given in Table 2.2 (overleaf). Details of primer pairs and annealing temperatures are given in individual chapters.

The presence of appropriately sized PCR product was established by agarose gel electrophoresis of samples using 100bp marker (Section 2.4.3.1) for approximate size determination.

Primer	Sequence	Annealing temperature (°C)	Target	Reference
8f*	AGA GTT TGA TCC TGG CTC AG	57.3	Bacterial 16S gene	Muyzer et al., 1993
341f	CCT ACG GGA GGC AGC AG	56.3	Bacterial 16S gene	Muyzer et al., 1993
907r*	CCG TCA ATT CMT TTG AGT TT	52.2	Universal 16S gene	Muyzer et al., 1993
1346r	CCG TCA ATT CMT TTG AGT TT	50.4	Bacterial 16S gene	Nubel et al., 1996
M13f	TGT AAA ACG ACG GCC AGT	50	M13 region (plasmid)	n/a
M13r	GGA AAC AGC TAT GAC CAT G	50	M13 region (plasmid)	n/a
16S_nested 1_f*2	TGG GGA ATC TTA GAC AAT GG	55.3	Bacterial 16S gene	n/a
16S_nested 1_r*2	TAA (CT)CT TGC G(AG)C CGT ACT	59.4	Bacterial 16S gene	n/a
GC-Clamp	CGC CCG CCG CGC CCC GCG CCC GCC CCG CCG CCC CCG CCC C	n/a	n/a	Muyzer et al., 1993

Table 2.2 Primer sequences utilised to amplify bacterial DNA. PCR reactions performed as described in Section 2.4.2.5. Generally a compromise annealing temperature was used following optimisation using a gradient block thermocycler.
* External primer for nested PCR, *2 Post DGGE primers sequenced bands only.

* External primer for nested PCR
*2 Post DGGE primers sequenced bands only

2.6.3 Denaturing gradient gel electrophoresis (DGGE) of amplified DNA

2.6.3.1 Gel preparation and running conditions

Generally a gradient of 20-60% was used. Briefly, solutions of 20% and 60% were made up as shown in Table 2.3 and 18 µL of TEMED and 120µL of APS added to polymerise the gels. Gels were allowed to polymerise for a minimum of two hours and electrophoresis performed at 60 volts overnight (gel, solution and buffer preparation given in Section 2.4.3.2).

Denaturant concentration	20% (low)	60% (high)
Volume of 0% stock	11.25 mL	3.75 mL
Volume of 80% stock	3.75 mL	11.25 mL

Table 2.3 Volumes utilised to prepare the denaturant concentrations at extremes of the denaturing gradient. Stock solution components are shown in Section 2.4.1.4

After electrophoresis DGGE gels were stained using 1µL stock SYBR GOLD I (10,000×) in 10 mL TAE (1×) buffer.

2.6.3.2 Staining and band excision

The stain was spread evenly over the surface of the gel using a sterile pipette tip and incubated in the dark for 1 hour. The gel was then rinsed with MilliQ water and transferred onto a dark-reader (Clare Chemical Research Inc. USA). Gel images were documented using the GeneGenius gel documentation system (SynGene) both before and after band excision.

Bands of interest were excised using Harris Unicore gel cutters. This gave a cylindrical cutting surface with an internal diameter of approximately 1.2 mm. The gel plug was placed into a pre-labelled microfuge tube and overlaid with 20 µL of MilliQ water. Plugs were incubated at 4°C overnight to elute the DNA by diffusion. Following elution the DNA solution was either stored at -20°C or subjected to PCR as described above (Section 2.6.2; primers 16S_nested1_f and 16S_nested1_r) prior to cloning and sequencing.

3 How do diversity, production and population dynamics of bacterioplankton from two contrasting marine environments (coastal and oceanic) change in response to manipulated zinc conditions?

3.1 Introduction

3.1.1 Trace metals in the marine environment

In biological systems, trace metals can be defined as those metals which occur as natural constituents of living organisms or tissues and may be required in minute quantities to support biochemical processes within the cell (Outten 2000). In marine systems trace metals are generally present at concentrations below 1µmol.kg^{-1} (P. Statham Pers. Comm.). Zinc, and other trace metals, are present naturally in rocks, soils and sediments and are released gradually through natural processes such as weathering and erosion. Marine sources of trace metals including zinc are summarised in Section 1.2.1. Although zinc is required in the cell for essential enzymes (including DNA polymerases) (Hase and Finkelstein, 1993; Panina *et al*., 2003) it can in certain states and concentrations be toxic and has been shown to bioaccumulate in the flesh of various filter feeding organisms (Lin and Hsieh, 1999; Ke and Wang, 2001). Zinc toxicity is primarily a result of oxidative damage or the substitution of zinc for other essential metal cations (which prevents the normal function of enzymes) and thus consumption of contaminated shellfish may have implications for human health (e.g. Nriagu and Pacyna, 1988; Kasprzak, 1995). This in turn has implications for the viability of oyster fisheries in waters receiving trace metal contaminated industrial effluent (e.g. Lin and Hsieh, 1999)

Anthropogenic activities have increased metal release into the environment since the industrial revolution (Ayres, 1992). The threat of metal pollution to aquatic ecosystems (and human health) due to greater volumes of biological waste and industrial activity is now widely recognised (Nriagu and Pacyna, 1988). As a result, government legislation regarding inputs to aquatic systems have been introduced e.g. EU Directive on Integrated Pollution Prevention and Control, 1996 (IPCC), Urban Wastewater Treatment Directive, 1994, and more recently the Water Policy Framework Directive, 1997. Such legislation has resulted in reduced contamination of UK

waterways via reduction of pesticide, fertiliser and sewerage inputs (EA report, 2002). The requirement for reduced levels of contamination from industrial sources has also directed the development of bioremediation techniques, the majority of which rely on the ability of bacteria to utilise a wide range of substrates, including metals, in their metabolic processes (Lovley, 1995; Lovley and Coates, 1997; Malik, 2004). This ability also confers a certain level of metal tolerance, the mechanisms of which are well documented in a small number of organisms (Nies, 1999). Metal tolerance is most often apparent in bacteria from seriously contaminated environments such as acid mine drainage (e.g. Bhagat *et al*., 2004) whereas the ability of coastal and oceanic bacteria to tolerate large inputs of zinc is largely undetermined. It is crucial to understand the affects of zinc and other metal influx on bacterial communities to enable accurate estimates of the implications of such contamination on cycling of essential elements such as carbon and nitrogen.

3.1.2 Bacterial Diversity

Bacteria are an essential part of many (marine) biogeochemical cycles (see Section 1.1.1.1 and references therein) and as such their diversity can have implications for the magnitude and rates of processes in such cycles (e.g Taroncher-Oldenburg *et al*., 2003). It has been shown that perturbations can affect the diversity of a bacterial community (Øvreas *et al*., 1998; Beaulieu *et al*., 2000; Hemida *et al.*, 1997; Baath *et al.*, 1998; Kelly 1999; Stephen *et al.*, 1999; Sandaa *et al.,* 2001) and observation of reductions in bacterial diversity under increased contaminant conditions support the use of bacterial diversity as an indicator of ecosystem 'health' (Ford 2000). Historically the assessment of bacterial diversity has been fraught with difficulty, largely due to the morphological simplicity of bacteria despite very different roles of different phylotypes in their environment. More recently, the widespread use of molecular techniques has alleviated some of the constraints on microbial ecology (Section 1.1.3) and allowed scientists to establish and monitor changes in genetic diversity (Ward *et al*., 1990) and place previously indistinguishable bacteria accurately into phylogenetic trees (Woese *et al*., 1990; Ludwig *et al.*, 1998; Rossello-Mora and Amann, 2001; Rappe and Giovannoni, 2003).

Molecular phylogenetic approaches have been shown to consistently detect different microbial phylotypes in many environments, and have facilitated the discovery of many novel, as yet uncultured, bacteria (Ward *et al.,* 1990; Giovannoni *et*

al., 1990; Suzuki *et al.,* 2001; Nedashkovskaya *et al.,* 2003; Macian *et al.,* 2005). Limitations of such approaches are well documented (Section 1.1.2.2), but despite these limitations they remain the best tools available to bacterial ecologists for rapid assessment of bacterial diversity and can be applied in a wide range of environments (Giovannoni *et al*., 1990; Fuhrman *et al*., 1993; Murray *et al*., 1996; Øvreas *et al*., 1997; Wise *et al*., 1997; Zimmermann 1997; Øvreas *et al*., 1998; Rath *et al*., 1998; Konopka *et al*., 1999; Zhongtang and Mohn 2001) A particularly powerful tool applied to microbial ecology is Denaturing Gradient Gel Electrophoresis (DGGE) (Santegoeds *et al*., 1996; Teske *et al.,* 1996; Øvreas *et al.,* 1997; Wise *et al*., 1997; Fandino *et al*., 2001; Zhongtang and Mohn 2001). DGGE allows microbial ecologists to consistently separate strands of amplified DNA at the level of single nucleotide changes on an acrylamide gel (Sheffield *et al*., 1989) and by doing so determine the minimum number of different phylotypes present in any given sample. This enables bacterial community structure to be tracked over both spatial and temporal scales (Muyzer *et al.,* 1993). The use of cell specific measurements such as leucine incorporation (Section 2.5.2) in concert with molecular techniques gives a clearer picture of how a bacterial community is behaving in terms of dominant bacterial phylotypes and the effect of variability in community composition on productivity.

The aim of the study described here was to determine the effects of the addition of typical estuarine (100 nM) and elevated (1 µM) concentrations of zinc on marine bacterial diversity, abundance and production. The zinc concentrations (100 nM and 1 µM) represent an increase of one and two orders of magnitude respectively when referenced against zinc concentration measured in the English Channel (Tappin *et al*., 1993). The relative increase is nearer two to three orders of magnitude *c.f.* the Western North Atlantic (Bruland and Franks, 1983). This was achieved by sampling naturally occurring bacterial consortia from two contrasting marine environments (Section 2.2) which were incubated with the elevated zinc concentrations described above (100 nM and 1 µM). Response of the bacterial community to zinc enrichment was assessed by following temporal changes in bacterial abundance, bacterial production, and variation in the distribution of dominant bacterial phylogenies within the samples.

3.2 Methods

3.2.1 Sampling regime

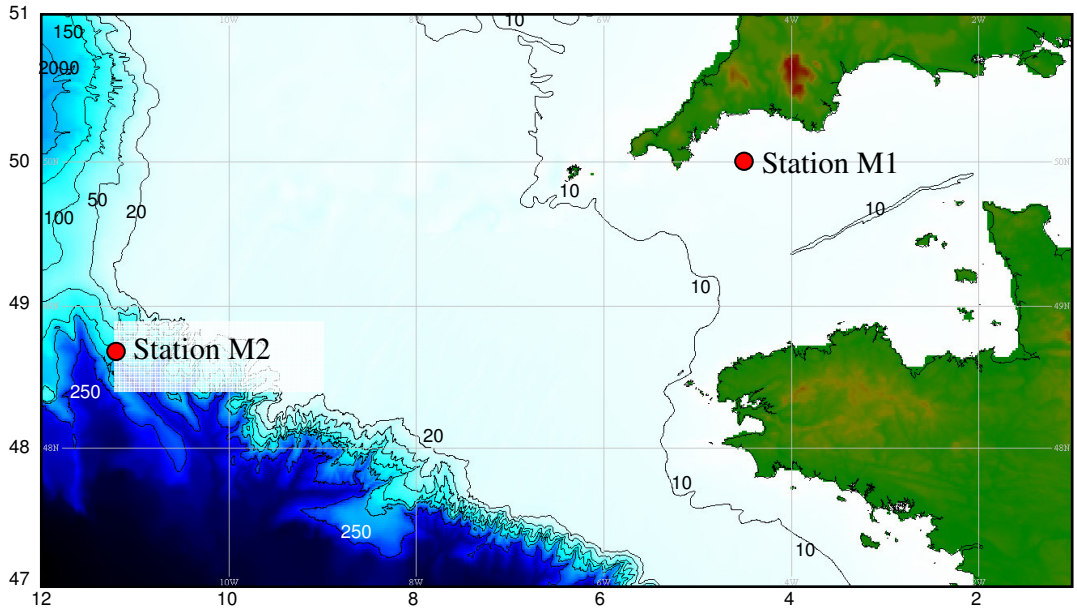


Figure 3.1 Map of sample sites showing depth contours at 100 m, 200 m, 500 m and 500 m intervals to 2500 m. Position of Stations M1 (50 m) and M2 (30 m) are shown by red circles. Parentheses indicate depth at which the water body was sampled.

Water samples were collected during R.R.S Discovery cruise D261. The cruise took place in the English Channel and Celtic Sea region and was conducted between the 1st and 14th of April 2002. Two contrasting water bodies were sampled: Station M1, a shallow, coastal site, 16km south of Plymouth sampled on the 2nd of April and Station M2 an off-shelf, deep water site (Figure 3.1), approximately 300 km from land sampled on the 4th of April. When on station, CTD profiles were used to establish the degree and depth of stratification. Chlorophyll fluorescence was used to determine the depth of the chlorophyll maximum. Water collection was performed using externally closing Niskin bottles (10 L) on Kevlar® hydroline. Both stations were sampled in the surface waters, Station M1 at 50 m and Station M2 at 30 m. Following deployment, the Niskin bottles were transferred to a trace metal clean containerised laboratory for manipulation. Incubations were initiated immediately after sampling with all manipulations performed using trace metal clean laboratory techniques.

3.2.2 Sample manipulation

Sterile 2 L pre-acid washed polycarbonate bottles were filled directly from the Niskin bottle taps and maintained at ambient sea temperature (10.7 and 12.1 ± 0.5^{o}C Station M1 and M2 respectively) in Thermotote portable incubators prior to the addition of dissolved zinc. For each sample one bottle was enriched with 1 µmol.L^{-1} Zn^{2+}, a second bottle with 100 nmol.L^{-1} Zn^{2+} and a third was untreated as a control. Zinc was added in the 2^{+} form dissolved in nitric acid (Spectrosol standard 1000 mg.L^{-1}). The pH was monitored prior to and following zinc additions and adjusted back to ambient levels, where necessary, with 0.1 M sodium hydroxide (AristaR). The bottles were incubated with stirring at ambient sea temperature (Table 2.1) in the dark. Sub-samples were taken immediately (T_0) and treated as follows for bacterial enumeration, production and diversity. Further sub-samples were taken over a period of 41 days (Table 2.1).

Samples for bacterial enumerations were fixed with 2.5% (final concentration) 0.2 µm filtered glutaraldehyde and stored at 4°C until counting. Samples were stained with 1 x SYBR Green I DNA stain in potassium citrate (Marie, 1997; Zubkov, 2001) and counted on the basis of SYBR Green fluorescence and forward and side scatter parameters on a Perkin Elmer FACSort flow cytometer (section 2.5.1).

Bacterial productivity was assessed using the rate of ^{3}H-leucine incorporation into bacterial protein. Incorporation rate was determined following the method of Smith and Azam (1992) with 20 nM tritiated leucine as substrate and 1 hour incubation at ambient temperature (Section 2.5.2).

Cell harvesting for molecular analyses was performed on 100 mL aliquots by filtration through 47 mm diameter, 0.2 µm pore size Nuclepore polycarbonate filters. DNA was extracted (Section 2.6.1.2) and precipitated from the aqueous phase using isopropanol (Section 2.4.2.4). DNA was resuspended in 25 µL of sterile ultrapure water (MilliQ) and stored at -20°C. PCR was performed on resuspended DNA template using the primers 341f (GC) and 907r (see Table 2.3 for sequence, reference and target regions) and an annealing temperature of 57°C. Presence and estimated size of PCR product was confirmed by 1% agarose gel electrophoresis (Section 2.4.3). PCR products were subjected to DGGE (Section 2.4.3.2) with a gel gradient of 20-60% at 60 V for 18-20 hours and stained with SYBR Gold II (Section 2.6.3).

3.2.3 Statistical analyses

The Students T-Test was used to determine the significance of variations in bacterial production and numbers between incubation conditions. One dimensional (1-D) gel analysis using the Genetool software package, was performed. Briefly, background correction was performed using a rolling disc and the number and position of individual bands / operational taxonomic units (OTUs) in sample fingerprints were determined. Multi-dimensional scaling was performed using this data to determine the similarity between samples from different time points and incubation conditions.

3.3 Results

3.3.1 Station Characteristics

3.3.1.1 Physico-chemical characteristics

The two stations were strikingly different in terms of physical and chemical characteristics. Station M1 was coastal, shallow and occasionally influenced by terrestrial run-off whereas Station M2 was deep, off the continental shelf and beyond influence from land (Figure 3.2). Both stations were mixed throughout the upper water column and showed little sign of stratification (Figure 3.3). Temperature at the stations varied from 10.7°C at Station M1 to 12.1°C at Station M2 (Figure 3.3). Nutrient data showed winter characteristics at Station M1 (figure 3.4) and slight depletion in nitrate and silicate consistent with pre-bloom conditions were detected at station M2.

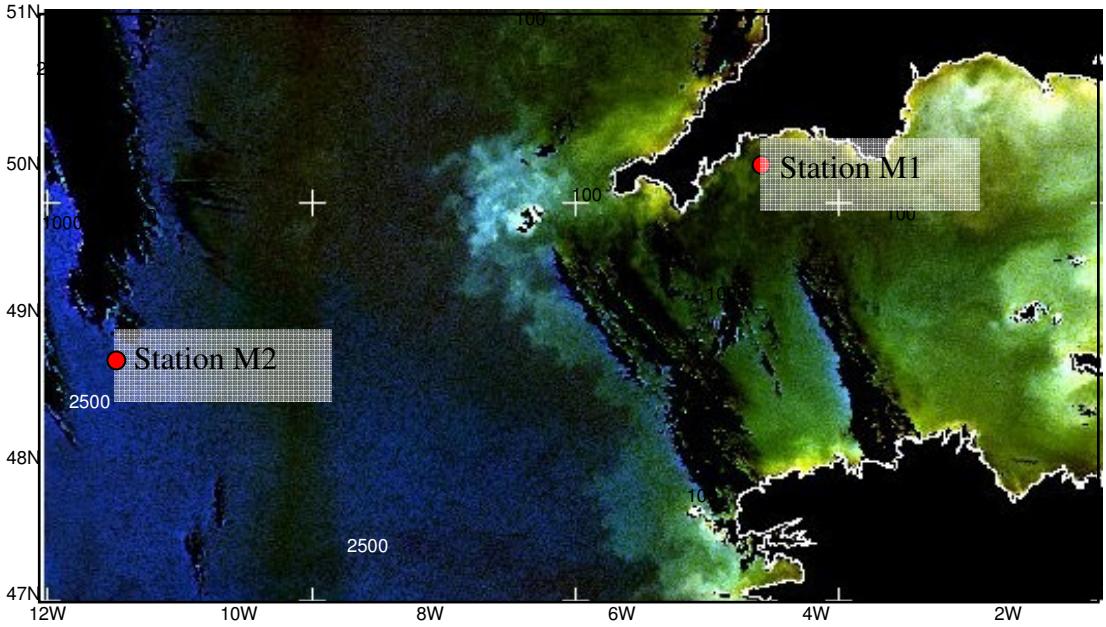


Figure 3.2 Remotely sensed enhanced true colour image showing position of sampling sites. Water leaving radiance at 555, 510, and 443 nm are displayed as the red, green and blue components of the image. Water leaving radiance was detected by the NASA Sea-viewing Wide Field-of-view Sensor (SeaWiFS) satellite in November 2003 (Peter Miller pers. comm.). Areas coloured black are where measurement was prevented by cloud cover or land (outlined in white), blue indicates areas of low turbidity, brown/green shows areas of high turbidity. Image provided by the Remote Sensing Group at Plymouth Marine Laboratory.

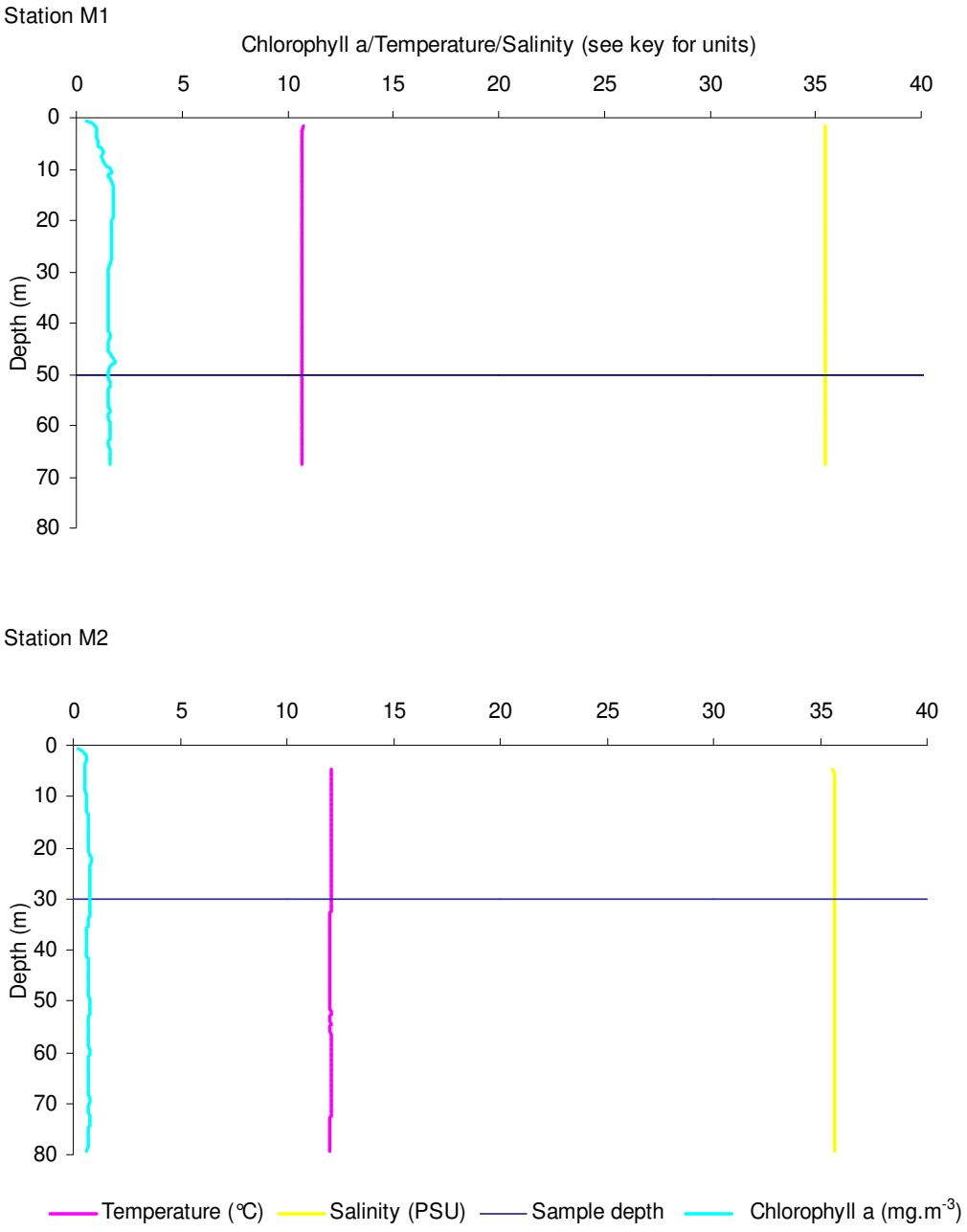


Figure 3.3 Temperature, salinity and chlorophyll a CTD profiles for stations M1 and M2. Chlorophyll *a* data obtained using the in-situ fluorometer chlorophyll method (BODC calibration number 2205 applied).

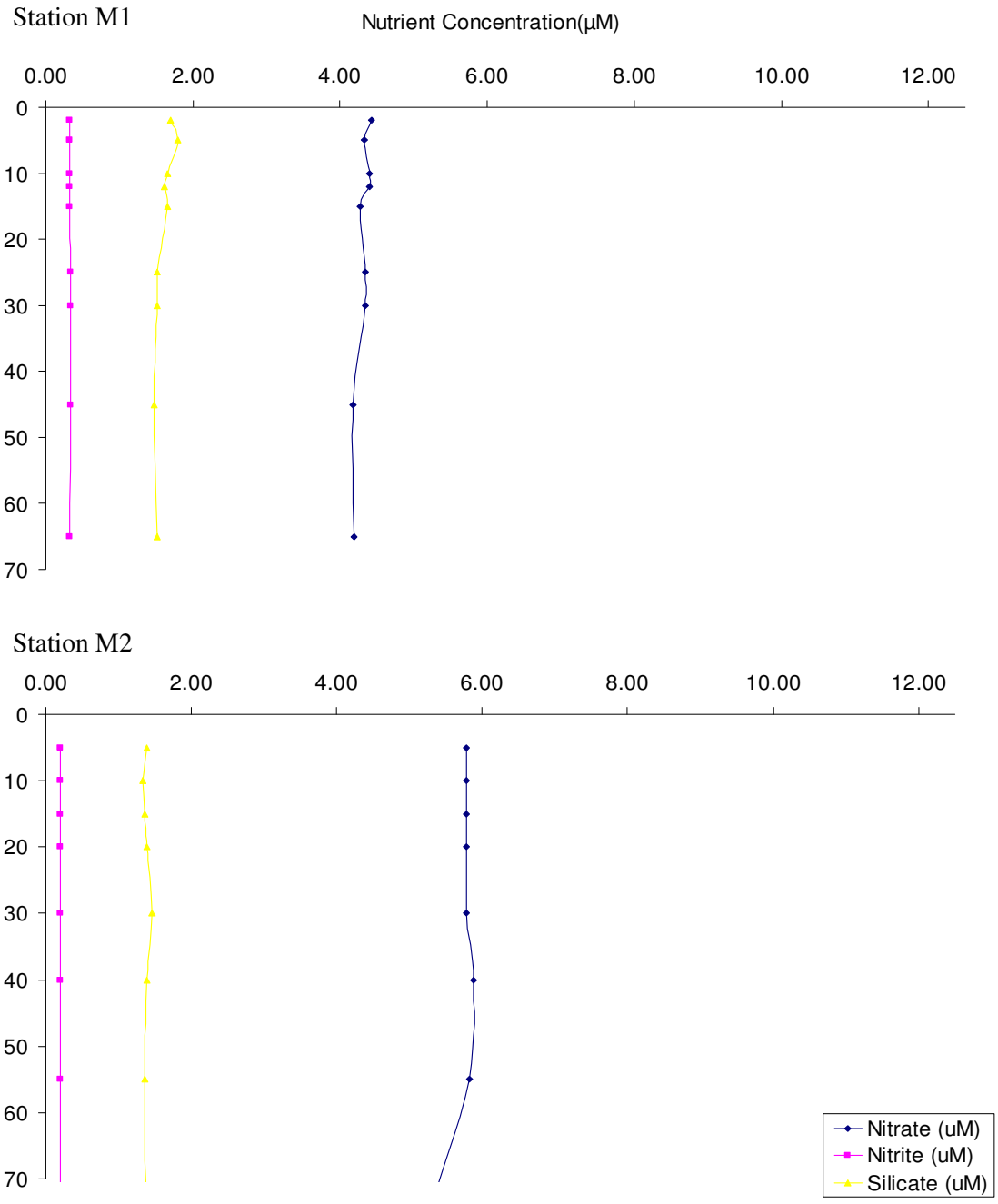


Figure 3.4 Profiles of nutrient concentration throughout the photic zone at stations M1 (coastal) and M2 (off-shelf). Station M1 shows typical winter characteristics. Depleted nitrite and silicate indicate pre-bloom conditions at Station M2.

Blondeau-Patissierre *et al*. (2004) measured chromophoric dissolved organic matter (CDOM) levels in samples taken on the same research cruise (D261). They found approximately twice the operational units of CDOM at Station M1 (1A in

reference: 0.103 m^{-1}) compared with station M2 (Station 620 in reference: 0.048 m^{-1}). Station M2 is typically influenced by CDOM from phytoplankton degradation products (fulvic acid – SCDOM slope 0.014 m^{-1} nm^{-1}) whereas Station M1 is likely to contain CDOM from both humic and fulvic acid origin (SCDOM slope 0.017 $m^{-1}nm^{-1}$) (Pers. Comm. G.Tilstone, PML). In addition Station M1 showed significantly higher SPM levels than Station M2 although these levels were below 5 µg/L (Pers. Comm. G. Tilstone and V. Martinez, PML).

3.3.1.2 Biological characteristics

The contrasting nature of these stations, in terms of physicochemical parameters, was also reflected by variation in phytoplankton standing stocks (as indicated by chlorophyll a concentration). Chlorophyll a concentration was significantly different between the two stations (T-Test, P value = 8.18×10^{-10}). In addition pigment analysis (Figure 3.5) suggested that different phytoplankton classes dominated each station (Pers. Comm. Denise Cummings, PML). At Station M1 fucoxanthin (Fuc) made up a large proportion of the pigment present, indicating the dominant algal class to be the diatoms. At Station M2 hexanoyloxyxanthin (Hex) and chlorophyll b (Chl b) dominated indicating the presence of a large proportion of prymnesiophytes (Hex) and green algae (Chl b) in the algal community at this station.

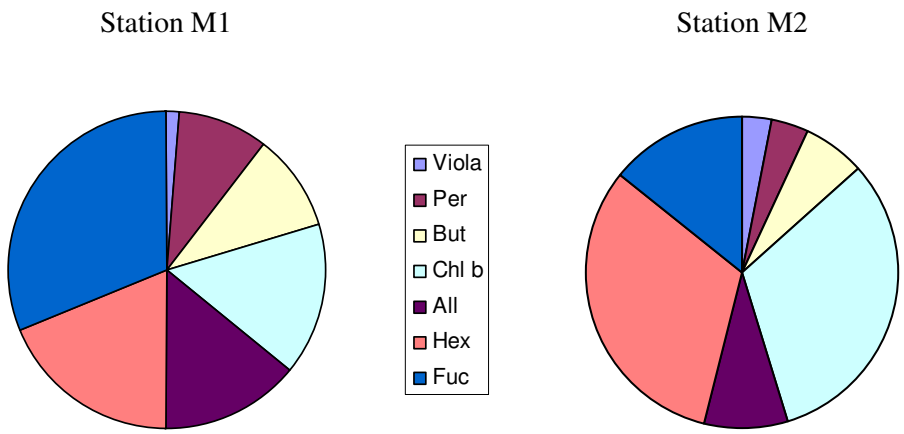


Figure 3.5 Dominant pigments detected at the sample depth from each station. Table A.2 (Appendix A) shows the marker pigments for the ten main algal classes.

Bacterial and primary production (Figure 3.6) show distinctly different distributions at each station. Station M1 showed maximum bacterial and primary

production in the upper water column (5 m) with rates decreasing rapidly with depth. In contrast station M2 showed high bacterial production in the surface 10 m with a second peak at 30 m (Figure 3.6). Primary production at Station M2 showed greatest production at 15 m and was notably higher than Station M1 at comparable depths in the water column.

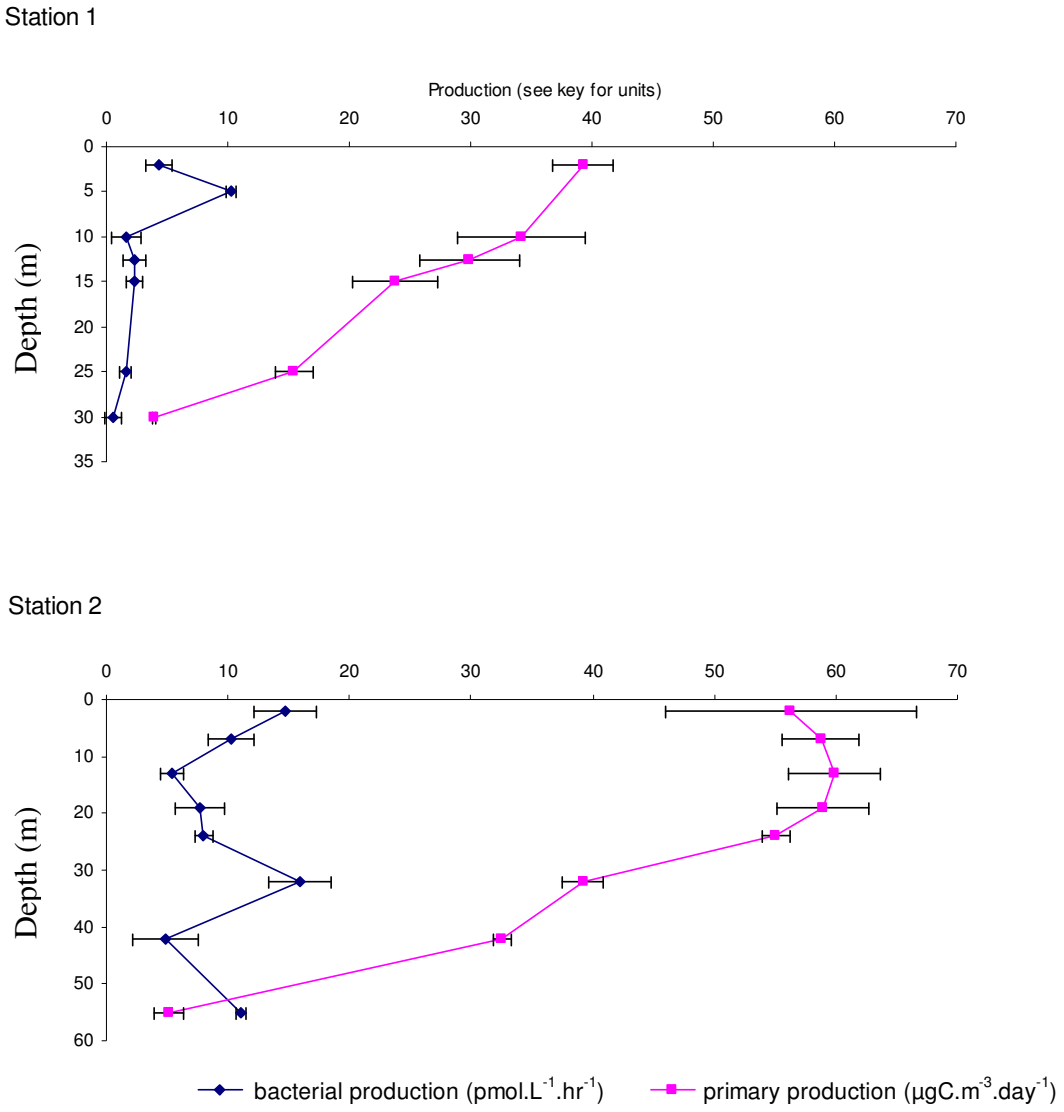


Figure 3.6 Variation in bacterial and primary production rates in the photic layers of stations M1 (coastal) and M2 (off-shelf). Bacterial production was determined using the leucine incorporation method and primary production determined using the standard JGOFS protocol (Susana Barquero-Molina, PML)

3.3.2 The effect of zinc addition on bacterial production

3.3.2.1 Station M1

Examination of the incubation time series data (Figure 3.7) showed different degrees and rates of effect depending on the concentration of zinc added. In the early stages of incubation (0 – 7 days), all samples followed a similar trend in bacterial production i.e. an initial increase followed by a decrease. Comparison of bacterioplankton production at each time point showed that the bacterial responses to the different concentrations of zinc were significantly different except for during the first 24 hours and at day 7 (Students T-test values $p \leq 0.05$). Throughout the majority (except T_0 and T_{end}) of the incubation period production in the 100 nM Zn^{2+} condition was not significantly different to that observed in the control samples. It was only in the final stages of incubation that any significant effect on bacterial production was observed with the addition of 100nM Zn^{2+}. Enhanced rates of leucine incorporation were observed between days 20 and 40 in the 1 µM Zn^{2+} incubation when compared to the control (Students T-Test, $p \leq 0.05$).

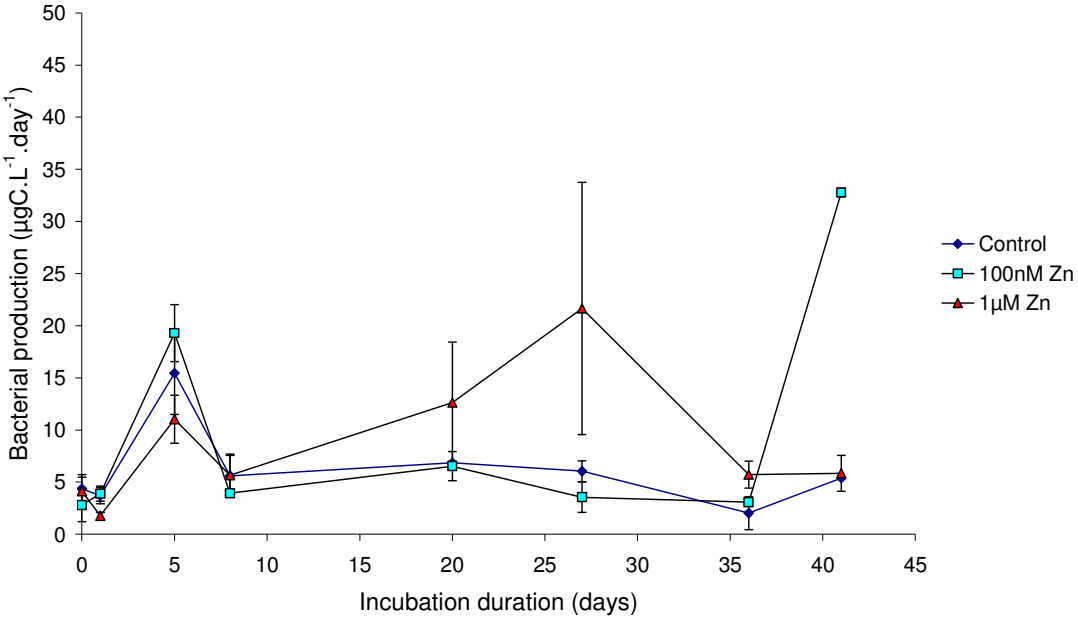


Figure 3.7 Production rates over time for bacterioplankton from Station M1 (coastal) incubations with and without additional Zn^{2+}. Error bars show standard deviation between replicates.

Integration of bacterial production (Figure 3.8) over the duration of the incubation showed that the addition of zinc resulted in an increase in bacterial production in both of the amended conditions. However this increase was only statistically significant in the 1µM Zn^{2+} condition ($p \leq 0.05$).

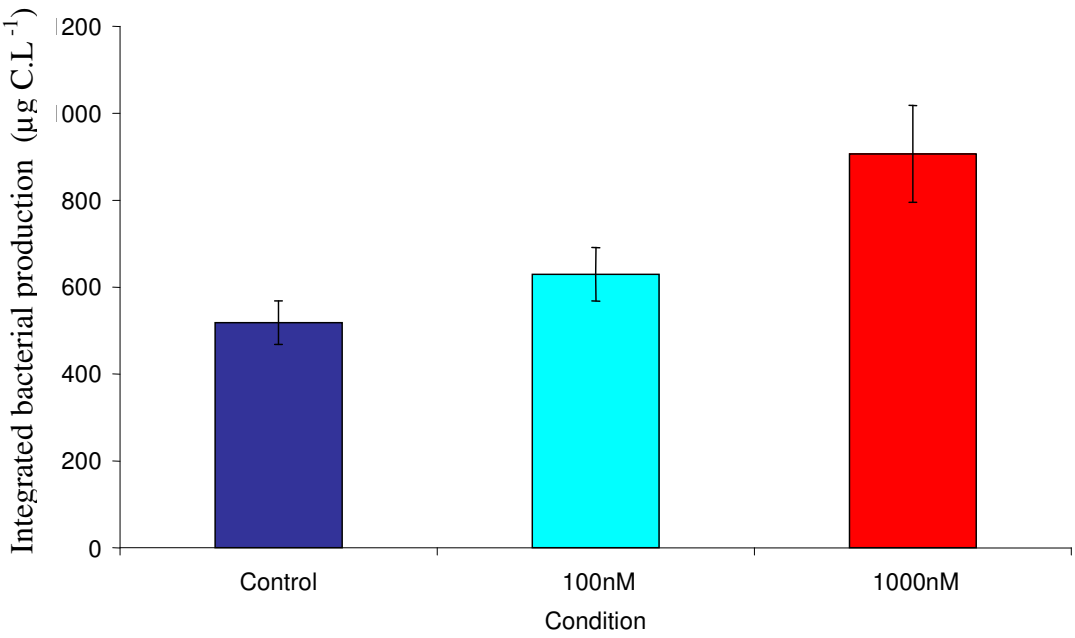


Figure 3.8 Bacterial production for samples from Station M1 (coastal) integrated over the duration of the incubation. Integrated values show the total amount of carbon fixed by bacteria over the duration of the incubation (42 days). Error bars show standard deviation.

3.3.2.2 Station M2

At station M2 the temporal trend of bacterial production observed in the enriched conditions differed from that observed in the control (Figure 3.9). In the control condition production increased until day 20. Following a subsequent decrease (similar to that observed at Station 1) a second, smaller increase was observed. With the addition of zinc however, an initial increase and subsequent plateau was observed. During the first week of incubation no significant difference was observed between the experimental conditions. By day 20 bacterial production in the control condition was significantly higher than in the zinc enriched conditions (Students T-test, $P \leq 0.05$). However, the high level of production observed at day 20 in the control was unsustainable and a rapid decrease was observed by day 27. Day 27 is the only

occasion when both enriched conditions were significantly more productive than the control ($P \leq 0.05$).

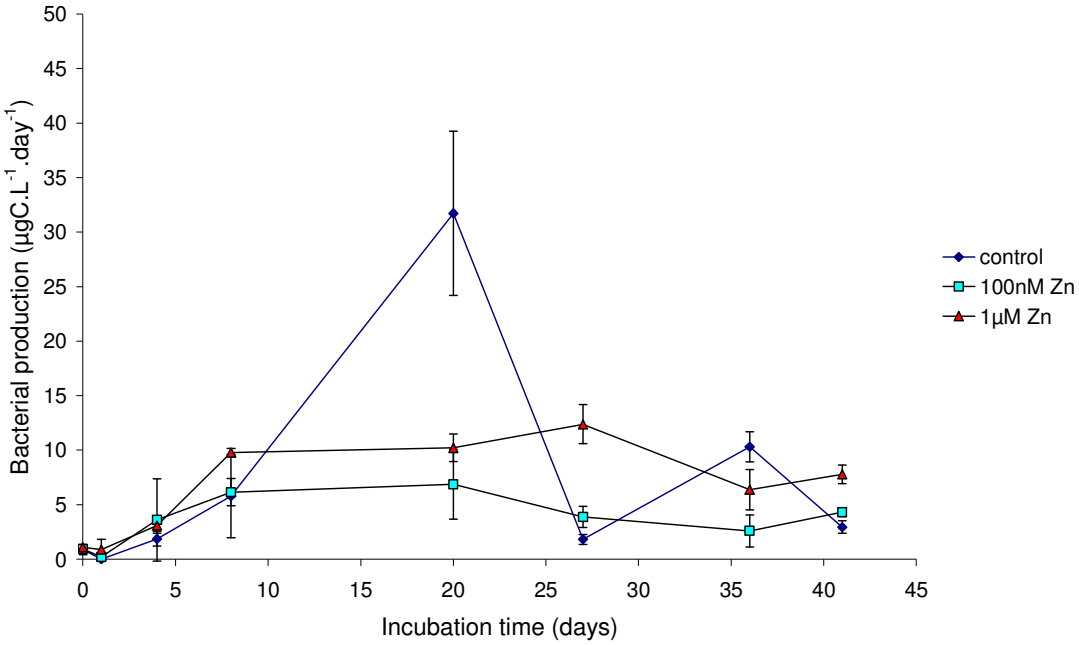


Figure 3.9 Production rates over time for bacterioplankton from Station M2 incubated with and without additional Zn^{2+}. Error bars show standard deviation between replicates. Key shows concentrations of zinc added.

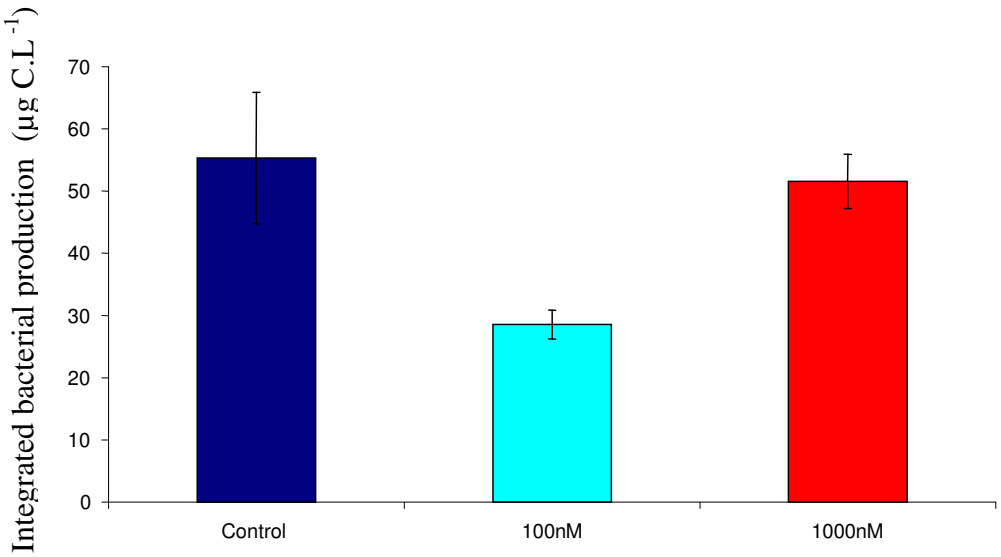


Figure 3.10 Bacterial production for samples from Station M2 (coastal) integrated over the duration of the incubation. Integrated values show the total amount of carbon fixed by bacteria over the duration of the incubation. Error bars show standard deviation.

The low bacterial production observed in the 100nM zinc enriched condition throughout the incubation was reflected by lower integrated (total) values of production than observed in the control (Figure 3.10). Conversely the total production in the 1µM zinc enriched condition was not significantly different to the control.

3.3.3 The effect of zinc on bacterial numbers

3.3.3.1 Station M1

Bacterioplankton in all experimental conditions from Station M1 followed the same general trend i.e. an initial increase in abundance in the first 5 days, followed by a sharp decrease and subsequent plateau (Figure 3.11). In the control condition abundance decreased further until day 27 at which time abundance slightly exceeded that originally observed. In the zinc amended conditions the decrease in abundance levelled slightly earlier (day 20) and in the 100 nM Zn^{2+} condition remained elevated when compared to the control for the remainder of the incubation. In the 1µM Zn^{2+} condition bacterial numbers were consistently lower than the control and 100 nM Zn^{2+} condition throughout the incubation. Conversely in the 100 nM Zn^{2+} condition bacterial numbers were enhanced relative to the control from day 27 onwards.

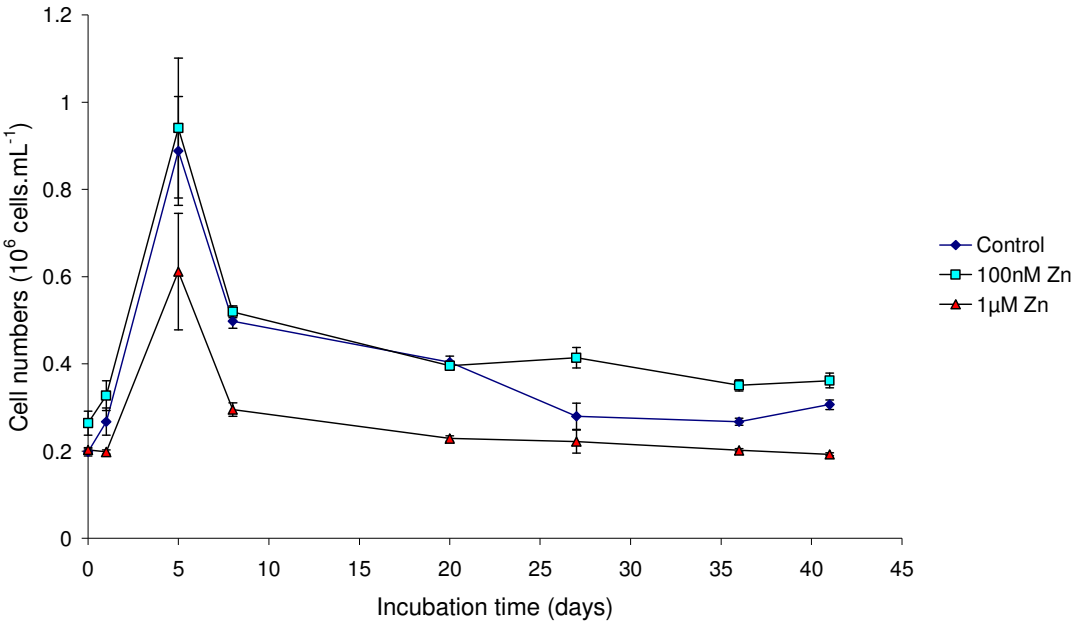


Figure 3.11 Variation in bacterioplankton abundance in samples from Station M1 over the duration of the incubation. Error bars show standard deviation between replicates.

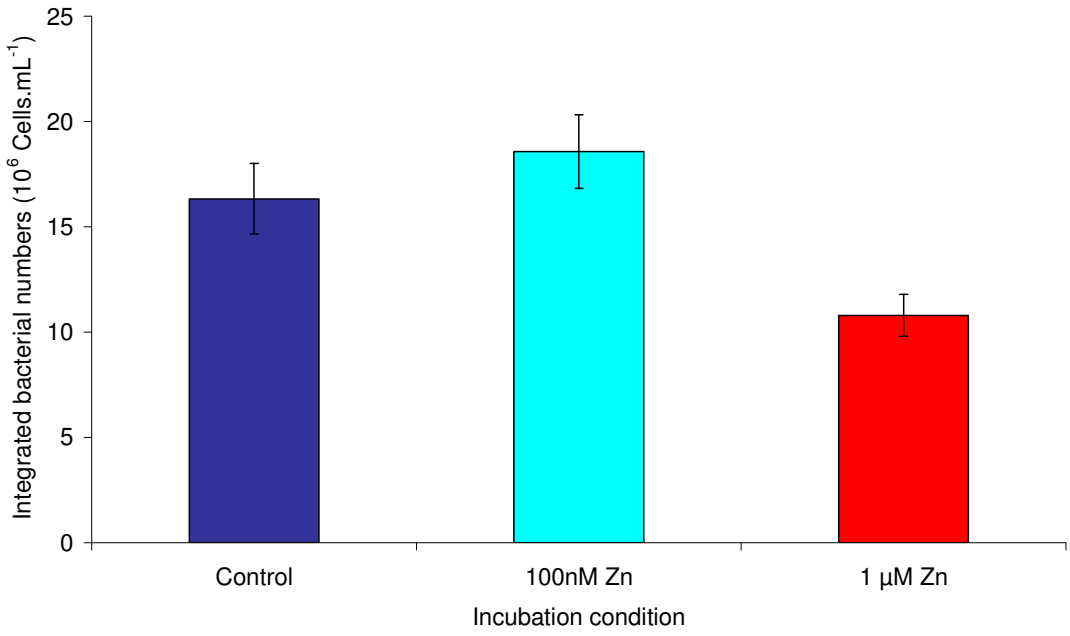


Figure 3.12 *Bacterial numbers in samples from Station M1 integrated over the duration of the incubation. Error bars show standard deviation*

Integration of bacterial numbers over the duration of the experiment confirmed that the addition of 1µM zinc resulted in overall suppression of bacterial numbers (Figure 3.12). In addition, despite elevated bacterial numbers c.f. control towards the end of the incubation period in the 100 nM Zn^{2+} condition, integration showed that the difference between overall numbers in the control and 100nM Zn^{2+} conditions was insignificant.

3.3.3.2 Station M2

The pattern observed in bacterial numbers at Station M2 was similar to Station M1 in that an increase in bacterial abundance was observed in the first seven days of incubation. The observed increase was followed by a decrease in numbers and subsequent plateau in the control and 1 µM Zn^{2+} condition. However, in the 100 nM Zn^{2+} condition bacterial numbers reached a plateau immediately following the increase and the numbers were maintained for the duration of the experiment (Figure 3.13). Bacterial numbers in the 1µM Zn^{2+} condition were suppressed throughout the incubation but again showed a similar temporal trend to the control.

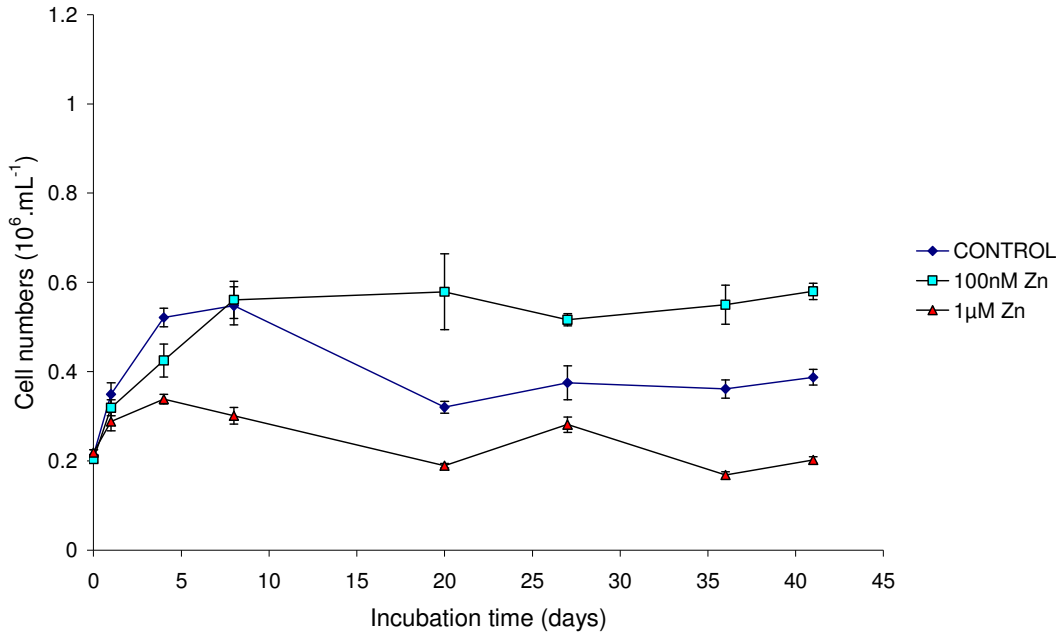


Figure 3.13 Variation in bacterioplankton abundance in samples from Station M2 over the duration of the incubation. Error bars show standard deviation between replicates.

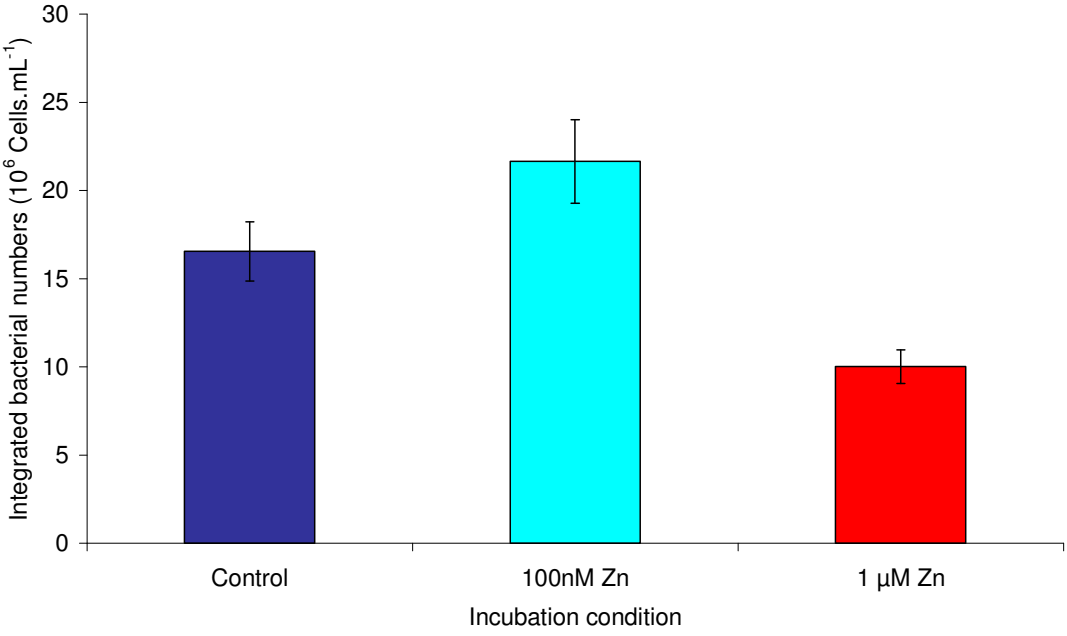


Figure 3.14 Bacterial numbers in samples from Station M2 integrated over the duration of the incubation. Error bars show standard deviation.

Integration of bacterial numbers over the duration of the experiment again confirmed that the addition of 1µM zinc resulted in overall suppression of bacterial numbers (Figure 3.14). In addition, consistent with the observation of elevated bacterial numbers c.f. *control towards the end of the incubation period in the 100 nM Zn^{2+} condition, integration confirmed that the overall numbers in the 100nM Zn^{2+} conditions were higher than those in the control.*

3.3.4 The effect of zinc on bacterial diversity

Fingerprints (DGGE) of samples from stations M1 and M2 (Figure 3.15 and 3.16 respectively) were analysed and presence and absence of bands migrating to a similar depth determined for each sample at each station. This data was used to create a similarity matrix and also to calculate the total number of bands per sample.

3.3.4.1 Number of phylotypes

Trends observed in phylogenetic richness at Station M1 (Figure 3.15) were different in both the 100nM and 1µM Zn conditions when compared to the control. The number of dominant phylotypes was similar in all conditions for the first five days of the incubation. The total number of phylotypes was generally higher in the Zn^{2+} enrichments in the first 20 days with the exception of a decreased number of phylotypes present at day 0 in the 1 µM Zn^{2+} condition (Figure 3.15), although the difference is negligible (1 band). At day 27 this pattern changed and the number of phylotypes present in the control increased and remained higher than the Zn^{2+} enriched conditions for the remainder of the experiment. By the end of the incubation (41 days) phylogenetic richness in all conditions had increased to levels greater than those observed at the beginning of the incubation except for the 100 nM Zn^{2+} condition.

At Station M2 DGGE analyses showed that after the first 24 hours the number of dominant bacterial phylotypes in the 100 nM Zn^{2+} condition was consistently equal to or greater than the control with the exception of the final time point (Figure 3.16). Phylogentic richness in the 1 µM Zn^{2+} condition was more variable in the later stages of the incubation (day 27 onwards) but lower than that observed in the other conditions.

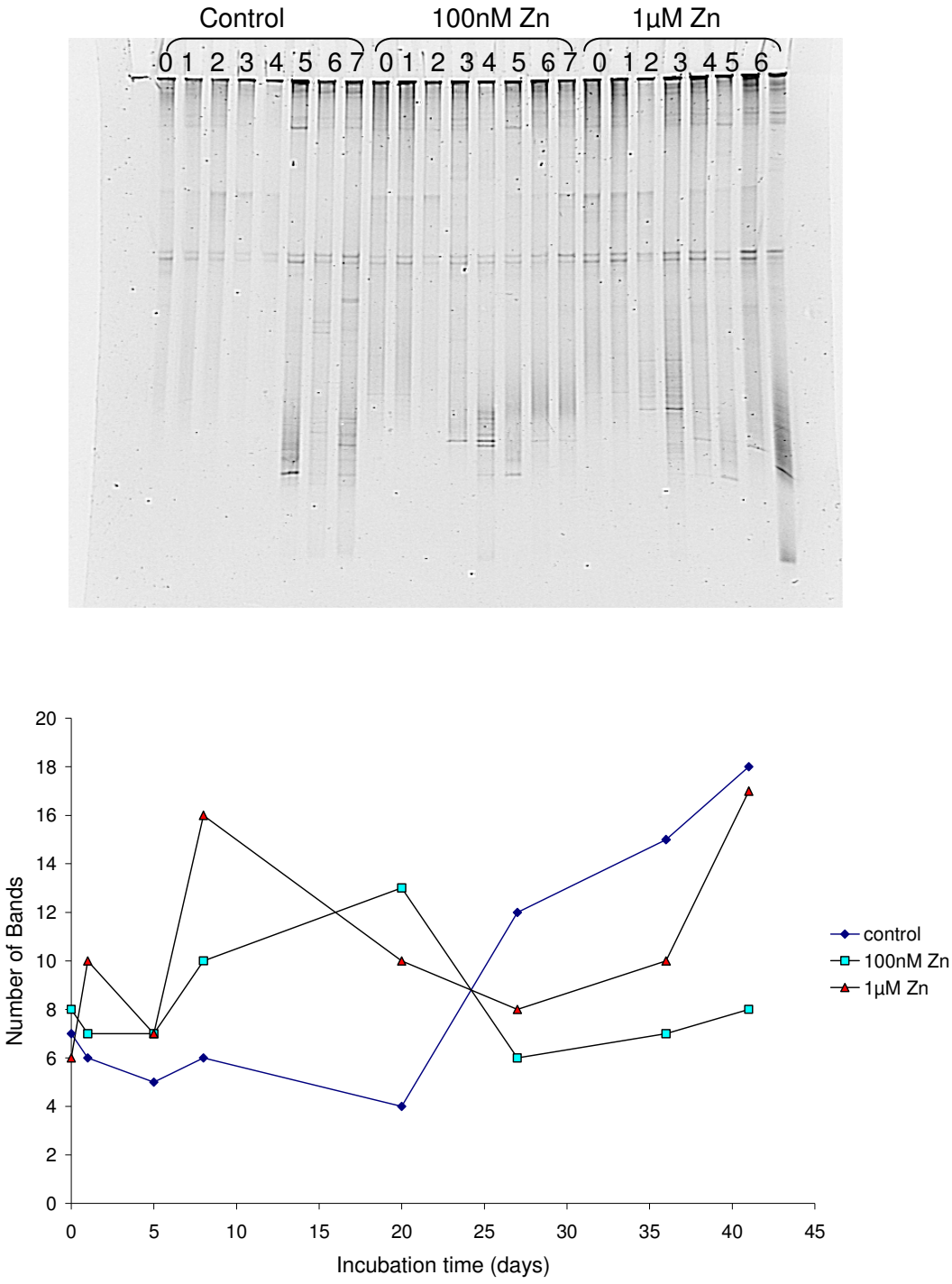


Figure 3.15 Number of dominant DGGE bands observed over the duration of the experiment in samples from Station M1. Upper image shows the DGGE gel, lower image shows graphical representation of the number of bands in each fingerprint.

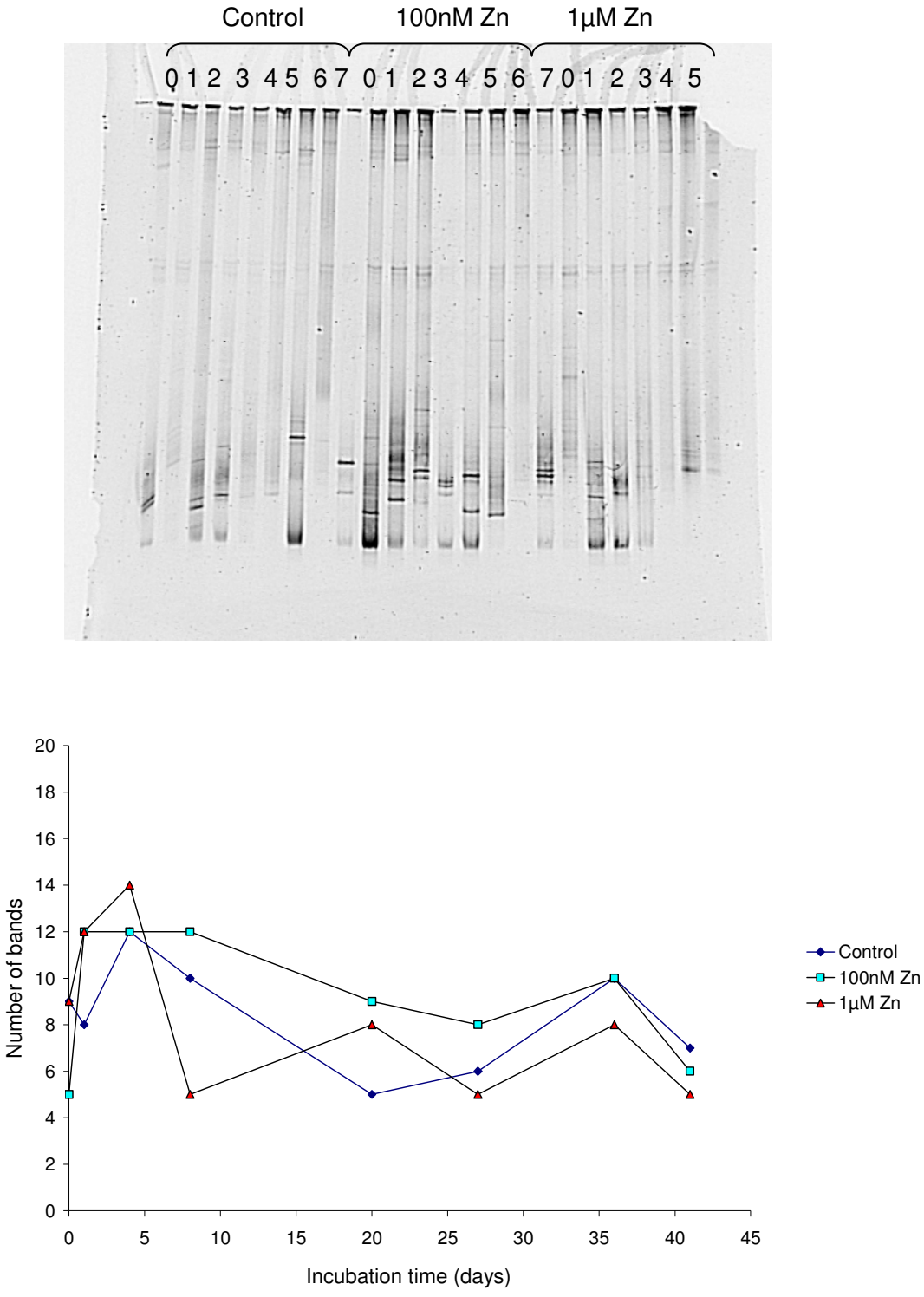


Figure 3.16 Number of dominant DGGE bands observed over the duration of the experiment in samples from Station M2. Upper image shows the DGGE gel, lower image shows graphical representation of the number of bands in each fingerprint.

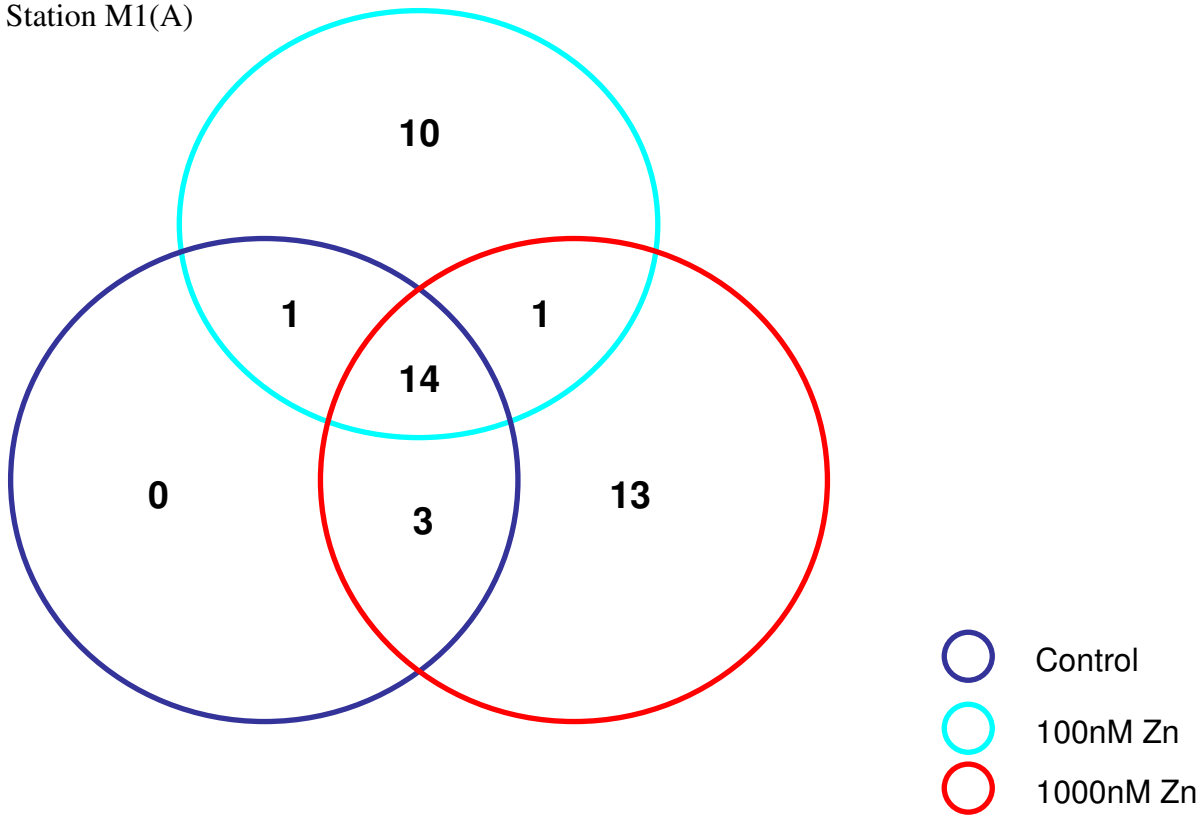
3.3.4.2 Distribution of bands

Investigation of the presence of major bands in the control and enriched conditions at any time during the incubation showed that at Station M1 the majority of bands were distributed between the control and 1 µM zinc conditions. None of the dominant bands were present solely in the 100 nM zinc condition (Figure 3.17A). However at Station M2 the bands were distributed more evenly between the conditions with the appearance of 4 bands specific to 100 nM Zn^{2+}. In addition there were fewer bands present in the 1 µM Zn^{2+} conditions (Figure 3.17B) at Station M2 than observed at Station M1.

3.3.4.3 Similarity of communities in each condition

Multi-dimensional scaling (MDS, PrimerE statistical package) indicated that the community at Station M1 had changed over the time course of the experiment, and that the communities from the different conditions overlapped (Figure 3.18). Furthermore, similarity matrices indicated that time may have been a more important factor than metal addition. The community present at the end of the incubation with 100nM Zn was more like the T_0 control samples than the T_{end} control. Samples from Station M2 clustered more firmly within the experimental conditions indicating that the treatment was the more important factor (Figure 3.18) controlling community dynamics throughout the incubation.

Station M1(A)



Station M2 (B)

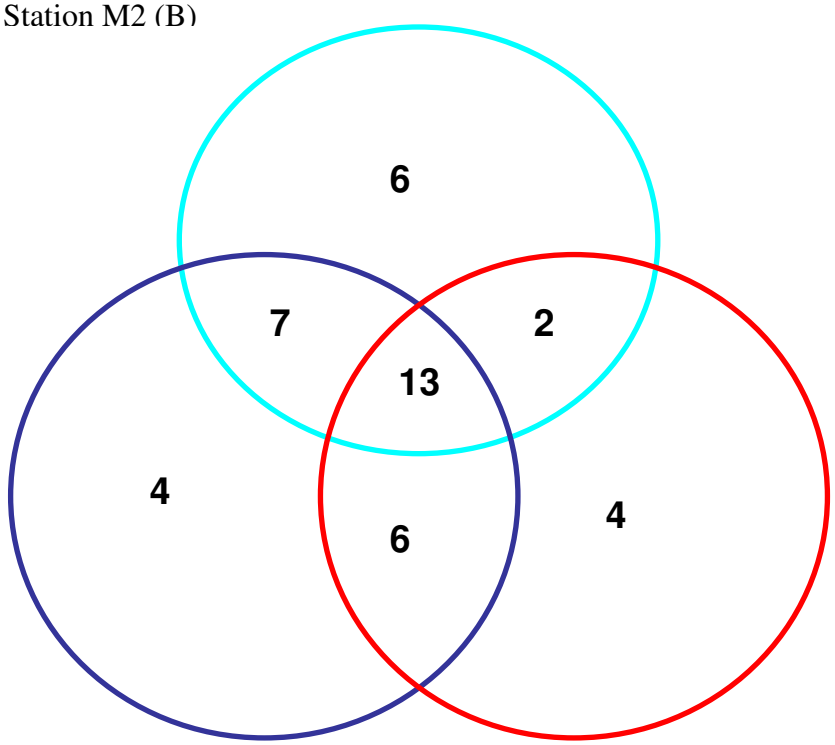


Figure 3.17 The number of dominant DGGE bands observed in each incubation condition, stations M1 and M2. Overlaps indicate presence of bands in multiple conditions.

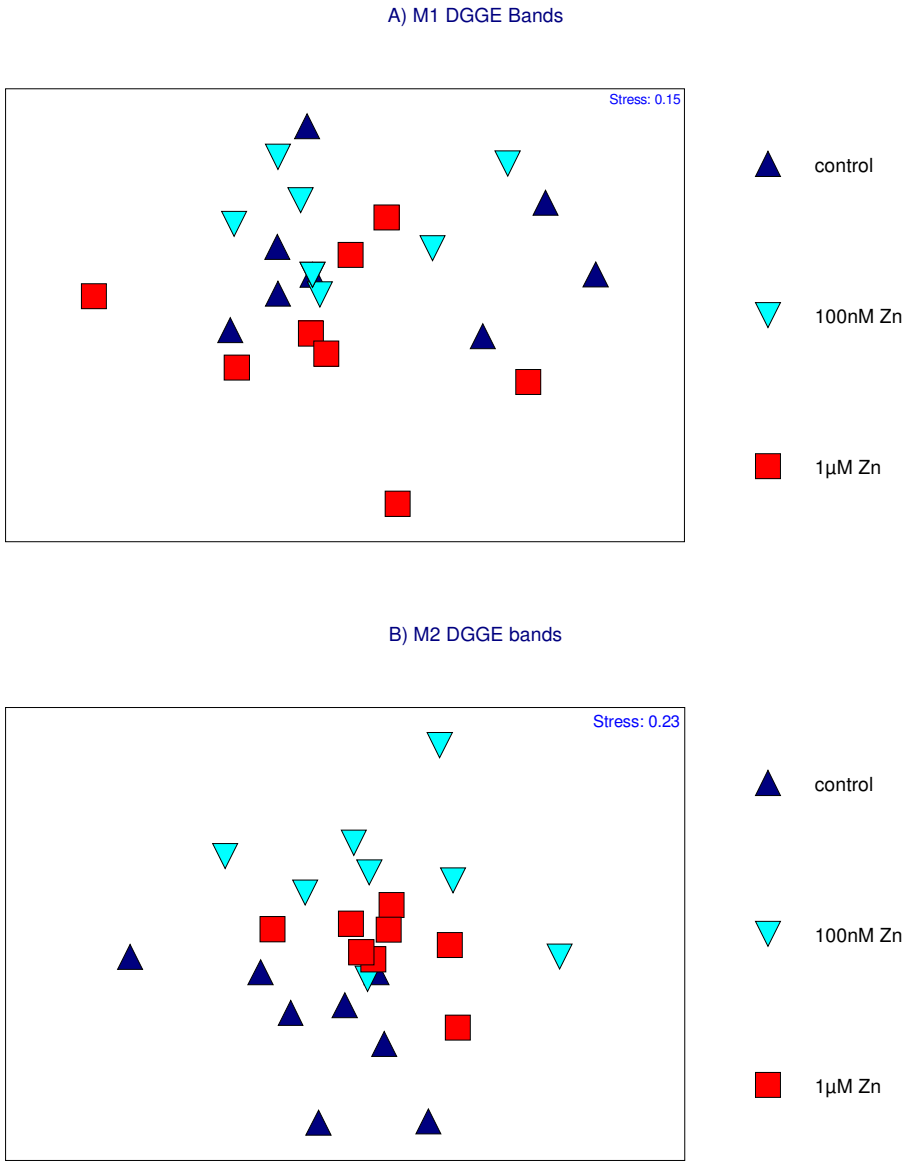


Figure 3.18 Ordination plots (MDS) showing the similarity between banding patterns of DNA fragments retrieved from A) Station M1 and B) Station M2.

3.4 Discussion

The response of marine bacterioplankton communities to elevated zinc concentration has been investigated in a coastal and an oceanic environment. The influence of zinc concentration, initial bacterial community composition and physicochemical conditions on bacterial response to zinc enrichment is discussed.

3.4.1 Direct effects

3.4.1.1 Zinc tolerant bacteria

Bacterial assemblage structure may change in the presence of elevated concentrations of zinc, both as a result of the presence of zinc tolerant phylotypes and the degree of zinc tolerance (or not) displayed by the remainder of the community. The different zinc tolerances observed at the two sites correspond with different bacterial abundance. One example of this is the different response of bacterioplankton to the 100 nM Zn enrichment; at Station M1 numbers are similar to the control in the 100 nM Zn condition until day 27 (Figure 3.11). In addition no phylotypes were unique to this condition (Figure 3.17A). In samples from Station M2 however, phylotypes specific to the 100 nM Zn^{2+} condition were observed (Figure 3.17B) combined with increased numbers *c.f.* control (Figures 3.13 and 3.14). At Station M1 the addition of 1 µM Zn^{2+} resulted in a comparatively large number of phylotypes (13 c.f. 4 at Station M2, Figure 3.17). The increase in phylotypes was accompanied by a decrease in bacterial numbers (Figure 3.11 and 3.12). Conversely, it also corresponded with increased leucine incorporation (Figure 3.9) throughout much of the incubation. This may indicate that the addition of 1 µM Zn^{2+} resulted in the death of less zinc tolerant bacteria which may previously have dominated the bacterial community. This would provide extra substrate and alleviate competitive inhibition of more tolerant bacteria thus explaining both the increase in diversity and productivity. At Station M2 there was a greater degree of overlap in conditions in which different phylotypes were detected (Figure 3.17B). In addition, fewer phylotypes were detected in the 1 µM Zn condition, possibly indicating that phylotypes detected at this station were generally less tolerant to zinc.

Leucine incorporation data indicates that whilst bacteria are present and incorporating leucine into cellular protein in the 1µM Zn^{2+} condition at Station M2 (Figure 3.9) those in the control were able to incorporate substantially more i.e. were more productive. This suggests that energy which would otherwise be used for creation

of cellular biomass may be required to support the zinc tolerance mechanisms of these bacteria under zinc stress. The patterns observed in leucine incorporation also suggest that in metal amended samples a period of incubation is required before a changed incorporation rate is observed, this hypothesis being supported by the gradual nature of the change in bacterial community observed by DGGE. DGGE images also indicate that the rate of community change is greater in the 1 µM Zn condition at both sites i.e. selection for certain bacteria occurs more rapidly (Figures 3.15 and 3.16).

However, it is generally in the second half of the incubation that increased leucine incorporation or indeed numbers are observed. This indicates a lag period, or perhaps acclimation period, after which the bacteria are able to tolerate and function under elevated Zn conditions. This theory is supported by work carried out by Bender *et al.* (1994) who showed that microbial mats incubated with elevated zinc were able to remain active in the presence of greater concentrations of metals than those without an equivalent acclimation period. So, whilst putatively metal tolerant phylotypes emerging here may always have been present, it is only due to the prolonged incubation that they have been able to become dominant members of the bacterioplankton community.

It is also possible that the background concentrations of zinc, i.e. pre-enrichment levels, affect the ability of bacterial consortia to tolerate elevated zinc. The concentrations of zinc previously encountered would be very different in the two environments sampled and this may explain the different tolerance regimes observed at the two sites. Coastal bacteria, particularly from a site such as Station M1, influenced sporadically by terrestrial run-off, would have been previously exposed to zinc levels approaching those observed in estuarine environments, whilst a degree of dilution would be expected these bacteria would certainly experience elevated zinc concentrations compared to those encountered by truly marine bacteria. Coastal zinc concentrations of 7.6nM have been detected in the English Channel (Tappin et al., 1993) whereas truly marine concentrations are more likely to be an order of magnitude lower than coastal values (Bruland and Franks, 1981). Previous work has shown that pre-exposure to metals can influence bacterial tolerance levels (Lehman *et al*., 1999; Diaz-Ravina and Baath 2001). Lehman *et al.* (1999) indicated that 'pollution adapted' bacterial consortia emerged when incubated with zinc above a threshold concentration of 1µM. This supports the designation of phylotypes present in the 1 µM zinc condition alone as zinc tolerant, it also implies that the 100 nM Zn concentration may have been

too low to elicit a response as was observed at Station M1 (Figures 3.7 and 3.11). Bacterioplankton from a truly marine site would be influenced more greatly by atmospheric deposition and more dilute concentrations of metals depending on currents and prevalent wind direction, this lack of 'pre-conditioning' may help to explain the distribution of phylotypes between conditions observed at Station M2 (Figure 3.17). It is possible that the bacterial consortia have evolved different coping strategies as a result of low environmental zinc concentrations and as such are less able to tolerate concentrations which are comparatively extreme. Analysis of DGGE gels indicates that different coping strategies are employed in the two environments. Oceanic bacterial communities are thought to be able, under zinc limiting conditions, to concentrate required zinc within the cell. It is possible that a similar strategy may be utilised in times of zinc stress to control the toxicity of zinc to the cell by removed damaging cations from the cytoplasm and exporting them from the cell (Nies, 1999).

3.4.1.2 Carbon source and enzymatic digestion

The availability of carbon sources may be critical in determining how a bacterial community responds to zinc amendment. For example humic material, which was highest at Station M1, is less available than freshly excreted phytoplankton derived carbon, which is the major source of DOC in oceanic regions such as Station M2 (Grossart and Ploug, 2000; Cherrier and Bauer 2004). Humic substances may affect the response to elevated zinc in a number of ways. They may complex with the zinc ions added in the experiment and make them less biologically available (Koukal *et al*., 2003). Alternatively humic substances may be a source of organic material which can be utilised for growth. The complex nature of the majority of humic molecules means they are highly refractory and thus not readily metabolised. It is usually assumed that the humic molecules have structures which are not accessible to degrading enzymes. However it is possible that, in metal-limited conditions, essential enzymes capable of at least partially digesting humic molecules may be inactive due to lack of essential metal co-factors. If zinc was limiting in these conditions the addition of zinc may result in greater microbial enzymatic activity and a greater proportion of such molecules may be metabolised.

Zinc is known to be an essential trace element (Madigan *et al*., 2003) and can be taken into the cell passively or via active uptake mechanisms (Nies, 1999). It is probable that elevated zinc concentrations would increase the rate and amount of

passive uptake. This would reduce the energy expended in producing zinc binding proteins thus making the formation of enzymes less energetically expensive. This theory is supported by the different rates of leucine incorporation in response to zinc enrichment observed at the two sites. It should also be noted that primary production was notably higher at Station M2, this would result in a greater pool of labile organic carbon, again reducing the requirement of the bacteria at this site for the production of energetically expensive enzymes, and thus their requirement for zinc.

Whilst carbon availability satisfactorily accounts for the increased production at Station M1 in the 1 µM Zn condition it does not explain why no such dramatic increase was noted in the 100 nM Zn condition. However, in conjunction with the possibility of humic material acting as ligands it is plausible that it is only in the presence of higher concentrations of zinc that it becomes available for potentially zinc limited bacteria.

3.4.2 Indirect effects

3.4.2.1 Reduced grazing

The effect of added zinc on the bacterial community may not be direct. Variations in both bacterial numbers and leucine incorporation may be influenced by the effect of zinc on bactivorous grazers. In fresh-water systems, Daphnia species have been shown to graze less when exposed to elevated zinc (Allen *et al.*, 1995). It is reasonable to assume that a similar effect may be observed with marine microzooplankton. Reduced grazing could account for the constant bacterial numbers observed in the 100 nM Zn condition at both stations. The control samples show typical bacterial growth curves followed by a decrease in bacterial numbers commonly associated with 'predator-prey' interactions. The constant bacterial numbers could be achieved by one of two mechanisms. The first could be the achievement of a steady state between growth and mortality (possibly due to increased growth as a result of zinc tolerance or alleviation of zinc limitation) and the second the possible inhibition of bactivorous grazers. The latter mechanism is supported by the relative numbers of bacteria remaining in the 100nM zinc treated conditions at both stations. The depleted number of bacteria observed in the 1 µM Zn conditions suggests that if grazing pressure was being reduced it would have to be coupled with reduced bacterial growth to explain the substantial reduction in bacterial numbers *c.f.* control. It may be that the 100 nM concentration of Zn had little or no negative affect on bacteria but had a deleterious

effect on the grazing community and that 1 µM Zn had a negative effect on both the grazers and their prey.

3.4.2.2 Phytoplankton viability

A second indirect effect may be that high concentrations of zinc affect phytoplankton viability. This may lead to release of labile carbon and nutrients either as a result of cell damage (or increased growth in photic conditions). Sunda and Huntsman (1992) showed that some phytoplankton species are tolerant of elevated zinc concentrations. A further factor influencing phytoplankton viability may be the incubation strategy. As described previously the incubations were carried out in the dark. It is possible that this played a more important role in this scenario than zinc tolerance. Different phytoplankton genera have different dark survival strategies. Diatoms were dominant at Station M1 as evidenced by a combination of microscopy and pigment analysis (C. Widdecombe pers. comm.). A study carried out using Diatom species from the North Sea showed that they had the ability to 'survive' without spore formation for up to 10 months and more than 80% of individuals remained active for as long as two months under similar incubation conditions (Peters, 1996; Murphy and Cowles, 1997). The observed differences in phytoplankton community may therefore affect the bacterial community response via either zinc tolerance or dark tolerance or a combination of the two i.e. whilst the phytoplankton may not be actively photosynthesising only those susceptible to zinc toxicity would senesce and leak nutrients into the surrounding media.

3.4.2.3 Incubation effects

In any incubation experiment such as this it is important to note that confinement within a small volume can affect the dynamics both between and within trophic levels. One example of this is the introduction of a surface, this can result in the formation of bacterial biofilms, this may be particularly important in the study of effects of trace metals. Biofilms are often associated with large quantities of exopolysaccharides, which can act as ligands and thus reduce toxic effects of metals. This would result in an increased difference in the response to the zinc concentrations investigated here. Thus the effects here should be considered minimal response to zinc enrichment. A second incubation consideration is the influence of stirring on grazing pressure. Grazing has been shown to be reduced in turbulent environments (Peters *et*

al. 2002; Malits *et al.*2004), which could account for some of the effects previously attributed to metal toxicity to grazing organisms. However, if this was the controlling factor the same response would be observed in both the 1 µM Zn^{2+} and Control conditions. This discrepancy suggests that the effects of Zinc addition play a more important role in controlling bacterial community dynamics than the incubation conditions. Stirring may also increase availability of nutrients (Malits *et al.* 2004; Delaney 2003), this may alter the dynamics between bacteria which are able to survive at low nutrient concentrations and those which rely on a feast or famine approach to nutrition. However, because comparisons discussed here are between conditions incubated in the same manner, conclusions drawn still show merit and improve the current knowledge of effects of zinc on bacterial communities in marine environments. The effect of incubation technique is explored in more detail in Chapter 4 by comparing variation in bacterial production and abundance over time with different incubation strategies.

3.5 Conclusions

The results presented here suggest that pre-exposure environmental conditions, including zinc concentration and the concentration and type of organic matter present, had a strong influence on the response of the bacterial community to zinc enrichment. It was also apparent that the spectrum of response varied depending on the initial composition of the bacterial community exposed to elevated zinc concentrations. Bacterial communities from the coastal environment (M1) were either tolerant or intolerant to zinc, independent of concentration, whereas bacteria from the oceanic environment (M2) displayed a range of tolerance levels dependant on the concentration of zinc added. In both environments a decrease in 'species' richness was observed with the addition of 1 µM zinc (although an initial increase was observed at Station M1), furthermore the richness of the bacterial community changed more rapidly in this condition. Conversely, the addition of 100 nM zinc resulted in increased species richness throughout the majority of the incubation at Station M2. In the oceanic samples (M2) the addition of 100 nM also resulted in increased productivity throughout the incubation, suggesting a degree of zinc limitation in this environment.

4 How do bacterial communities respond to incubation strategies inducing aggregate formation?

4.1 Introduction

4.1.1 Formation and importance of aggregates

Aggregates form as a result of the interactions between particles and both biological and physical parameters. Variation in salinity affects the formation and strength of ionic charges between particles and thus the probability of aggregation, this phenomenon is particularly important in terms of the formation of estuarine turbidity maxima zones (TMZ) (Section 1.2.1.2; Gregory and Duan 2001). Aggregate formation can also be positively influenced by biological processes such as production of mucopolysaccharides by bacteria and higher organisms (Busch and Stumm 1968; Alldredge and Silver 1988; Biddanda 1988; Vandevivere & Kirchman 1993; Heissenberger *et al*., 1996). These and other extracellular polymeric substances (EPS), including those secreted by benthic diatoms on the surface of intertidal regions (Austen *et al*., 1999) and free DNA (Alldredge and Silver 1988) increase particle stickiness, which further increases particle size, which in turn increases frequency or likelihood of collisions. A second biological mechanism involves the production of faecal pellets by higher organisms (reviewed by Turner 2002) which are essentially ready-made aggregates which provide a rich source of nutrients for microbial organisms as they sink through the water column. A third biological influence on aggregation is the production of Larvacean houses which provide an ideal starting point for aggregate formation (Alldredge 1972; Hansen *et al*., 1996).

Aggregate formation in estuaries has a number of implications for the remineralisation of organic carbon (Section 1.3.2.1). Firstly, the colonisation of particles by bacteria creates a short-cut in the food chain for grazing organisms by providing suitably sized substrate (Lampitt *et al.,* 1993), secondly, the formation of particles results in increased residence time of organic matter in the estuarine system (Geyer 1993; Jay and Musaik 1994; Uncles *et al.*, 1994) and thirdly, the colonisation of those particles (which are not ingested) results in an increase in the proportion of organic matter mineralised before it is flushed from the estuary (Grossart and Ploug 2001; Simon *et al*., 2002). Furthermore, particles released from the confines of the

TMZ (and become marine aggregates) contribute significantly to the removal of carbon from said systems via the biological pump.

4.1.2 The effects of aggregate formation on estuarine bacterial communities

The formation of aggregates in aquatic environments affects bacterial communities dramatically. Aggregates provide a microhabitat rich in organic matter and nutrients (Shanks and Trent 1979; Simon *et al*. 2002) and support the development of mutually beneficial bacterial consortia. Phytoplankton and microscopic organisms associated with aggregates develop a highly efficient nutrient transfer process in the early stages of colonisation (Patterson *et al*., 1993). In addition, the production of hydrolytic enzymes by aggregate associated bacteria can benefit free-living bacteria via the provision of labile organic matter in the surrounding water mass (Patterson *et al*., 1993, Unanue *et al*., 1998). Bacterial production on aggregates is generally equal to or in excess of the production of their free-living counterparts (Turley and Stutt 2000). This, in combination with increased numbers of bacteria on aggregates, where enrichment factors vary from 0.6 to 5 700 (Simon *et al*. 2002), can result in aggregate associated bacteria being responsible for upwards of 30% of total bacterial production in estuaries (e.g. Crump and Baross 1996). Furthermore, in estuaries with high concentrations of suspended particulate matter (SPM) as much as 90% of the bacterial community can be associated with aggregates (Simon *et al*. 2002).

Studies of artificial aggregate formation support the use of natural substrates for assessment of aggregate effects. Whilst they do not have the advantage of being clearly defined in terms of organic matter and nutrient content they do provide useful and relevant information regarding bacterial communities (Unanue *et al*., 1998; Grossart and Ploug 2000; 2001). The majority of such studies have utilised rolling bottles or up-flow systems to simulate environmentally relevant conditions. This study was performed using an 'aggregate roller' designed to stimulate the formation of aggregates from natural substrates found in the Fal Estuary (Cornwall, UK).

4.1.3 The Fal estuary

The Fal Estuary is tidal for a distance of 18 km inland and has a shoreline totalling approximately 115 km in length. The estuary has historically been a site of trace metal contamination due, initially, to influx from active tin mines and, more recently, from old mine adits and spoil heaps. The Fal Estuary is also currently still

affected by residual metal contamination from a pollution incident originating from the abandoned 'Wheal Jane' tin mine in 1992. Trace metal concentrations in the sediments of Restronguet Creek are extremely high in comparison to other estuaries in the Southwest (Grossart 2001). Despite this contamination the Fal has been designated a site of special scientific interest (SSSI) and an area of outstanding natural beauty (AONB) by English Nature. The area has also been designated a special area of conservation (SAC) (http://www. cornwall.gov.uk/Environment/sachome.htm) on the basis of the habitats provided by the estuary complex for marine wildlife. The estuary's mudflats and salt-marshes provide a feeding ground for a number of species of wading birds.

The purpose of this study was to investigate the effects of incubation strategy on bacterial community structure and activity.

4.2 Methods

4.2.1 Water collection and incubation strategy

Water (60 L) was collected at a salinity of 15 from Restronguet Creek, a tidal tributary of the Fal Estuary, on the 24th of March 2003. Collection was performed directly into sterile, 20 L polycarbonate carbuoys which were rinsed twice with water from the target environment prior to sampling. Bottles containing water were transferred to a CT lab set to ambient estuarine water temperature (12°C) at Plymouth Marine Laboratory within two hours of collection and samples taken immediately for analysis of T_0 parameters. Bottles were incubated either on aggregate rollers ('Rolled condition' - Section 2.4.4), stirred on magnetic stirrers ('Stirred' condition) or incubated without agitation ('Still' condition). Samples were incubated in the dark and sub-samples collected for assessment of bacterial abundance, productivity and diversity at 8 time-points over a total duration of 21 days. Visible aggregates were present in the rolled sample from day 2 onwards (Figure 4.1). No visible aggregates were detected in either the Stirred or the Still conditions. Aggregates were amorphous and fragile, thus attempts to sample intact aggregates for image analysis were unsuccessful.

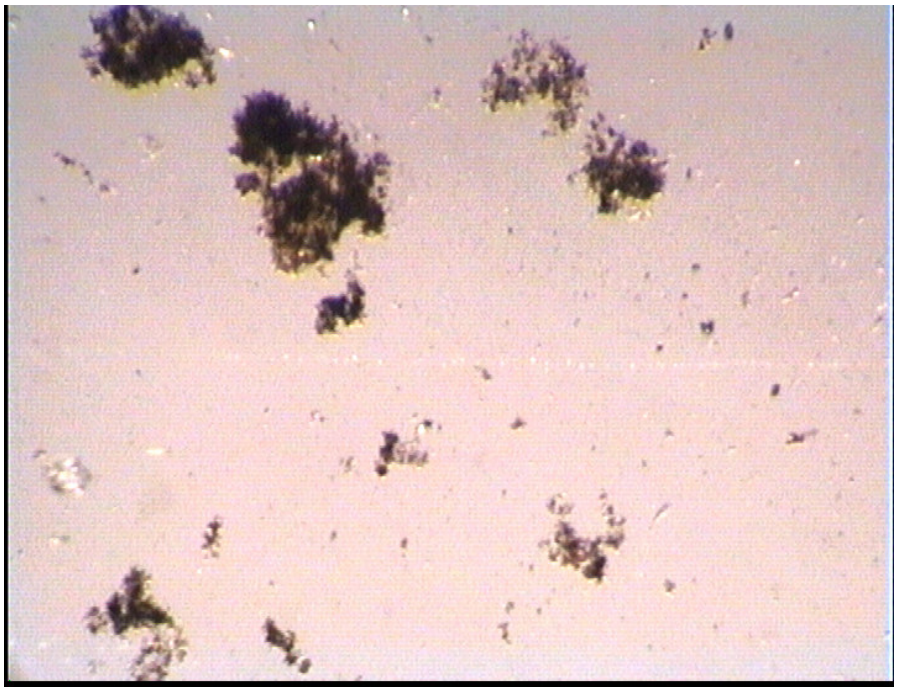


Figure 4.1 Visible aggregates formed in the Rolled condition, present from day 2.

4.2.2 Analyses of bacterial community dynamics and diversity

Triplicate sub-samples (900 µL) for determination of bacterial abundance were stained using SYBR Gold II (Section 2.2.4.2) and cell numbers determined using flow cytometry. Replicate (4 × 1 mL) sub-samples were used to determine productivity using the incorporation of tritiated leucine into cellular protein (Section 2.2.4.2). Bulk bacterial biomass was collected by filtration of 100-250 mL aliquots of incubated samples through 47mm, 0.2µm pore size polycarbonate filters. In addition particles (slurried aggregates) were harvested at each time point for aggregate associated diversity analyses (Section 2.6.1.1). DNA was retrieved from the filters and particles using the CTAB method, extracted with chloroform:iso-amyl alcohol and precipitated with isopropanol (Section 2.6.1). In some cases co-precipitation with Pellet Paint™ was performed to ensure maximum recovery of precipitated DNA. Diversity analyses were performed using PCR-DGGE (Sections 2.4.2 and 2.4.3).

4.2.3 Statistical analyses

Statistical analyses were performed using PRIMER-E (5) software. Briefly, a Bray-Curtis similarity matrix was created and one-way analysis of similarity (ANOSIM) performed to determine the significance of differences between conditions. Descriptive statistics in the Excel package were used to identify trends in bacterial abundance and production. DGGE gels were analysed using Genetool software. Background correction was performed using a rolling disc and the number and peak height of individual bands (operational taxonomic units; OTUs) determined. This information was used to determine relative band intensity and Shannon diversity indices for each sample. Shannon diversity (H') was calculated using the following formula:

$$H' = -\sum (P_i [\ln P_i])$$

When P_i is the proportion of the total peak height contributed by each band (OTU) to the total peak height.

4.3 Results

4.3.1 Effects of incubation technique on bacterial abundance

Bacterial abundance increased in all incubation conditions in the first 24-48 hours (Figure 4.2). In the Stirred and Rolled conditions the increase was described by exponential growth in the first 24 hours ($R^2 = 0.998$ and 0.997 respectively). In the still condition the increase in the first 48 hours was linear ($R^2 = 0.993$). The similarity in trend observed in the Rolled and Still conditions continued for the remainder of the experiment (Figure 4.2) with a decrease followed by a slight increase after which the abundance in both conditions decreased until it was very similar to T_0 values. Bacterial abundance in the Stirred condition, however, was significantly different from the other conditions from 4 days where it peaked, until the end of the incubation. Abundance in the Stirred condition remained high until 7 days, after which it decreased to approximately half the T_0 value. Minimum and maximum abundance were both observed in the Stirred condition ($0.504 \pm 0.001 \times 10^6$ and $3.14 \pm 0.054 \times 10^6$ cells mL^{-1} respectively). No significant difference between conditions was observed over the total duration of the incubation (ANOSIM, P = 0.36, PrimerE analysis of similarity between multiple samples).

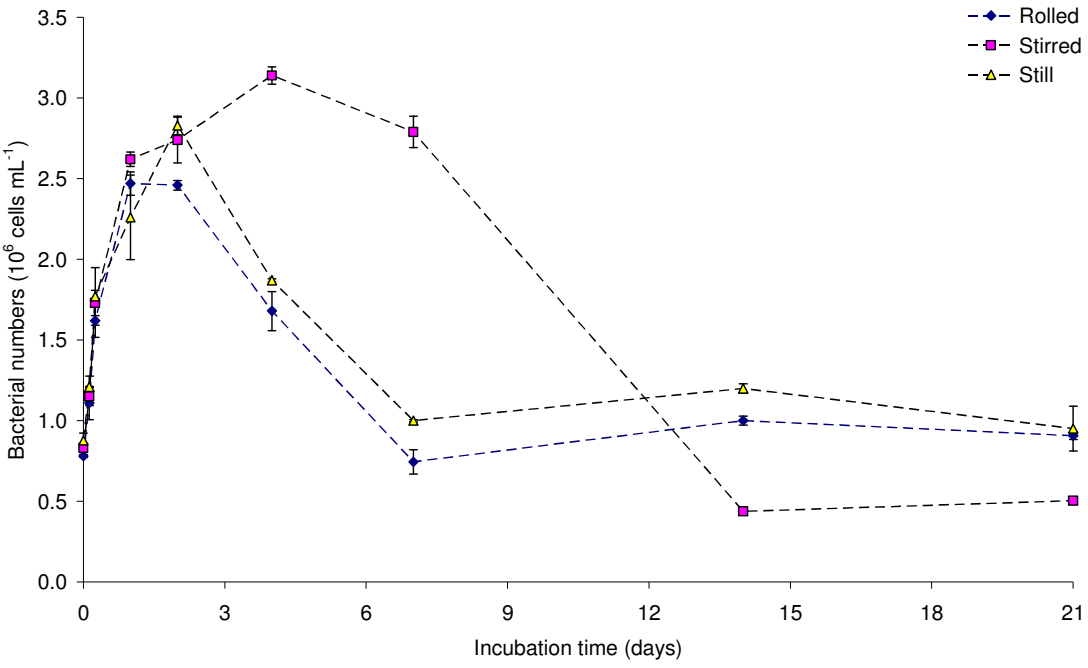


Figure 4.2 Changes in bacterial abundance over time with different incubation strategies. Error bars indicate standard deviation between replicates.

4.3.2 Effects of incubation technique on bacterial productivity

An increase in production was detected in all conditions in the first 1-2 days (Figure 4.3). In the first day (24 hours) production in all conditions increased in the same way, thereafter the pattern of increase was more similar in the Rolled and Stirred conditions up to and including 2 days of incubation. Production increased more rapidly between days 1 and 2 in the Still condition *c.f* the remaining conditions. Following the initial increase bacterial production decreased in both the Rolled and the Still conditions (Figure 4.3) until day 14 at which point the production in the Still condition started to increase and the production in the Rolled condition continued to fall. Production in the Stirred condition remained high until day 7 after which point it decreased to approximately double the 0 hour value.

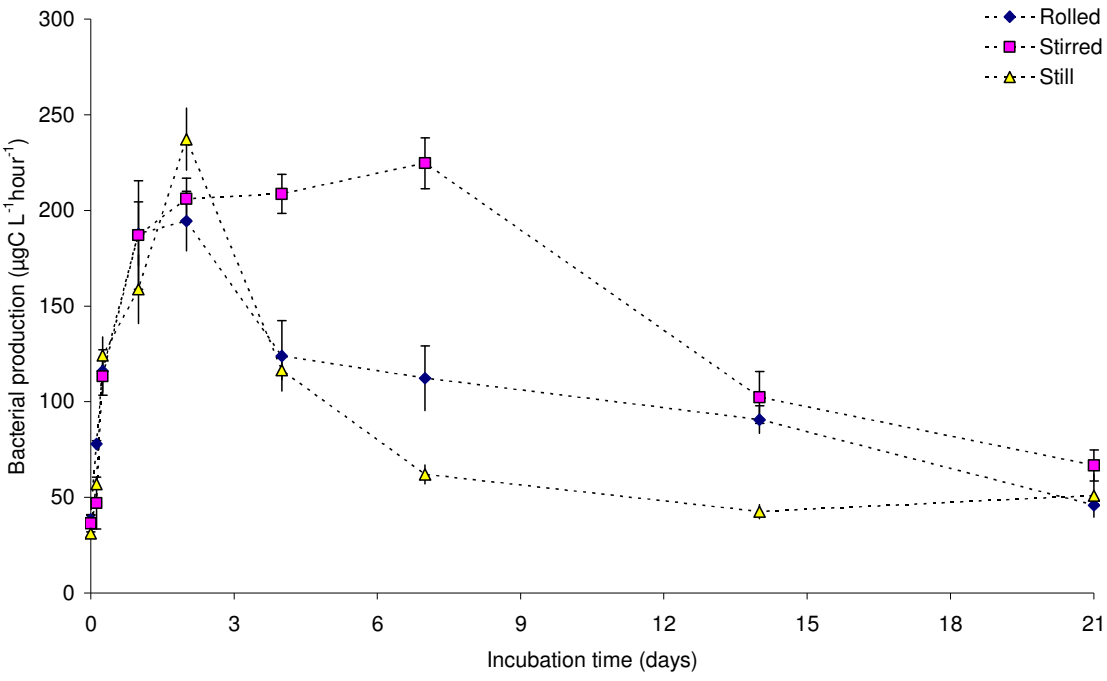


Figure 4.3 Changes in bacterial production over time with different incubation strategies. Points show averages of replicates, error bars indicate standard deviation.

Minimum and maximum production (not including T_0) was observed in the Still condition (31.1 ± 2.0 and 237.3 ± 16.22 µgC. L^{-1} $hour^{-1}$ respectively). No significant

difference was observed between conditions over the duration of the incubation (ANOSIM, P = 0.71). However, production was significantly different between all conditions at 7 days (T-tests, all P values below 0.01).

4.3.3 Normalised bacterial production

When production data is normalised to cell numbers the extent of the effect of incubation technique becomes more apparent (Figure 4.4). Cell specific production was similar in all conditions for the first 4 days of incubation. A rapid initial increase in production per cell was observed in the first 6 hours in all cases. Production rates remained stable and roughly equal (± 15%) between 6 hours and day 4 in all conditions after which the cell specific production trends diverged. Production in the Rolled condition doubled within 72 hours then decreased slowly for the remainder of the incubation. In the Still condition production per cell decreased initially and then varied around the 0 hour value (± 45%) for the remainder of the incubation. Production in the Stirred sample increased exponentially ($R^2 = 0.97$) between day 4 and day 14 after which production decreased until day 21.

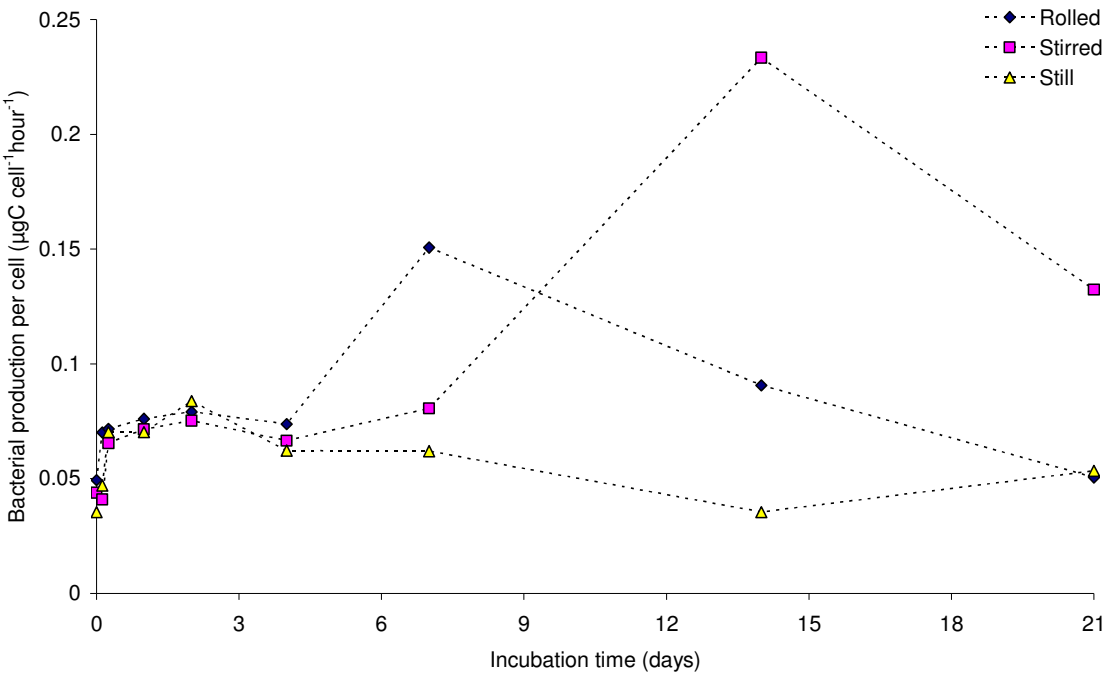


Figure 4.4 Bacterial production per cell over time with different incubation strategies.

Cell specific production integrated over the duration of the experiment (Figure 4.5) showed that bacteria in the Stirred condition were more productive on a cell to cell basis than the bacteria in the Still condition (T-test $P < 0.01$), this was particularly apparent during the latter part of the experiment. No significant difference was observed between the Rolled and Stirred cell specific production over the total duration of the experiment, nor between the Rolled and Still conditions. However when assessed for day 4 onwards a significant difference was detected between the Stirred and Still conditions (T-test, $P<0.05$).

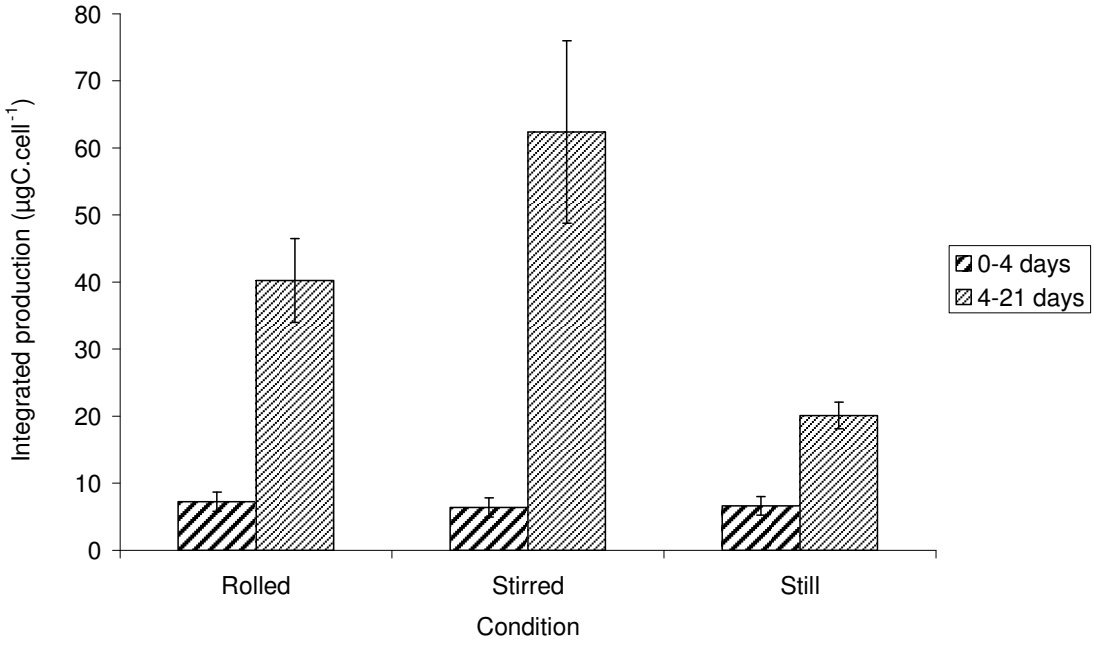
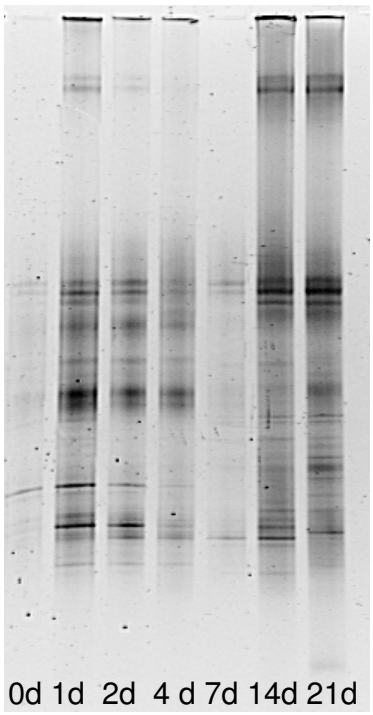


Figure 4.5 Bacterial production normalised to bacterial abundance and integrated over time. Comparison of production between the first four days and the remainder of the experiment (see key for time period represented). Error bars show standard deviation.

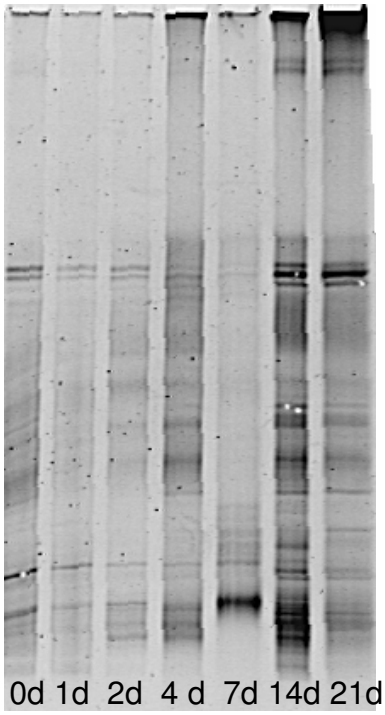
4.3.4 Denaturing gradient gel electrophoresis analyses

DGGE of PCR-amplified DNA was performed to determine changes in diversity over the time-course of the experiment (Figure 4.6). DGGE showed that there was little variation in the number of operational taxonomic units present between 1 and 7 days (Figure 4.6a, b and c). Furthermore, the dominant bands remained the same throughout the majority of the incubation. Diversity increased over time - the average Shannon statistic (diversity, H') was 1.1 at the beginning of the incubation whereas at the end of the incubation the average diversity was 2.76. Comparatively small variations in diversity (H') were observed in the early stages of incubation (between day 1 and day 7) in the Stirred and Still conditions (H' = 1.81 - 2.41 and 2.06 - 2.77 respectively). Diversity indices for samples from the Rolled condition increased from H' = 1.30 to 1.45 (day 1 and day 2 respectively) in the early stages of incubation. However, after 4 days diversity in this condition was almost double (H' 2.25).

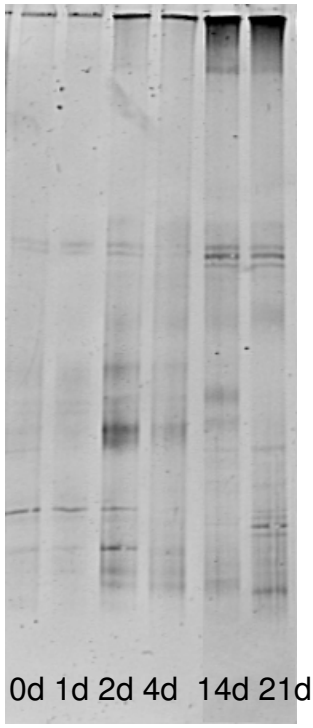
Diversity in the rolling bottle was assessed on a whole water basis (encompassing free-living and attached communities) for the purposes of initial comparisons between incubation methods, in addition, bacterial communities associated with aggregates within the bottle (Figure 4.6d) were assessed. The DGGE profile of the aggregate associated bacterial community appeared to remain fairly consistent throughout the incubation although some variation in relative band intensity was observed (Figure 4.6d). Diversity indices calculated for the bacterial community associated with aggregates at each time point varied between a minimum of H' = 2.35 and maximum of H' = 2.75.



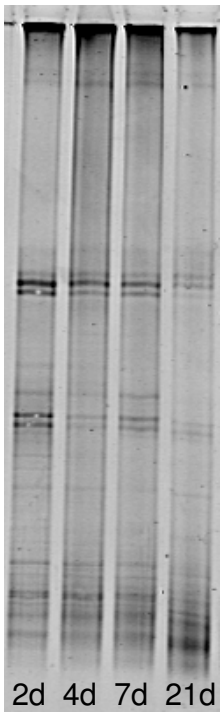
a) Still



b) Stirred



c) Rolled



d) Rolled (aggregate attached)

Figure 4.6 DGGE images showing 0 day to 21 days community profiles for Still, Stirred and Rolled conditions (a-c). DGGE image showing the community profile for bacteria associated with temporarily suspended particles in the Rolled condition (d). Incubation time in days is shown by the text at the bottom of individual gels.

4.4 Discussion

The presence of aggregates is a key feature of many estuarine environments. Incubation strategies simulating the formation of aggregates have been widely used since the seminal use of aggregate rollers by Shanks and Edmondson (1989). However, in light of the effects on bacterial communities attributed to containment of bacteria (Chapter 3), the primary goal of this experiment was to determine changes in bacterial community structure and function induced by such incubation strategies.

4.4.1 Effects of incubation strategy on bacterial community dynamics.

Bacterial abundance (Figure 4.2) followed similar trends in the Still and Rolled conditions. The pattern of abundance suggests an initial affect, possibly due to alleviation of nutrient limitation (Lochte and Turley 1988). This is attributed to phytoplankton mortality arising from incubation in the dark. This initial increase was short-lived in both the Rolled and Still conditions, whereas bacterial abundance not only increased most in the Stirred condition but it also remained elevated for longer than the analogous increase observed in the Rolled and Still conditions. One explanation for this difference comes from the study of effects of turbulence on bacterioplankton. Turbulence has been shown to affect bacterial numbers both directly, via increased nutrient availability (Malits *et al.*, 2004; Delaney 2003) or indirectly, through alleviation of grazing pressure (Peters *et al.*, 2002; Malits *et al.*, 2004). The increase observed in the Rolled and Still conditions occurred after 48 hours suggesting a rapid response to the increased availability of nutrients. The equally rapid decrease (within 24 hours) to levels approximating those observed at 0 hours suggests that the effect was short-lived and that the grazing population was able to respond equally rapidly to increased food availability. This theory is supported by work performed by Beardsley *et al*. (2003) who observed a similar pattern of abundance with confined, untreated bacterioplankton communities. The authors also noted a subsequent increase in heterotrophic nanoflagellates, similar to the grazing population which is likely to be present in our samples. This theory is also supported by the sustained elevation of bacterial abundance in the more turbulent (Stirred) condition previously suggested to have a more relaxed grazing pressure (Peters *et al.*, 2002; Malits *et al.*, 2004). A second explanation for this clear difference between incubation techniques is the putatively more favourable oxygen diffusion conditions resulting from the agitation of the sample. This possibility is supported by the elevated bacterial production observed

in both the Stirred and Rolled conditions *c.f.* the Still condition. Further support for this theory is provided by Ploug (2001) who noted that increased bacterial production values detected in upflow incubations of aquatic aggregates were likely to be a result of favourable diffusion conditions.

Cell specific production (figure 4.4) in the first part of the incubation (up to and including day 4) was similar in all conditions. This could be attributed to the strength of the response to possible increased nutrient levels as a result of confinement and incubation conditions. Only after the effects of this initial nutrient influx had diminished (approximately 4 days) did the longer term differences between conditions become apparent (Figure 4.4). Bacterial production remained comparatively constant in the Still condition whereas an increase in per cell production was observed in both the Rolled and Stirred conditions. In addition to the favourable oxygen diffusion conditions suggested by Ploug (2001) it is well documented that the formation of aggregates can result in increased bacterial production (for a review see Simon *et al.,* 2002). One suggested reason for this phenomenon is the increased production of extracellular enzymes. Visible aggregates (e.g. Figure 4.1) were first seen in the Rolled condition at day 2, and an increase in cell specific bacterial production was observed between days 4 and 7. Bacteria associated with aggregates are able to utilise products resulting from their enzyme activity efficiently during the early stages of aggregate formation, following which the enzymatic digestion of organic matter is thought to provide substrate for free-living bacteria in the surrounding water column (Unanue *et al.,* 1998[a]; Grossat and Ploug 2000). This suggests that the observed increase may have been related to the production of enzymes by aggregate associated bacteria. Furthermore, because bulk water samples were taken for the analysis of bacterial production it is likely that the increased bacterial productivity and hydrolysis associated with aggregate formation (Section 1.3.2.1; Turner 2002) would contribute to the observed increase in production in this condition. It is also likely that the increase in diversity observed after 4 days of incubation in the Rolled condition was linked to the formation of aggregates.

4.4.2 *Diversity change over time*

4.4.2.1 Incubation effects

Little change in diversity (Figure 4.6) was detected in the early stages of incubation (up to day 7) in the Still and Stirred conditions. Massana *et al.* (2001) observed a decrease in bacterial assemblage diversity following a ten day incubation having removed grazers from the initial samples by filtration. Bactivorous grazers have been shown to play an important role in controlling dominance of certain groups of bacteria (Gasol *et al.,* 1999) and it is thus not surprising that the authors found the final bacterial assemblages to be dominated by rapidly growing opportunistic organisms. A similar phenomenon has been observed as a result of substrate enrichment (Pernthaler *et al.,* 2001), suggesting another mechanism of community change resulting from pre-filtration. The samples from the incubation experiment described here were not pre-filtered and thus were not exposed to such selective pressures in the early stages of incubation. Bacterial diversity in the Rolled samples increased at day 4 when compared to the remaining conditions, it is possible that the formation of aggregates contributed to this increase by providing a suitable surface for bacterial attachment. This would enable biofilm forming bacteria to become more dominant and thus detectable by PCR-DGGE.

Towards the end of the incubation (days 14 and 21) diversity was seen to increase in all conditions with the dominant phylotypes remaining fairly constant. The long term nature of the increase in diversity suggests that it may result from increased numbers of slow growing bacteria to detectable levels. Certainly in both the Rolled and Stirred conditions the homogenous distribution of nutrients throughout the sample would support the growth of these types of bacteria (Pernthaler *et al.,* 2001). One widely acknowledged flaw in the use of DGGE as a tool for analysing diversity is the inability to detect numerically minor parts of the community (<0.5-1% of the community, Muyzer *et al.*,, 1993; Casamayor *et al.*,, 2000). The constancy of the dominant bands further supports the suggestion that the variation in diversity was attributable to the minor components of the bacterial community.

Throughout the incubation the dominant bands remained constant (Figure 4.6), the constancy observed may be a result of the elevated metal concentrations observed in this estuary exerting a more powerful selection pressure than the effects of confinement or indeed incubation strategy. Incubation experiments performed to assess the effects

of metal enrichment on bacterial communities have historically resulted in decrease of phylogenetic and/or phenotypic diversity (Hemida *et al.* 1997; Baath *et al.*, 1998[a,b] ; Kelly *et al*., 1999; Stephen *et al.*, 1999; Sandaa *et al.,* 2001). This supports the suggestion that the effects of the incubation strategies employed here have less impact on bacterial community than is expected to occur with metal enrichment. Furthermore, because the initial affect of substrate enrichment is believed to be short-lived (<2 days), longer term enrichment incubations should enable valid conclusions about the effects of trace metals on bacterial communities to be drawn.

4.4.2.2 Bacterial community fingerprints associated with particles from rolling bottles

Denaturing gradient gel analysis of the bacterial community associated with temporarily suspended particles showed a more stable bacterial community fingerprint in the aggregate attached portion of the community. Although a change in relative band intensity was observed, which implies a shift in dominant phylotypes, diversity indices stayed fairly constant (H' = 2.35-2.75) throughout the incubation. A similar phenomenon has been observed *in situ* (Crump *et al.,* 1999) where 75% of clones associated with estuarine aggregates were specific to that environment whereas a similar proportion of the free-living bacterial community consisted of a mixture of marine and terrestrially derived clones. The authors suggested that this could be attributed to the physical dynamics of such particles within estuaries. An alternative reason for the small variation in diversity over time observed here could be the mechanisms by which bacteria locate and attach to aggregates. The micro-environments associated with aggregates provide comparatively stable conditions in terms of substrate availability (when compared to patchy nutrient availability in aquatic environments). Thus following a period of community development, little change in community is apparent over short time scales. Longer term studies, however, have shown a shift in the bacterial community on aggregates as the organic matter associated with them becomes more refractory (Grossart and Ploug 2000). The small variations observed here support the suggestion that the effects of incubation and indeed substrate type are likely to exert a smaller selection pressure than the established effects of metal enrichment (Chapters 5 and 6).

4.5 Conclusions

The results of this study demonstrated a marked increase in both bacterial abundance and productivity at the beginning of the incubation, probably a result of confinement and incubation in the dark. It was also shown that stirring of the sample resulted in enhanced productivity and numbers for a longer duration than the initial increase, a phenomenon attributed to a more favourable oxygen distribution.

Whilst a change in bacterial community composition and community dynamics were observed as a result of incubation technique, our results showed that the dominant bacterial community (particularly within aggregates) was relatively stable for the duration of the experiment. Some change in bacterial community would be expected to occur over such time scales as that employed here even *in situ*. Furthermore, because the remainder of this study investigates the effects of metals on the bacterial community the effects conferred by metal toxicity are expected to outweigh any changes occurring as a result of confinement. These conclusions suggest that the incubation of experimental samples on aggregate rollers is a suitable approach for the investigation of effects of trace metals on natural bacterioplankton communities. It has also become apparent that the role of aggregates in mediating the response of bacterial communities to change should be investigated with regards to trace metals. In addition this study supports suggestions (Chapter 3) that the length of the incubation may play a crucial role in the assessment of trace metal effects and suggests that the incubations performed be no shorter than 7 days to elucidate changes in the less dominant bacterial community.

5 Can the bacterial community from a pristine estuarine environment adapt to changes in trace metal concentration?

5.1 Introduction

5.1.1 Importance of bacteria in estuarine systems

Estuarine systems are biologically, chemically and physically dynamic (Almeida *et al.*, 2002, Uncles *et al.* 2002). Constant changes in salinity and nutrient availability occur over tidal cycles as a result of saline intrusion, and seasonal cycles due to river flow variability (Josselyn and West, 1985). Bacterial communities in estuaries can be divided into two categories, as demonstrated by work performed in the Columbia River Estuary (Crump *et al*., 1999). The first are the free-living bacteria, a highly variable portion of the community influenced by influx of bacteria from coastal, riverine and terrestrial sources which can account for as much as 48% of the diversity of the free-living community (Crump *et al.* 1999). The second, comparatively stable, portion of the estuarine community is made up of bacteria associated with aggregates (Crump *et al.* 1999). Phylotypes associated with estuarine aggregates in the Columbia River Estuary tended to be poorly represented in either the particle attached or free-living bacteria from the riverine and marine communities (Crump *et al.* 1999). Whilst this indicates the development of communities specific to aggregate environments, these two communities are not distinct and aggregate associated bacteria can also contribute to the free-living communities via disaggregation and detachment processes (and vice versa) (Riemann and Winding 2001). Aggregates are created by shear forces within estuaries (Law *et al*., 1997) and are believed to enhance bacterial numbers and production (Crump and Baross 2000). In estuaries with high particulate loadings an estimated 90% of the bacterial community can be associated with aggregates (Simon *et al.,* 2002). These authors have also suggested that more than 30% of total bacterial production in estuaries is consistently associated with aggregates. The sinking and re-suspension of aggregates in the tidal cycle extends their residence time in the estuarine system (Jay and Musiak, 1994). Consequently the particulate organic matter, nutrients and associated colonies of bacteria and microzooplankton remain in the estuary for longer periods (Crump and Baross 2000; Uncles *et al.* 2002).

Bacterial activity is believed to enhance remineralisation processes on aggregates (Smith *et al.,* 1992; Turley 1992) via the production of extracellular enzymes which break down the biological 'glue' (e.g. transparent exopolymeric particles) responsible for enhancing aggregation (Smith *et al.* 1992; Grossart and Simon 1998; Engel 2000). The production of extracellular enzymes such as aminopeptidases, phosphatases and glucosidases results in the rapid hydrolysis of aggregate associated biopolymers and can account for up to 87% of the decrease in particulate organic nitrogen (Grossart and Ploug 2001) observed in estuaries as a result of biological activity. The reduction in stickiness, as a result of enzyme activity, may prevent further aggregation or result in the disaggregation of large particles. The latter may extend the length of time aggregates remain suspended in the water column. These processes also provide substrate for free-living bacteria (Grossart and Simon, 1998; Kiorboe *et al.* 2001; Kiorboe and Jackson 2001), and food for the estuarine grazing community (Alldredge and Silver 1988). The extended residence time of particles facilitates the bacterial remineralisation of a significant proportion of the organic matter in estuarine systems before it meets the sea. Furthermore, aggregate-associated bacteria enhance the ability of an estuary to support higher trophic levels (Lampitt *et al.* 1993, Grossart *et al.* 1998) and are responsible for significant recycling of organic matter in such environments.

5.1.2 Relationships between bacteria and trace metals

Investigations into the relationship between bacteria and trace metals have concentrated predominantly on single cultures of bacteria (for a review see Nies, 1999) or specific environments such as contaminated sediments (Cummings *et al*., 2003), sewerage sludge treatment plants (Rudd *et al.* 1984) or sewage treated agricultural soils (Moffett *et al.*, 2003) which are historically metal impacted. In addition, extensive research has been carried out to determine the action and identification of bacteria relevant to acid mine drainage and tailings treatment (Garcia *et al*. 2001; Bhagat *et al*. 2004) where the influence of bacteria can have commercial advantages for both retrieval (Rawlings 2002; Chen, 2004) and remediation (Barkay and Schaefer 2001; Valls and de Lorenzo 2002; Gadd 2004) of metals. Bacteria which are renowned for their ability to remove metals from solution such as Sulphate Reducing Bacteria (SRB) have been studied extensively and information regarding their mechanism(s) of action is widely available (e.g. Garcia *et al*. 2001; Bhagat *et al*. 2004). Conversely, the ability

of bacteria found in natural aquatic environments to tolerate and/or respond to an influx of elevated concentrations of trace metals is largely unknown, despite the obvious ecological importance.

Although a number of metals are required for the effective functioning of bacterial cells (Madigan *et al.* 2003), the presence of functional metals (in high concentrations) or non-functional metals can be detrimental or indeed toxic to bacteria (e.g. Knight *et al*., 1997; Stephen *et al*., 1999) and have been shown to influence a number of microbially mediated processes (Babich and Stotzky, 1985). Modes of toxicity include displacement or substitution of non-functional metal ions for functional ions, resulting in compromise of cell membranes, enzyme function and nutrient transport systems (Sunda, 1988; Bruland *et al.,* 1991). A second mode of toxicity is the alteration of the redox state of the cell resulting in oxidative stress (Bruins *et al*., 2000).

5.1.3 The Erme estuary

The Erme estuary located in the South Hams of Devon is a pristine environment named as one of the four cleanest estuaries (with regard to trace metals) in the U.K. (EA report 2001). It has been designated as an area of outstanding natural beauty (AONB) and is classified as a site of special scientific interest (SSSI) (www.southdevonaonb.org.uk). The only large discharges into the estuary originate from Holbeton sewerage works, which were updated recently to include secondary treatment and ultraviolet disinfection. A smaller input originates from the Flete estate which surrounds the estuary. The estuary is described as a 'drowned ria' and has large expanses of sandy bed exposed at low tide. A saline intrusion extends approximately 5.6 km upriver.

The main goal of this research was to examine the response of bacteria from a 'pristine' environment to exposure to comparatively high concentrations of trace metals. The response has been characterised in terms of change in bacterial numbers, production, and phylogenetic diversity.

5.2 Methods

5.2.1 Water collection and manipulation

Samples were collected from the Erme Estuary (Devon, UK) at a salinity of 14.9. At the time of sampling (June, 2003) water temperature was 16.6°C and pH was 7.8. The estuarine water was collected directly into a large volume container (40 L) and distributed into eight (4 L) polycarbonate bottles. The bottles were transported back to the laboratory and transferred (within 2 hours of sampling) to a constant temperature room set to ambient estuarine water temperature (16.6°C) and allowed to equilibrate (approx 1 hour).

5.2.2 Metal addition

The addition of trace metals to water samples taken from the Erme Estuary was designed to be representative of the concentrations detected in heavily contaminated estuarine systems such as the Fal Estuary (Chapter 4). Trace metals (Spectrosol standards 1000 mg.mL^{-1}) were added to the sample bottles in the concentrations and volumes shown in Table 5.1. Bottles were mixed by repeated inversion and the pH adjusted to ambient estuarine equivalent (7.8) with NaOH where necessary. A final

Condition	1	2	3	4	5	6	7	8
Metal (nmoles.L^{-1})	Control (0)	Zn^{2+} (1000)	Zn^{2+} (15000)	Cd^{2+} (18)	Cu^{2+} (950)	Ni^{2+} (200)	Pb^{2+} (300)	Zn^{2+} (15000) Cd^{2+} (18) Cu^{2+} (950) Ni^{2+} (200) Pb^{2+} (300)
Volume of standard added (µL)	0	261.6	3 920	8.1	248.4	47	248.6	As per conditions 3-7
pH adjustment	no	no	yes	no	no	no	no	yes

Table 5.1 Conditions 1-8 showing type and species of metal added to each bottle. Concentration increase in nmoles L^{-1} given in parentheses. Condition 3 (15000 nM Zn^{2+}) was selected to reflect highly contaminated conditions with regard to zinc. Condition 8 will be described as the 'cocktail' hereafter.

bottle was left unenriched as a control. Sub-samples (section 2.1.2) were taken immediately for analysis of initial parameters. The bottles were incubated on aggregate rollers at ambient temperature (16±1°C) in the dark for a total of 21 days. Sub-samples were collected at eight time-points during that period.

5.2.3 Sub-sampling and analyses

Triplicate aliquots of 1 mL were fixed with gluteraldehyde (2.5% final concentration) and stored at -20°C for determination of bacterial numbers. Enumeration was achieved using flow cytometry (Section 2.5.1). Bacterial production was determined using incorporation of ^{3}H-leucine into protein (Section 2.5.2).

Bacterial biomass was collected by filtration of 250 mL aliquots of incubated samples through 47mm, 0.2µm pore size polycarbonate filters. DNA was retrieved from the filters using the CTAB method, extracted with chloroform:iso-amyl alcohol and precipitated with isopropanol (Section 2.6.1). The resulting DNA was amplified using nested PCR with the external primers 8f and 1346r and internal primers 341f (GC) and 907r (Section 2.6.2). DNA fragments were analysed by DGGE (Section 2.6.3). Bands of interest were extracted from the gel (section 2.6.3), cloned and sequenced (sections 2.4.4 and 2.4.5 respectively) and sequences submitted to the BLAST search programme of the NCBI website to ascertain closest matches. Chimeric sequences were identified using the RDP-II check chimera facility and excluded from further analyses. Phylogenetic analysis and tree construction was accomplished using the RDP-II website Phylip interface.

Aggregates were collected using a wide bore 10 mL pipette tip and allowed to settle into a 90mm Petri dish containing sample water. Digital photographs were taken and analysed using image analysis of parameters pertaining to aggregate size and shape (Image ProPlus imaging system).

T-tests were performed to determine the significance of differences between conditions (Minitab). A similarity matrix was created using bacterial production and abundance data which was then subjected to multi-dimensional scaling (MDS) to examine the relationship between samples. MDS is an ordination technique which separates samples on the basis of the level of similarity (i.e. the distance between samples) which is determined using the Bray Curtis similarity co-efficient. MDS and similarity calculations were performed using PRIMER-E software.

5.3 Results

5.3.1 The effect of trace metal addition on bacterial numbers

The response to metal addition elicited two modes of observed response:

- **Type 1** response to metal addition was broadly similar to the control (Figure 5.1). Bacterial numbers in the control and condition 2 (1 µM Zn^{2+}), 4 (Cd^{2+}) and 6 (Ni^{2+}) either increased or remained static in the first 6 hours of the incubation and were maximal at day 3. Bacterial numbers decreased to levels approximating those observed at T_0 in all conditions (except for condition 4 (Cd^{2+}) in which bacteria were approximately half as abundant) in the latter stages of the incubation.
- **Type 2** is characterised by the increase in bacterial numbers in conditions 3 (15 µM Zn^{2+}), 5 (Cu^{2+}), 7 (Pb^{2+}) and 8 (cocktail) towards the end of the incubation (Figure 5.2). Bacteria in Type 2 samples either decreased in abundance or remained at approximately T_0 values in the first 24 hours of incubation.

An increase in abundance from T_0 was observed within the first three days of incubation in all cases (Figures 5.1 and 5.2). The pattern preceding this increase varied between conditions. In condition 2 (1 µM Zn^{2+}) an initial increase in numbers was followed by a decrease and a second increase of greater magnitude than the first. This closely resembled the trend observed in the control condition (Figure 5.1). In conditions 4(Cd^{2+}), 6 (Ni^{2+}) and 7 (Pb^{2+}) (Figures 5.1, 5.1 and 5.2 respectively) a single peak in abundance was observed. Thirdly in conditions 3 (15 µM Zn^{2+}), 5 (Cu^{2+}) and 8 (cocktail) an initial decrease was observed in the first 24 hours (Figure 5.2). In the latter stages of the incubation (from day fourteen) bacterial abundance decreased (Type 1) or increased (Type 2) to levels approximating the T_0 value.

Bacterial numbers ranged from 1.2 ± 0.05 to 6.84 ± 0.66 x10^6 mL^{-1} in control incubations. Overall the minimum bacterial numbers were observed in the cocktail at day eleven (0.15 ± 0.03 x 10^6 mL^{-1}) and maximum numbers were achieved in the 15 µM Zn condition at day three (7.19 ± 0.72 x 10^6 mL^{-1}), both of which displayed Type 2 patterns of abundance. Minimum and maximum values observed in Type 1 samples were 1.13 ± 0.14 x 10^6.mL^{-1} (Condition 4, Cd^{2+}) and 5.98 ± 0.60 x 10^6 mL^{-1} (Condition 6, Ni^{2+}) respectively. Bacterial numbers in conditions 2 (Zn^{2+} [1 µM]) and 8 (cocktail) were significantly lower than the control ($P<0.05$).

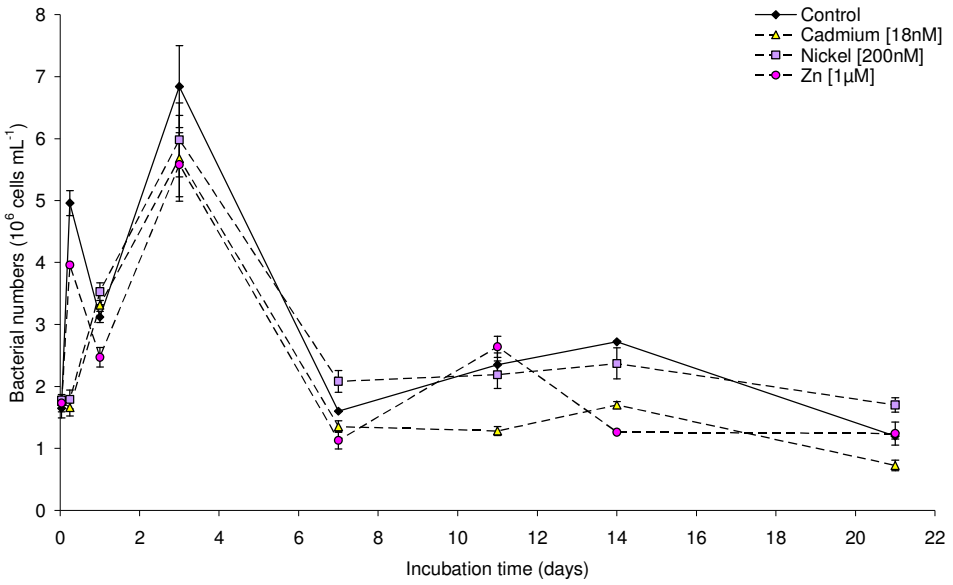


Figure 5.1 Type 1 effects of metal addition on bacterial abundance patterns over the duration of the incubation experiment. Points are averages of replicate measurements. Error bars show standard deviation.

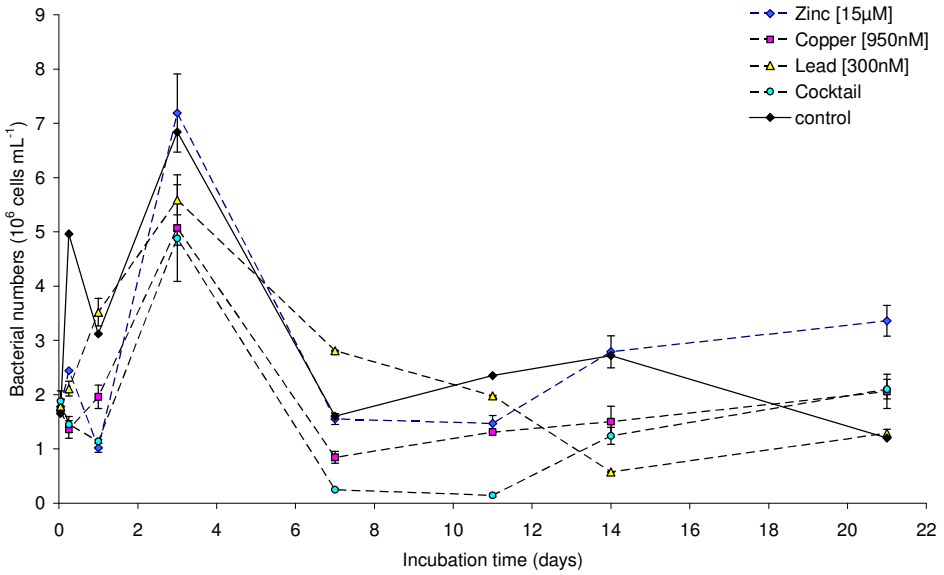


Figure 5.2 Type 2 effects of metal addition on bacterial abundance patterns over the duration of the incubation experiment. Points are averages of replicate measurements. Error bars show standard deviation.

5.3.2 The effect of trace metal addition on bacterial production

The same distinct responses were apparent in the change in bacterial productivity in response to metal addition. Bacteria displaying a Type 1 response reached maximum production, or within 5% of their maximum productivity, in the first 24 hours (Figure 5.3). Bacteria from the Type 2 group demonstrated a peak in productivity at day 3 following varying degrees of inhibition in the first 24 hours of the incubation.

Bacterial production in control incubations ranged from an initial rate of 205.1 (±16.0) µg $C.L^{-1}.d^{-1}$ to a maximum of 2216.5 (±184.5) µg $C.L^{-1}.d^{-1}$ and a subsequent minimum of 37.8 (±5.0) µg $C.L^{-1}.d^{-1}$ in the final stage (21 days) of the incubation. This minimum at 21 days was also observed in all Type 1 conditions and the Pb^{2+} (300 nM) condition (Type 2).

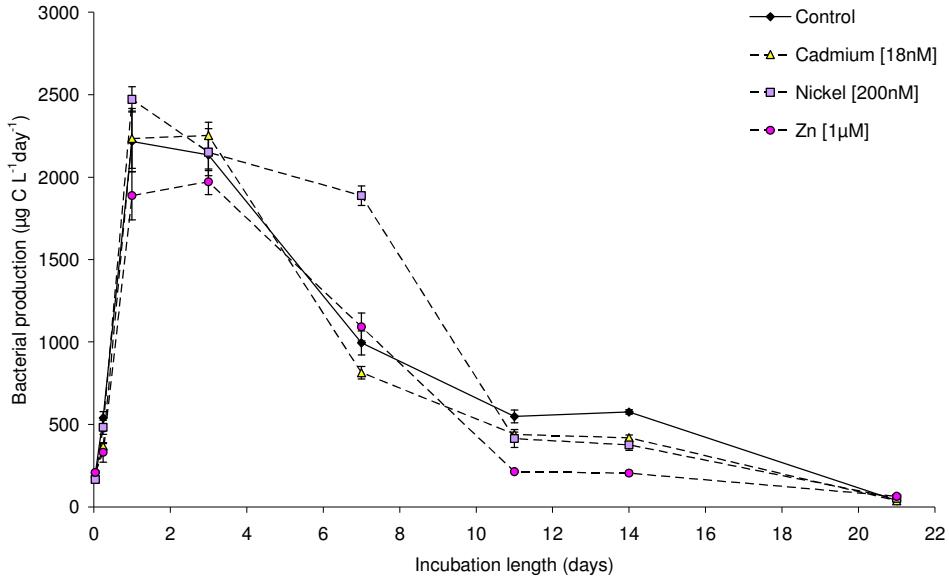


Figure 5.3 Type 1 effects of metal addition on bacterial production over the duration of the incubation experiment. Points are averages of replicate measurements. Error bars show standard deviation.

In the first 24 hours of incubation the response of the bacterial community to Type 2 metal addition was clearly more profound than to any of the Type 1 metals (Figure 5.4). No increase in production was observed in the first 6 hours in the Zn^{2+} (15 µM), Cu^{2+} or cocktail conditions *c.f.* >100% increase in the control condition (inset, Figure 5.4; Lag phase Table 5.2). After 1 day of incubation production in the Zn^{2+} (15

µM) and Cu^{2+} conditions had increased to 44% and 60% of the control value respectively, in contrast production in the cocktail condition at this point had not increased (Table 5.2). After 3 days of incubation production maxima in the Pb^{2+}, Zn^{2+} (15 µM) and Cu^{2+} conditions exceeded that of the control condition whereas production in the cocktail had reached a maximum of 60% of the control value.

Condition	Control	Zinc (15µM)	Copper	Lead	Cocktail
Length of lag phase (days)	0	0.25	0.25	0	1
Production at day 1 (% of control production)	100	44	60	87	1

Table 5.2 Type 2 bacterial production in the first 24 hours of incubation.

Following the peak in production at day 3 a decrease was observed in all conditions except for the cocktail. Notably, the rate of decrease in bacterial production between days 3 and 11 was slower (*c.f.* control) in the Ni^{2+} condition (Type 1) and to a lesser extent in the Pb^{2+} condition.

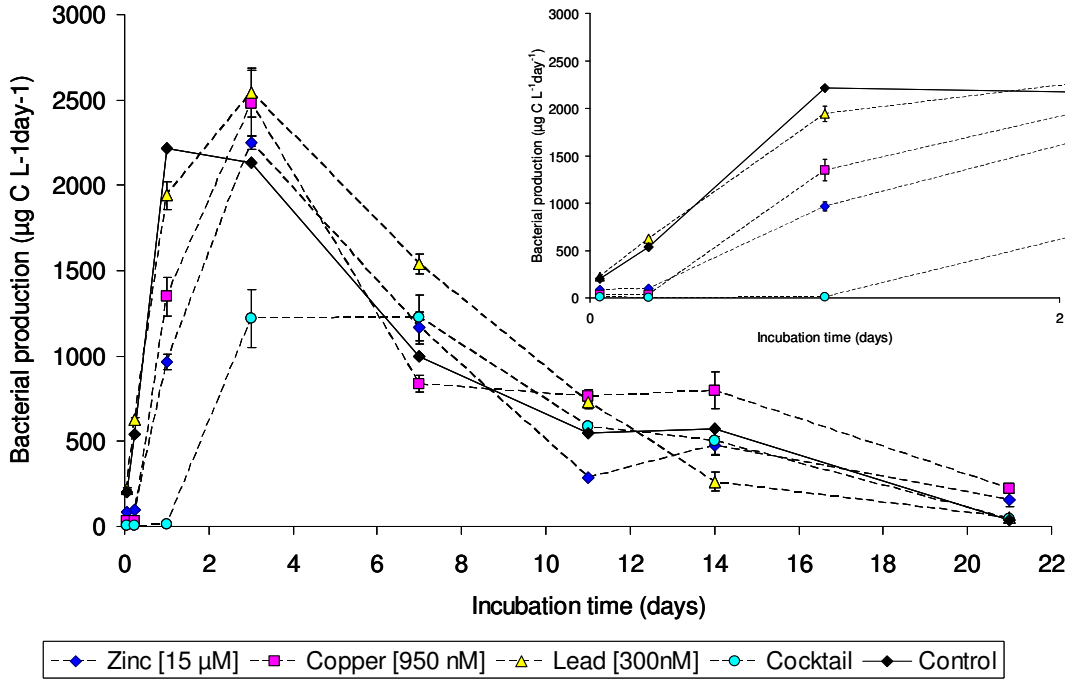


Figure 5.4 Type 2 effects of metal addition on bacterial production over the duration of the incubation experiment. Inset shows the first 3 days of incubation. Points are averages of replicate measurements. Error bars show standard deviation.

The minimum and maximum productivity was observed in type 2 samples (as observed in bacterial numbers). The minimum production rate was observed in the cocktail (7.3 ±1.9 µgC.L^{-1}.d^{-1}) and maximum production (2545.2 ±144.5 µg C L^{-1} d^{-1}) in the 300 nM Pb^{2+} condition.

5.3.3 The relative importance of metal additions / incubation effects

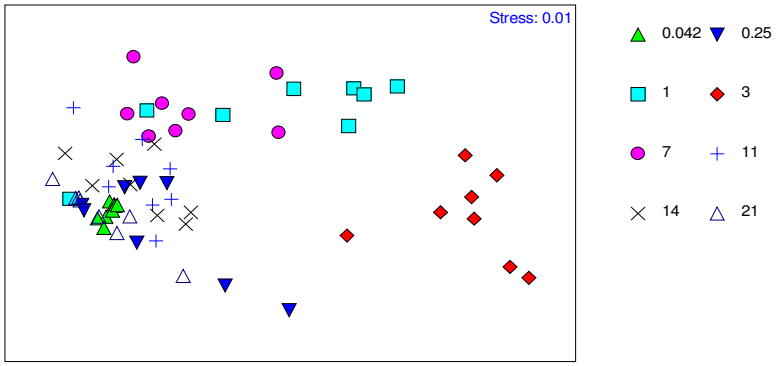
The measured bacterial abundance and production parameters were used to create ordination plots to assess the degree of similarity between conditions. Time of incubation was shown to be the most important factor when describing the similarity between conditions (Figure 5.5). At T_0 (immediately following metal addition) all conditions plotted within the same cluster. At 6 hours the control and condition 2 (1µM Zn^{2+}) samples differed from the remainder of the conditions. At day 1 samples from conditions 3 (15µM Zn^{2+}), 5 (Cu^{2+}) and 8 (cocktail) clustered separately, these samples displayed the Type 2 response in terms of bacterial production and numbers. At day 3 the majority of conditions were closely related with the notable exception of condition 8 (cocktail) which was consistently different to the other conditions from this point until the end of the incubation (Figure 5.5). After seven days conditions 6 (Ni^{2+}), 7 (Pb^{2+}) and 8 (cocktail) plotted separately from the remainder of conditions. From day 14 onwards most samples grouped closely together. The samples which showed the greatest difference to the control (as highlighted by MDS analysis) were selected from samples taken at three and seven days for further investigation focusing on bacterial diversity (DGGE).

5.3.4 Denaturing gradient gel electrophoresis of samples showing the greatest response to trace metal enrichment.

Denaturing gradient gel electrophoresis was performed to identify the bacterial populations present in samples which were distinct from time groupings (see above). The gel image (Figure 5.6) shows the dominant bands (phylotypes) in these samples. At day three, a particularly tight MDS cluster (Figure 5.5), the bacterial community from condition 8 (cocktail) was clearly different to the control and condition 3 (15 µM Zn^{2+}) communities. Dominant bands observed in the upper half of the control lane became insignificant in the cocktail condition. The community which emerged in the cocktail condition after 3 days was dominated by a number of phylotypes observed with low intensity in the control condition (bands 1, 2, 3 and 5, cocktail, day 3). At day 7

samples from conditions 6 (Ni^{2+}), 7 (Pb^{2+}) and 8 (cocktail) were distinct from time grouping of samples (Figure 5.5 A). However at this time differences between sample and control DGGE profiles were only observed in the less dominant bands. Bands indicated were excised for sequencing to identify the dominant phylotypes.

A



B

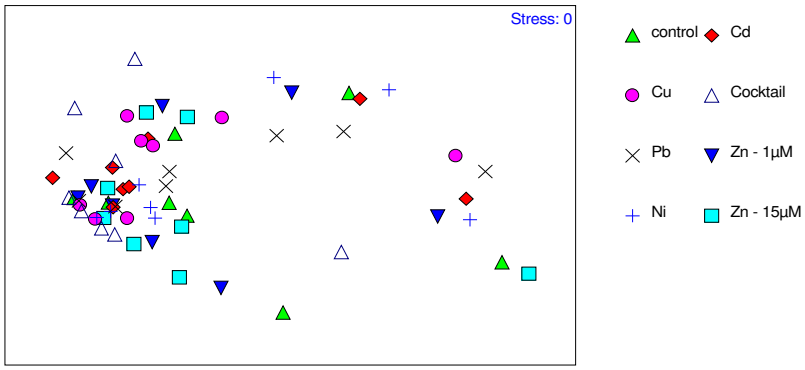
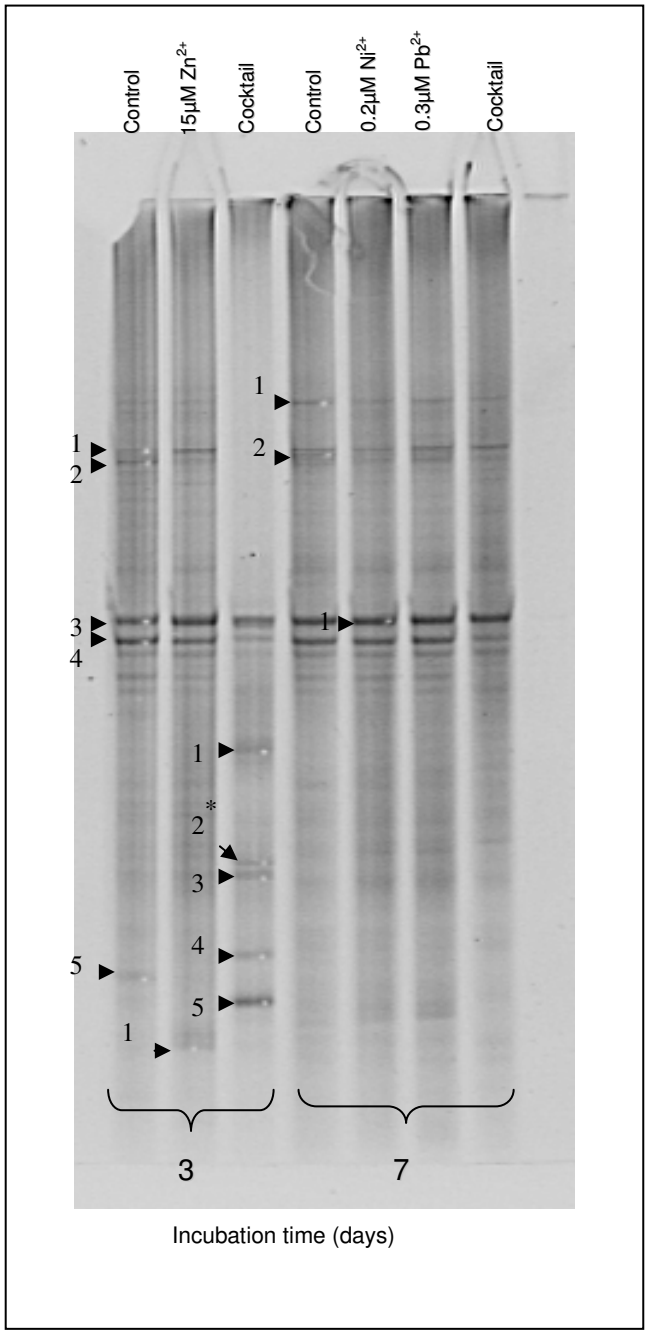


Figure 5.5 Multi dimensional scaling (MDS) plots showing spatial representation of the relationship between samples. The spatial arrangement i.e. distance between points is based on differences in production and numbers between samples taken from each time point in hours (A) and from each condition (B). The points on each plot are the same and the symbols represent either the time or the condition (shown in the key).



The stress levels show the likelihood of any other ordination result based on the number of iterations performed.

Figure 5.6 DGGE profiles of samples showing greatest response to trace metal enrichment. Image labels show conditions and incubation time, arrows show bands taken for phylogenetic analysis. Note, cocktail, 3 days, band 2 (*) was identified as a possible chimera and not included in further analysis.

All bands indicated in Figure 5.6 were successfully sequenced. One band (Cocktail, T3, band 2) was identified as a possible chimera and as such has not been included in these analyses. All remaining sequences belonged to a single group within the alpha-proteobacteria family; the Rhodobacteraceae (Figure 5.7). Dominant phylotypes after 3 days in condition 8 were shown to be most closely related to *Ruegeria* and uncultured marine alpha proteobacteria by BLAST analysis (Altschul *et al*., 1990).

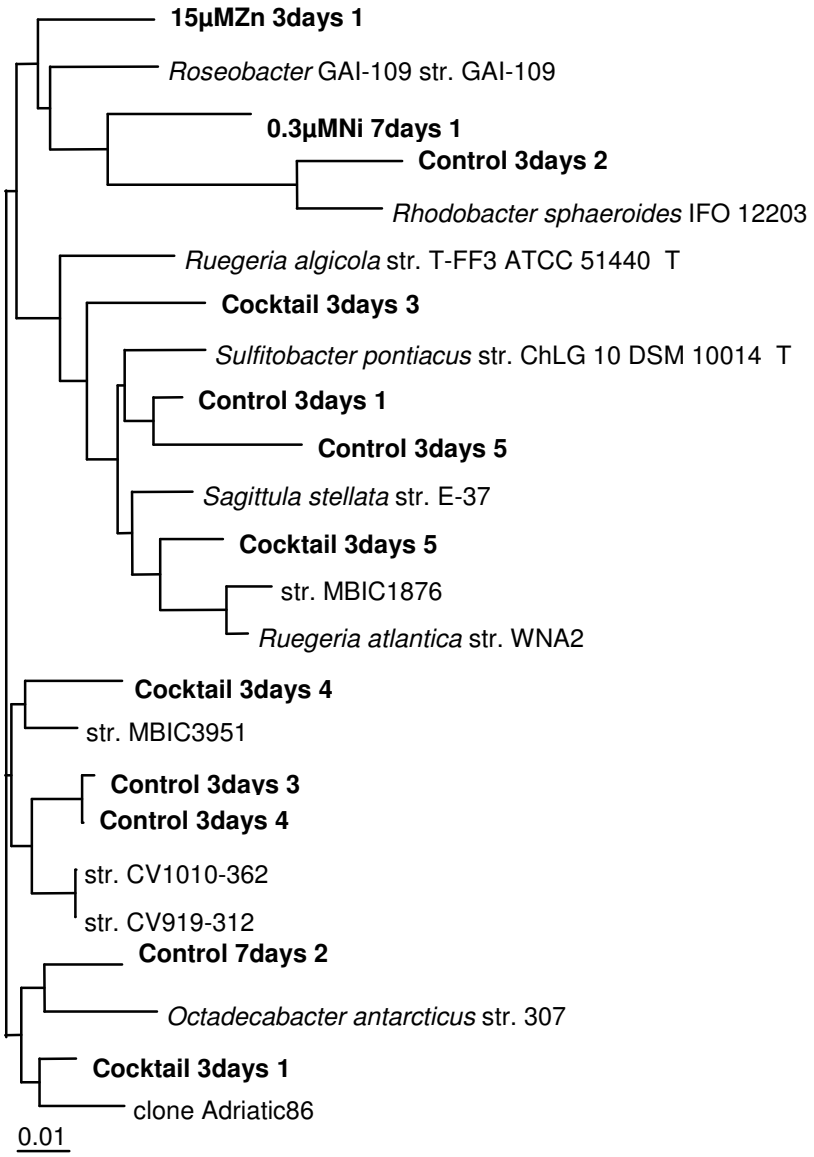


Figure 5.7 Phylogenetic tree showing the relationship between bands selected from samples showing greatest response to trace metal enrichment. Scale bar shows substitutions per base. Labels refer to condition, duration and DGGE band number.

5.3.5 The effect of metal addition on aggregate formation

The appearance of aggregates was different with the addition of different metals (Figure 5.8). Image analysis showed that the size distribution parameters and shape (roundness) were the parameters affected most by the addition of metals. In all metal enrichments the percentage of large particles (>1000µm) was lower than in the control. Roundness of the particles was similar in the control and most metal conditions (Figure 5.8 A-G), however with the addition of a cocktail of metals the roundness was much higher (approximately 40 ×) than the control and remaining conditions (Pers. comm. J. Dixon, PML).

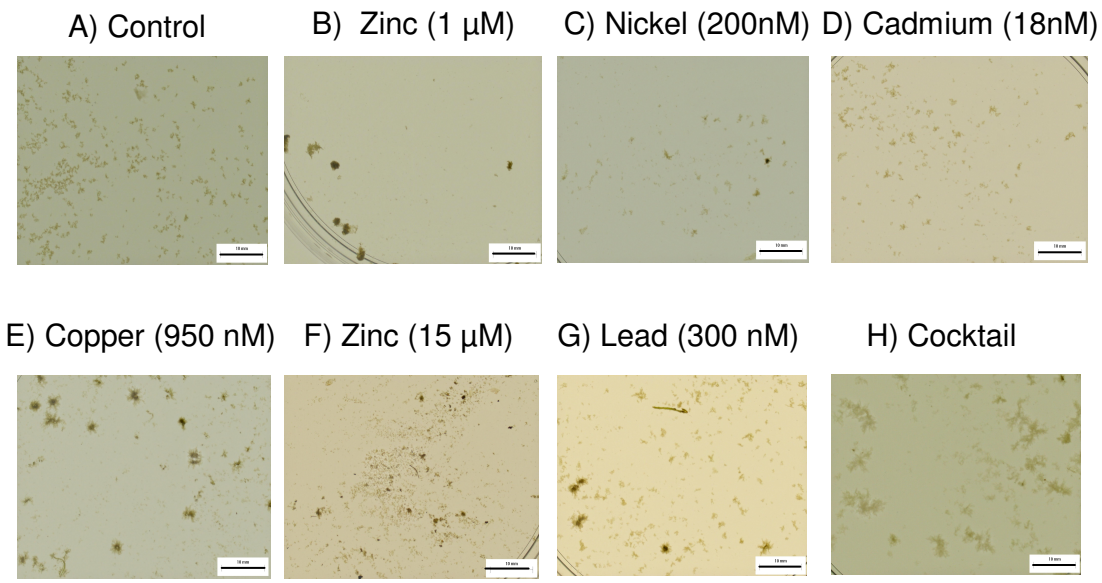


Figure 5.8 Images of aggregates formed after 5 days incubation. Scale bars show 10mm.

5.4 Discussion

The effect of a suite of trace metals on bacterial communities from a pristine estuary was determined. Effects were identified based on changes observed in community dynamics and diversity of the bacterioplankton in response to metal enrichment. As was observed in both chapters 3 and 4 an immediate increase in bacterial numbers was apparent, presumably in response to confinement (see Sections 3.4.2.3 and 4.4). Nevertheless, as concluded previously, the differences observed between the experimental conditions in the corresponding time period (0-2 days) indicate differential response depending on metal added. This emphasises the impact of metal enrichment on the ability of the bacterial community to take advantage of the presumptive pulse of nutrients. The remainder of this discussion accounts for the differences observed between metal enriched bacterial populations on short and long time scales.

5.4.1 Community dynamics of bacteria exposed to metals.

Analyses of time series data have suggested two distinct patterns of response to metal enrichment in terms of bacterial community dynamics. These are referred to in the results section as Type 1 and Type 2, and appear to be differentiated by the toxicity level of the metals to which they were exposed. Dynamics in the Type 1 conditions show greater similarity to the control conditions indicating a low level of toxicity whereas Type 2 conditions appear to elicit a more significant change in community dynamics (*c.f.* control). The response to Type 1 and Type 2 metals can be further categorised as short (<24 hours) and long term (>7days) effects.

5.4.1.1 Short term effects

In the first 6 hours bacterial numbers in Type 1 conditions (Figure 5.1) either increased as per the control (1µM Zn^{2+}) or remained within 5% of initial (T_0) values (Ni^{2+} and Cd^{2+}). This suggests that 1µM Zn^{2+} is too low to cause a response within this time frame. This suggestion is supported by MDS analysis (Figure 5.5) which showed that the bacterial communities in the control and 1µM Zn conditions were different to the remaining conditions and similar to each other in the early stages of incubation. Diaz-Ravina (1996) showed that soil bacteria did not respond to added zinc concentrations below 2mmol. Kg^{-1} soil (dry weight) and it is possible that the 1µM

concentration added here was too low to have any noticeable effect. This is supported by the similarity between temporal trends in bacterial numbers and production between control and 1µM Zn condition and thus placement of the 1µM samples close to the control and within time groups (Figure 5.5). However, the comparatively high numbers of bacteria and concentration of organic matter in soil *c.f.* estuarine water makes comparison between these studies speculative. Bacterial numbers in samples in which (little) change was detected (Ni^{2+} and Cd^{2+}) increased to approximately control values within the first 24 hours. This short time lag suggests a minimal toxic effect which was quickly overcome, furthermore, bacterial numbers in all Type 1 conditions showed similar trends to those observed in the control from this point onwards. Production in Type 1 conditions (Figure 5.3) was equal to that in the control during the first 24 hours again suggesting a minimal inhibitory effect within this time frame.

One possible explanation for differences observed in the first 24 hours between the Type 1 and 2 responses is that members of the phytoplankton community of the Erme may have been affected differently by the metals added. Type 1 conditions (Cd^{2+}, Zn^{2+} (1 µM) and Ni^{2+}) introduced metals which are known to be biologically required by phytoplankton cells. Both Zn^{2+} (Morel *et al.* 1994) and Cd^{2+} (Cullen *et al.* 1999; Lane and Morel 2000) are known to be components of carbonic anhydrase enzymes which are required for uptake of inorganic carbon. Ni^{2+} is also instrumental in phytoplankton production via its role in the enzyme urease which is essential for nitrogen uptake when urea is the sole source of nitrogen (Price and Morel 1991; Morel *et al.* 1991). The introduction of these biologically required metals may have resulted in an increase in the production of biological polymers designed to sequester such metals internally or within the immediate area of phytoplankton cells (Croot *et al.*, 2000). This would support the initial increase in bacterial numbers and production observed in this study which, with the exception of the Zn^{2+} (15µM) condition, were greater (abundance) or observed earlier (production) in the Type 1 conditions. This theory is put forward with particular reference to the early stages of incubation (first 24 hours) after which point it is likely that the influence of incubation in the dark would result in phytoplankton productivity being reduced to maintenance levels (0.5<10%; Jochem 1999). This hypothesis is supported by an increase in production of extracellular polysaccharides observed when diatoms and dinoflagellates were incubated in the presence of Cd^{2+} (Pistocchi *et al.* 2000). The same authors also noted no reduction in growth of phytoplankton at concentrations of Cd^{2+} up to 2 mg L^{-1} (an

order of magnitude higher than the concentration employed in this study), indicating that phytoplankton mortality was not a factor in the increased growth observed in this condition.

Type 2 conditions elicited a greater response than Type 1 conditions, this was apparent in both the bacterial numbers and production. In the first 6 hours bacterial numbers increased slightly (Zn^{2+} 15µM and Pb^{2+}) or decreased (Cu^{2+} and cocktail). In the conditions where an increase was observed the increase amounted to 10% and 20% of the increase observed in the control (Pb^{2+} and Zn^{2+} respectively). This suggests inhibition of bacterial growth by these metals. In those conditions where a decrease was observed (Cu^{2+} and cocktail) bacterial abundance was reduced by 20-25%, indicating an immediate toxic response (e.g mortality or reduced growth) to these conditions. The reduction in numbers was greater in the cocktail indicating that it was not only the Cu^{2+} content of the cocktail mixture that was responsible for the observed response. Assessment of the response of bacterial communities to Type 2 metals is further complicated by an increase in bacterial numbers observed between 6 and 24 hours in the Cu^{2+} condition (15% *c.f.* T_0) which is not reflected in the cocktail condition in which numbers decreased a further 15% (*c.f.* T_0). This suggests a delayed effect of some components of the metal mixture, a decrease in numbers of 60% in the Zn^{2+} (15µM) condition was also observed in this time period. This may implicate zinc toxicity in the decrease in numbers in the cocktail condition. However, the magnitude of the decrease is smaller in the cocktail suggesting a possible antagonistic affect of other metals within the mixture. This possibility supports observations made by Ince *et al.* (1999) who found that antagonistic interactions between binary mixtures of metals were the most common 'toxic interactions' observed when pairs of metals were tested for toxicity using standard toxicity tests (prokaryotic).

Bacterial production rates also indicate toxicity of Type 2 metals (Figure 5.4). With the exception of the Pb^{2+} condition, production rates were maintained at T_0 levels for the first 6 hours (Table 5.2). Production subsequently increased in the Cu^{2+} and Zn^{2+} (15µM) conditions, albeit to less than 60% of that observed in the control, after 1 day of incubation. The bacterial community in the cocktail condition again showed the greatest inhibition with no increase in production detected for the first 24 hours of incubation (Table 5.2). After three days of incubation production in all Type 2 conditions exceeded that in the control with the exception of the cocktail which, at its peak, achieved only approximately 65% of the production observed in the control. It is

possible that after this time the bioavailability of the metals was reduced, perhaps as a result of ligand production by either bacteria or phytoplankton present in the bottles (Gordon et al., 2000). This theory is supported by the results of laboratory experiments using cultures of *Emiliania huxleyii* and algal exudates which showed that metal impacted (increased Cu, Pb, Cd, Zn, Fe, Mn, Ni and Co) *E. huxleyii* were able to reproduce more successfully when exudates from *Enteromorpha* were added to the culture medium (Vasconcelos *et al* 2002).

It is also possible that metal toxicity was reduced as a result of phytoplankton mortality which would release cellular components into the surrounding media. One such example is that Cu^{2+} has been shown to reduce growth of phytoplankton (Pistocchi *et al.* 2000) at concentrations as low as 0.01mg L^{-1} (significantly lower than the concentration utilised in this study). Resulting cell debris would not only complex a proportion of the free metal ions it would also provide the extra substrate required for the increased production *c.f.* the control observed after 3 and 7 days of incubation. The theory that the addition of trace metals in this study may have resulted in the death of and subsequent release of organic matter from less metal tolerant algal species found in the Erme estuary is supported by work carried out in the Tinto and Odiel rivers (Spain). The authors suggest that trace metals may play a role in determining the composition of algal or phytoplankton communities (Lopez-Archilla and Amils 1999), the metal rich portion of the Tinto and Odiel rivers was found to be home to a considerably different phytoplankton community to other similar but non-metal-impacted rivers. However, it should be noted that the rivers discussed were also of consistently low pH which would favour acidotolerant/philic algal species irrespective of trace metal conditions. Rijstenbil and Gerringa (2002) demonstrated, using laboratory cultures, that copper concentrations above 157 nM stimulated production of phytochelatins and increased the cell-wall and internal copper binding capacity in a diatom (*Ditylum brightwellii*). This suggests that in addition to the release of organic substrate discussed above phytoplankton may also act to reduce the availability of non-required metals in surrounding water.

Both MDS (Figure 5.5) and DGGE (Figure 5.6) analyses showed incontrovertibly that the addition of a cocktail of trace metals had a more profound effect on bacterial communities than the addition of individual metals in the first 3 days. MDS showed that samples from the cocktail condition clustered separately from the remaining conditions from 24 hours until 11 days after the start of the incubation

and DGGE supported this observation and demonstrated dominance by a different group of bacteria in samples taken at day 3 from the cocktail condition when compared to the control and 15µM Zn^{2+} conditions. This indication of an enhanced response to multiple metals is supported by work performed by Wei *et al* (2003) using marine phytoplankton cultures and a natural algal assemblage from a pristine coastal site. Wei *et al* (2003) added Cd, Cu and Zn both singly and as a mixture and followed the production of phytochelatin (a metal toxicity response). Phytochelatin production varied considerably between metals and concentrations, however, the addition of a mixture of trace metals resulted in greatly suppressed phytochelatin production. The concentration produced closely resembled particulate phytochelatin observed in the field suggesting that investigations of mixtures of metals have greater environmental relevance.

A further consideration in this study is the formation of aggregates from the natural substrate found in the Erme Estuary. Previous work (Grossart and Ploug 2001) has shown that in the first stages of aggregate formation bacteria associated with aggregates are able to remineralize organic matter extremely efficiently. It is possible that the initial rapid increase in bacterial numbers and production was related to the formation of such aggregates and the production of extracellular enzymes by associated bacterial consortia. However, the toxic effects of metals would be likely to reduce the hydrolytic activity of bacteria associated with aggregates in the Type 2 conditions. These suggestions; firstly that an efficient hydrolysis of organic matter was occurring in the early stages of aggregate formation in Type 1 conditions and secondly, that hydrolysis is likely to be inhibited in Type 2 conditions, may be supported by the appearance of the aggregates formed (although it is difficult to interpret the appearance of the aggregates in a meaningful way without the aid of more detailed measurements). In Type 1 conditions the aggregates appear pale and less dense than those formed in Type 2 conditions (Figure 5.8). This may suggest reduced coherence of the aggregates in the Type 1 conditions *c.f* the Type 2 conditions. This could also be due to extracellular enzyme production, which may be inhibited in Type 2 conditions. The aggregates observed in the cocktail condition were amorphous and much larger (Figure 5.8). This could be attributed to a decrease in the amount of extracellular 'glue' produced by bacteria (Engel 2000) most likely as a result of metal toxicity. Alternatively, because visible particles are the product of aggregation of smaller particle, it is possible that the adsorption of metals to the surface of smaller particles in

the cocktail condition has resulted in the formation of metal bridges between the smaller particles. Conversely, the particles in the other conditions are likely to be cemented by sticky biological polymers.

5.4.1.2 Long term effects of metal enrichment

The affects of metal enrichment in the first 3 days of incubation provide important information about the immediate response of bacterial communities to metal stress. This information would be of greatest importance for assessment of implications of metal contamination in closed systems which rely on bacteria for remediation of waste such as sewage. It is likely that assessment of impacts on ecosystem health would be better informed by the long term effects which are of importance in terms of loss of functional groups and implications for the cycling of organic matter and nutrients. Long term response of bacterial communities in this study to enrichment with Type 1 metals were assessed in terms of bacterial production and numbers.

In all Type 1 conditions bacterial production was lower than observed in the control condition from day 11 onwards (Figure 5.3). This could be a result of decreased efficiency of nutrient assimilation, possibly as a result of metal substitution in active sites of enzymes. Alternatively the decreased production could be due to toxic effects of metals following the release of metals from ligands / aggregate surfaces by the hydrolytic action of bacteria (Chen *et al.*,1995[b]) This suggestion is supported by the magnitude of the initial increase in production matching the subsequent decrease observed. This suggests that the labile nutrient supply induced as a result of confinement has been exhausted and thus more refractory organic molecules become the main source of substrate. The digestion of such molecules may release metals which were previously sequestered leading to reduced productivity of the bacterial populations.

Bacterial production in Type 2 conditions decreased in all conditions between days 13 and 21. However, in contrast to Type 1 conditions, bacterial production was similar to the control with the exception of the Cu^{2+} condition which was greater than the control and the Pb^{2+} condition which was lower than the control. The production in the Pb^{2+} condition had been higher than the control from day 3 until day 11 hence the subsequent lower production may simply be due to the relative amount of substrate available towards the end of the incubation. The Cu^{2+} condition however was elevated from day 11 until the end of the incubation (c.f. control). This could be due to

decreased grazing activity as a result of metal toxicity, a phenomenon previously observed in freshwater microbial food webs (Havens, 1994).

Bacterial abundance in Type 1 conditions (Figure 5.1) decreased from day 13 until the end of the incubation, with the possible exception of the 1µM Zn^{2+}condition in which bacterial numbers remained more or less static. A similar decrease was observed in the control condition. This decrease in numbers is most likely to be a result of the activity of grazers on a population of bacteria which has become nutrient limited. The bacterial response to the addition of Cd^{2+} is notable due to the predominantly lower numbers observed *c.f.* the control throughout the incubation, particularly from day 7 onwards (Figure 5.1). This suggests decreased growth as a result of cadmium addition, an observation supported by congruent lower production observed in this condition. This strongly suggests that a significant part of the bacterial community was sensitive to Cd^{2+}. Furthermore, the difference observed between the Cd^{2+} enriched and control condition suggests that no opportunistic growth occurred to take advantage of substrate made available as a result of the demise of this group of bacteria. This suggests that the Cd^{2+} sensitive bacteria may have been utilising substrate which was refractory to the remaining community, thus indicating the possible loss of a functional group.

In the latter stages of incubation an increase in bacterial abundance in Type 2 conditions was observed. This phenomenon may be a result of the formation of bacterial biofilms either on the surfaces provided by aggregates or alternatively the internal surface of the incubation vessels. The formation of biofilms and associated EPS creates a diffusive gradient which enables bacteria within the inner layers of the biofilm to escape toxic effects of metals (Tietzel and Parsek, 2003). However, the nature of such biofilms means it is necessary to assess the likelihood of bacteria within the biofilms being enumerated by the methods utilised here. It is possible that the movement of water within the incubation bottles could disrupt the biofilms resulting in the inclusion of component bacteria in bacterial counts, it is also acknowledged that a constant process of attachment and detachment occurs between aggregates and the surrounding water column which would also influence bacterial counts.

Furthermore, it is possible that the production of EPS by biofilm forming bacteria and subsequent digestion thereof would provide an additional source of labile substrate which would support growth by the free-living bacterial community. Such a phenomenon has been observed previously associated with aggregate associated bacteria (Unanue *et al.*, 1998[a.b]). This theory is supported by sequence analysis which

confirmed the presence of bacteria commonly associated with biofilm formation in the incubation vessels.

Whilst it is likely that bacteria would also associate with surfaces under control conditions, it is feasible that the addition of Type 2 metals stimulated the formation of biofilms as a mechanism to avoid metal toxicity thus accounting for the different temporal trends observed in the control and Type 2 metal conditions. An alternative explanation for the latter stage increase in bacterial numbers is that a period of acclimation is required before bacteria are able to demonstrate tolerance to trace metals. One possible reason for this is sequestration of Type 2 metals in a different way to Type 1 metals. Copper and lead can be biologically transformed to oxalate crystals with low solubility (zinc can also form oxalates but they are far more soluble than the lead oxalate (Elliott and Shastri 1999). Nickel also forms oxalate crystals and is frequently present as phosphate. Cadmium is biologically precipitated as sulphides (anoxic environments), phosphates and carbonates and zinc removal from solution is predominantly by biosorption. It is possible that the formation of more refractory crystalline precipitates may remove metals from solution more efficiently and for longer than the comparatively more labile precipitates formed by Cd, Ni and Zn. This discrepancy could account for the increase in abundance observed in the latter stages of the incubation in Type 2 conditions via alleviation of metal toxicity and, in addition, may explain why production in the Ni^{2+} enrichment did not decrease at the same rate observed with the addition of other Type 1 metals.

MDS and DGGE analysis suggest that over longer time periods the bacterial communities became more similar to the original community in terms of abundance, productivity and composition. MDS (Figure 5.5) showed that from 7 days onwards sample attributes were similar to those observed in the early stages of the incubation with the exception of samples from the Ni^{2+}, Pb^{2+} and cocktail conditions (7 days). DGGE analysis showed that the bacterial community from the exceptions listed above were dominated by phylotypes which migrated to a similar depth in the DGGE gel as those which dominated in the control condition. Comparison between the DGGE profiles of samples from the cocktail condition at days 3 and 7 indicated recovery of phylotypes which were inhibited in the early stages of the incubation (Figure 5.6). The reversion of the bacterial community to one more similar to the control indicates that the initial toxicity levels had decreased by day 7, possibly through sequestration of free metal ions as discussed previously or via the formation of biofilms associated with

particle or incubation vessel surfaces. It is also possible that bacterial metal tolerance levels increased over time, perhaps through expression of genes involved in metal tolerance or selection for tolerant individuals within phylotypes.

5.4.1.3 Dominant bacteria in samples most impacted by metal enrichment.

Analysis of phylogeny (Figure 5.7) showed that all dominant bacteria found in DGGE separated samples (selected by virtue of their differences to control conditions) belonged to the Rhodobacteraceae clade of the α-proteobacteria. Surprisingly these bacteria also dominated in the control condition suggesting that it was the less dominant bacterial community which varied with the addition of trace metals. This theory is suggested by the marked increase in dominance of phylotypes previously present at low levels in the cocktail condition (day 3, Figure 5.6). All samples were dominated by a small number of bacterial phylotypes whose closest relatives include; *Sagittula stellata*, a bacterium capable of transforming lignin and hydrolysing cellulose (Gonzalez *et al*. 1999); *Rhodobacter sphaeroides*, a bacterium able to photosynthesize, utilise aerobic and anaerobic modes of respiration, fix molecular nitrogen and detoxify a number of metal oxides and oxyanions (Moore and Kaplan, 1992); *Roseobacter*, a bacterial genus instrumental in the degradation of dimethylsulfoniopropionate (DMSP) and other organic and inorganic sulphur compounds in marine environments (Gonzalez *et al*. 1999). This bacterial family (Rhodobacteraceae) can utilise a wide variety of substrates including sulphite, methanesulphonic acid, DMSP, methylamine, methyl bromide, lignin and aromatic compounds (Gonzalez *et al*. 1997; 1999; Pukall *et al*., 1999). It is clear that this group of bacteria are important in terms of carbon and sulphur cycles and as such their apparent ability to maintain productivity in the presence of elevated metals has implications for the biogeochemical cycling of such elements. Whilst this bacterial group is frequently found in marine or estuarine environments it is important to note that only a small proportion of the total bands were sequenced (based on their apparent dominance) and it should not be assumed that this bacterial group was present to the exclusion of other groups. Nonetheless this study suggests that members of the Rhodobacteraceae are more able to tolerate a cocktail of trace metals than other estuarine bacteria.

5.5 Conclusions

The observations presented in this chapter allowed the division of the metals added into two groups based on the response of the bacterial community. Observed differences in response (both in bacterial production and numbers) supported the division into Type 1 and Type 2 metals. This study also demonstrated that there are two major time-scales of response, short-term (<24 hours) and long term (>7 days), suggesting that the length of incubation has important implications for assessing response of bacterial communities to metal enrichment. The dominance of the *Rhodobacteraceae* (in the communities which responded differently to their temporal counterparts in different conditions) suggests a degree of metal tolerance common to members of this metabolically diverse group of bacteria. Particularly in light of the ability the bacteria in these communities to maintain comparable levels of production to that observed in the control. The formation of aggregates with very different appearances in the different conditions suggested an impact of metal enrichment on aggregate formation. The importance of aggregates in estuarine and marine systems suggests that this phenomenon should be investigated further.

It was apparent that the effects of metal enrichment on bacterial community were not adequately investigated using the techniques used in this study. Whilst this study provides a general picture of the effects of Type 1 and Type 2 metals on bacterial communities in a pristine estuary, information pertaining to substrate use, changes in metal concentration / speciation, numbers and activity of bacteria associated with aggregates, would enhance our understanding of the effects of metal enrichment.

6 Effects of trace metals on bacterial community structure associated with size-fractionated suspended particulate matter (SPM) - does RNA give a clearer picture of community change than DNA?

6.1 Introduction

6.1.1 Trace metals in estuaries

Anthropogenic sources of trace metal contamination in estuarine environments are many and varied (Cobelo-Garcia *et al.* 2004). They range from diffuse sources such as the use of fertilisers on agricultural land, to point source industrial inputs from industries such as smelting. The distribution and toxicity of trace metals once within an estuary is highly dependant upon a number of physicochemical variables including salinity, pH and suspended particulate matter (Achterberg *et al.* 2003; Hatje *et al.* 2003). The relationship between metals and particulate matter has been shown to depend on the size and composition of the particles in question. For example, metal sorption is dependant upon the organic matter content of the particle (Turner *et al.* 2004). Furthermore, the formation of iron and manganese oxides / oxyhydroxides on the surface of particles can subsequently increase the deposition of other metals (Millward and Moore 1982; Turner *et al.* 2004). The association of metals with SPM and ligands is thought to reduce their biological availability and in doing so reduce toxic effects (Chen *et al.* 1999). It is reasonable to hypothesise that the metal compliment of each size fraction of estuarine particulate matter will be different, thus bacteria within each size fraction are likely to respond differently to increased metal concentrations.

6.1.2 Analysis of aggregate associated microbial communities

The bacteria associated with aggregates, particularly in estuarine environments, have been shown to play an important role in the cycling and remineralisation of organic matter (Section 1.3). A number of studies have been performed to investigate the bacterial communities on and off estuarine aggregates (e.g. Delong *et al.* 1993; Rath *et al.* 1998; Acinas *et al.* 1999; Bidle and Fletcher 1995; Crump *et al.* 1999). Such studies tend to conclude that the aggregate associated community is different to the free-living community but with some overlaps. These may be attributed to either

insufficient separation due to sampling technique or to attachment and detachment processes. Experimental determination of aggregate association ranges from sampling of individual aggregates to size fractionation by filtration. The division of SPM into size fractions is arbitrary as particles are present in a spectrum of sizes. However sampling by filtration is less labour intensive than collection of individual aggregates *in situ* and does not require specialised equipment such as SCUBA (used for collection of visible aggregates such as marine snow).

6.1.3 Comparisons of rRNA and rDNA derived denaturing gradient gel fingerprints

Denaturing gradient gel electrophoresis is a technique which has been successfully employed in numerous aquatic environments to determine the diversity of bacterial communities (Section 1.1). The main benefit of fingerprinting methods such as DGGE is the ability to analyse a large number of samples in a relatively short period of time and at relatively low expense (*c.f.* clone library preparation and sequencing, see Table A.2 for a more comprehensive list of advantages and disadvantages of the DGGE method).

DGGE is routinely performed on PCR amplified fragments of the 16S rRNA gene (rDNA), although more recently a number of workers have investigated the application of DGGE using reverse transcribed rRNA and compared it with DNA-derived DGGE (e.g. Teske *et al.*, 1996[a,b] ; Griffiths *et al.*, 2000; Duineveld *et al.* 2001; Norris *et al.*, 2002; Girvan *et al.* 2003; Ebie *et al.*, 2004). Such investigations have, with few exceptions, attributed the bacterial population elucidated by rRNA derived DGGE as being the more active fraction of the community. This assumption has been based on the observations that i) all phylotypes represented in the cDNA fingerprint are also present in the DNA fingerprint and ii) the rRNA derived fingerprint is generally less complex than the fingerprint derived from rDNA.

6.1.4 The Tamar estuary

The Tamar estuary complex (location indicator: 50:26:114 N, 04:11:42 W) is a drowned river (Ria) which is tidal to approximately 30kM, the lower reaches form extensive tidal mudflats which border on saltmarsh communities. The estuary was classified as a special protection area in the summer of 1997 due to a wide variety of infaunal communities and the role of the estuary as a feeding and roosting area for large numbers of wintering and passage birds. The most notable metals present are lead,

arsenic and copper, derived from abandoned mines and waste tips in the mineralised part of the catchment (Environment Agency, 1999). As a result of tidal resuspension of bed sediments the Tamar has a pronounced turbidity maxima zone (TMZ; Uncles *et al*., 1994). Within that TMZ the loading of suspended solids ranges from 100 to 1000mg per litre.

The aims of this study were two-fold. Firstly to determine if bacterial communities associated with different size fractions of the suspended particulate matter responded differently to trace metals. Secondly to compare inferences about community change drawn from DNA and RNA derived DGGE community profiles. To achieve this, the effect of metal addition on bacterial community diversity has been analysed using cDNA and DNA template for PCR-DGGE of different size fractions. These were operationally defined as aggregates (>20 µm), microaggregates (2 - 20 µm) and free-living (0.2 - 2 µm).

6.2 Methods

6.2.1 Water collection and incubation strategy

The Tamar estuary was sampled from the PML research vessel *Tamaris* on the 16th of March 2004. Water from the surface of the estuary (salinity 15) was pumped directly into three 20 L low density, acid washed, sterile polycarbonate bottles. Samples were transferred to a CT (10±1°C) laboratory at PML for manipulation. Metal additions were made to two sample bottles within 2 hours of sample collection. The first addition was zinc (1µM) and the second was the same cocktail of metals used in Chapter 5 (Erme estuary: Zn^{2+} (15 µM), Cu^{2+} (950 nM), Cd^{2+} (18 nM), Ni^{2+} (200 nM) and Pb^{2+} (300 nM)). The third bottle was unamended as a control. All samples were incubated on aggregate rollers, in the dark, at ambient estuarine temperature (10±1°C). Sub-samples were collected at T_0, and days 5 and 9 of the incubation (hereafter T_5 and T_9 respectively).

6.2.2 Sub-sample collection

Sub-samples for determination of initial diversity were collected by filtration of 250 mL aliquots through a 0.2 µm pore size polycarbonate filter. Sub-samples were collected at subsequent time ponts for size fractionated analysis of bacterial diversity. Aliquots (250 mL) were sequentially filtered under low pressure through 20 µm, 2 µm and 0.2 µm pore size polycarbonate Nuclepore filters. Filters were snap frozen in liquid nitrogen and stored at -80°C. Sub-samples (100 mL) for analysis of absorbance were taken at two time points by decanting into 250 mL plastic beakers which were covered using aluminium foil. The filter sizes were selected to represent the fraction of the estuarine bacterial community associated with aggregates (>20 µm), microaggregates (20-2 µm) and the colloidal / free-living (2-0.2 µm) fraction of the community.

6.2.3 Analysis of sub-samples

6.2.3.1 Absorbance

Measurement of absorbance was used to quantify the difference in DOM absorbance of the sample water after 5 days and 9 days of incubation. Samples were collected by decanting into plastic beakers and covered using foil to prevent

contamination. Blanks were prepared by centrifugation (RCF= 16 000, 60 minutes, 4°C) to remove temporarily suspended particles. The supernatant was decanted into plastic containers and samples and blanks processed immediately for cDOM analysis according to the method of Tilstone *et al.* (2002). Briefly, quartz cuvettes were rinsed using approximately 10 mL sample blank (supernatant) at room temperature and the absorbance of the blank measured between 350 nm and 800 nm. The cuvette was then filled with the corresponding sample and scanned for absorbance over the same absorbance range. The equipment stability was checked by performing a scan with room temperature MilliQ water. The remaining samples were analysed as described above alternating blank and sample scans with MilliQ scans.

6.2.3.2 Nucleic acid extraction

Samples for diversity analyses were defrosted on ice prior to nucleic acid extraction. The CTAB method had previously been shown (data not presented here) to successfully extract good quality RNA and so was applied as described previously (Section 2.4) with RNase free reagents, plastic and glassware (see appendix 1). Nucleic acids were precipitated with 2.5 volumes of ethanol with Pellet Paint® (Novagen, Merck Biosciences, UK) as co-precipitant to maximise recovery. Following a 70% ethanol wash the dry nucleic acids were resuspended in RNase free Milli Q water (40 µL). Presence of nucleic acids was confirmed using agarose gel electrophoresis or analysed using a micro-chip reader (Agilent 2100 Bioanalyzer; performed by Mike Allen, PML). Samples for DNA analysis were stored at -80°C prior to PCR. Samples for RNA analysis were treated with RNase free DNase prior to first strand synthesis.

6.2.3.3 DNase treatment and first strand synthesis.

Aliquots (40 µL) of total nucleic acids were incubated for 30 minutes with RNAse free DNAse RQ1 (5µL) and 10 × reaction buffer (5 µL) at 37°C. The DNase was deactivated by addition of provided stop solution (5 µL) and incubation at 65°C for 10 minutes prior to PCR. Aliquots (5 µL) were used to confirm degradation of DNA by agarose gel electrophoresis prior to synthesis of cDNA. Reagents for DNase treatments were obtained from Promega.

cDNA first strand synthesis was achieved by combining aliquots (2µg) of RNA with 0.5µg random hexamers in 0.5 µL PCR tubes on ice. The mixtures were made up

to a maximum volume of 15µL with RNase free Milli Q and heated to 70°C for 5 minutes to melt secondary structure prior to transfer to ice.

Master mix:

5µL	M-MLV 5x reaction buffer
5µL	10mM dNTPs
25U	RNasin Ribonuclease inhibitor
200U	M-MLV RT
+ RNase free water to 25µL final volume.	

Master mix (25 µL) was added to each sample on ice, and mixed well prior to incubation at 37°C for 60 minutes. A control reaction without enzyme was run in parallel. The resulting product was then used as template in PCR.

6.2.3.4 Polymerase chain reaction - denaturing gradient gel electrophoresis

The polymerase chain reaction (PCR) was performed to amplify cDNA and DNA template using primers 341f (GC) and 907r (see Table 2.3 for sequence, reference and target regions) and an annealing temperature of 57°C . Products were electrophoresed (1% agarose) to confirm presence and size of product and assess the efficacy of the DNase treatment (Section 6.2.3.3). DGGE was performed to compare the RNA derived and DNA derived PCR products for each condition, each size class and each time point. Gels were stained and visualised (Section 2.5.4), and bands were selected for cloning and sequencing.

6.2.3.5 Cloning and sequencing

Excised fragments were reamplified using primers 16S_nested 1_f and 16S_nested 1_r (Table 2.3) and an annealing temperature of 57°C. Products were ligated into pGEM-T Easy vector, transformed into E. *coli* (DH5 α) and sequenced (sections 2.4.4 and 2.4.5 respectively). Sequences were submitted to the BLAST search programme of the NCBI website to identify phylogenetic affiliation of the fragments. Chimeric sequences were identified using the RDP-II check chimera facility and excluded from further analyses.

6.2.3.5 Statistical analysis

Statistical analyses were performed using PRIMER-E (5) software. Briefly, MDS and cluster analyses (Bray-Curtis similarity coefficient) were performed to determine the similarity of results obtained from DNA and cDNA derived DGGE gels (initial analysis using Genetool software) and also to compare the response of bacteria in different size fractions to metal enrichment. Background correction was performed using a rolling disc and the number and peak height of individual bands (OTUs) determined. This information was used to determine relative band intensity and Shannon diversity indices (H') for each sample.

6.3 Results

6.3.1 Molecular analyses

6.3.1.1 Analysis of DGGE derived from samples collected at the beginning (T_0) of the incubation

DGGE analysis of samples from T_0 showed that both the extraction and amplification of nucleic acids were reproducible between samples (Figure 6.1). Within each template type the same bands were present in replicate amplifications. The cDNA fingerprints were less complex than analogous DNA fingerprints and some variation in intensity was apparent.

Multi-dimensional scaling was used to show the similarity of fingerprints derived from samples taken at T_0 (Figure 6.2). Samples clustered according to template type, with the DNA derived fingerprints showing greater similarity to each other than the cDNA derived fingerprints (Figure 6.2A, >72% and >40% Bray Curtis similarity respectively). No clustering according to incubation conditions was apparent at this time with either template (Figure 6.2 B).

Diversity at T_0 was invariably higher in DNA derived fingerprints than those obtained from cDNA. This is supported by diversity statistics (H') which were always higher from DNA derived fingerprints than those derived from cDNA, regardless of incubation condition (Figure 6.3). Some variation in diversity was observed between replicates.

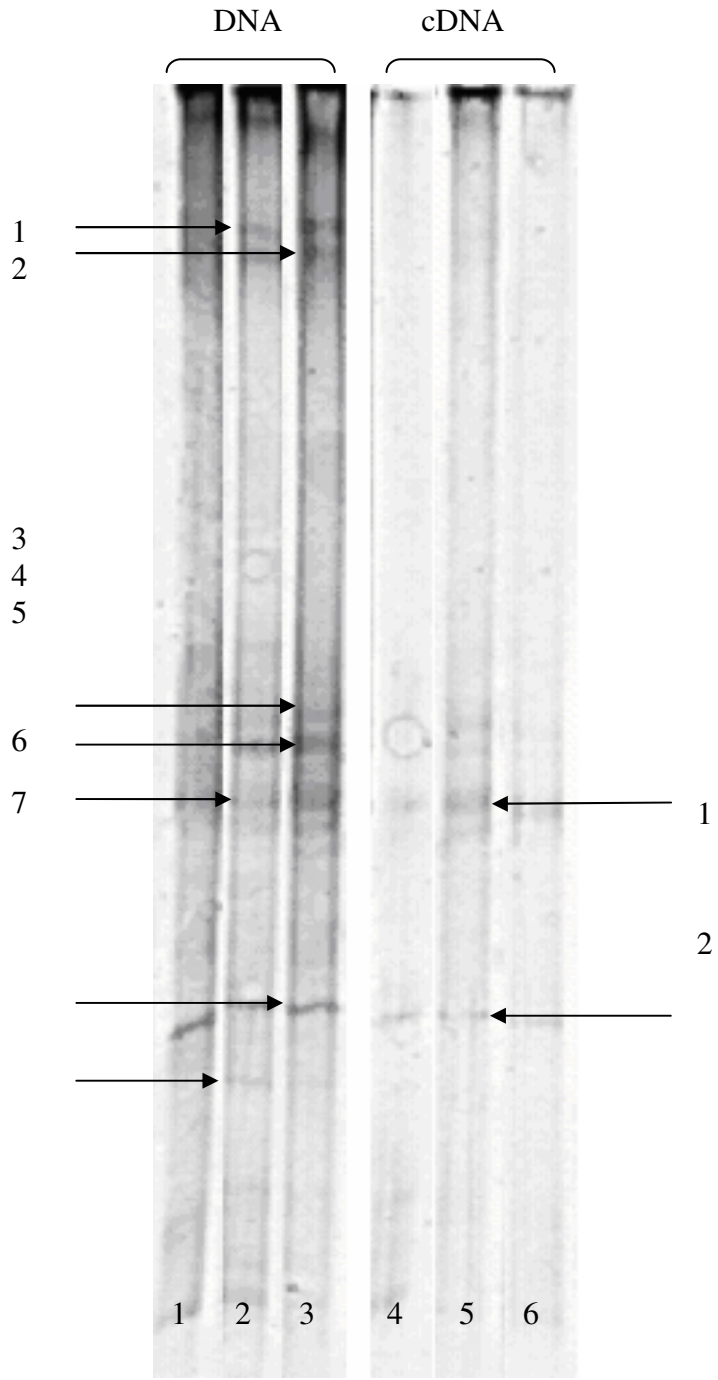


Figure 6.1 DGGE fingerprint of PCR products of samples taken from the Tamar estuary (T_0). Samples in lanes 1 and 4 were taken from the control; lanes 2 and 5 taken from the 1µM Zn condition; lanes 3 and 6 taken from the cocktail condition. Arrows indicate sequenced bands. DNA / cDNA indicate template for PCR.

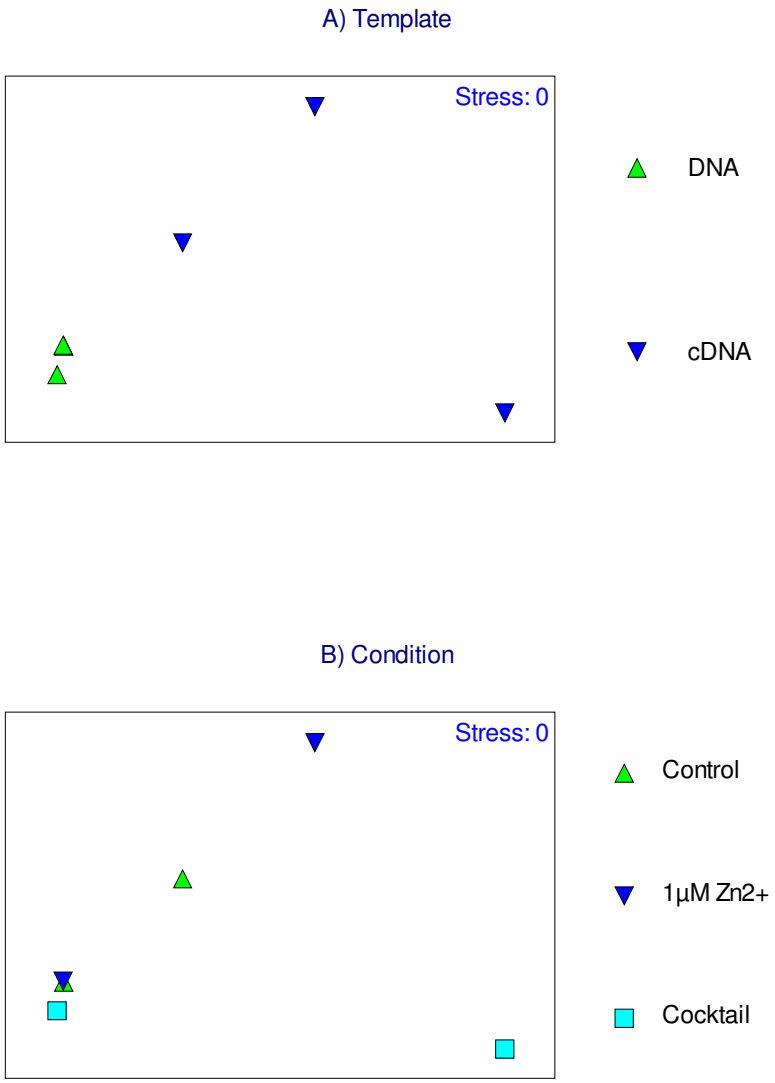


Figure 6.2 Multi dimensional scaling plots showing similarity between DGGE fingerprints at T_0 displayed as A) cDNA and DNA and B) Incubation conditions (see key for details).

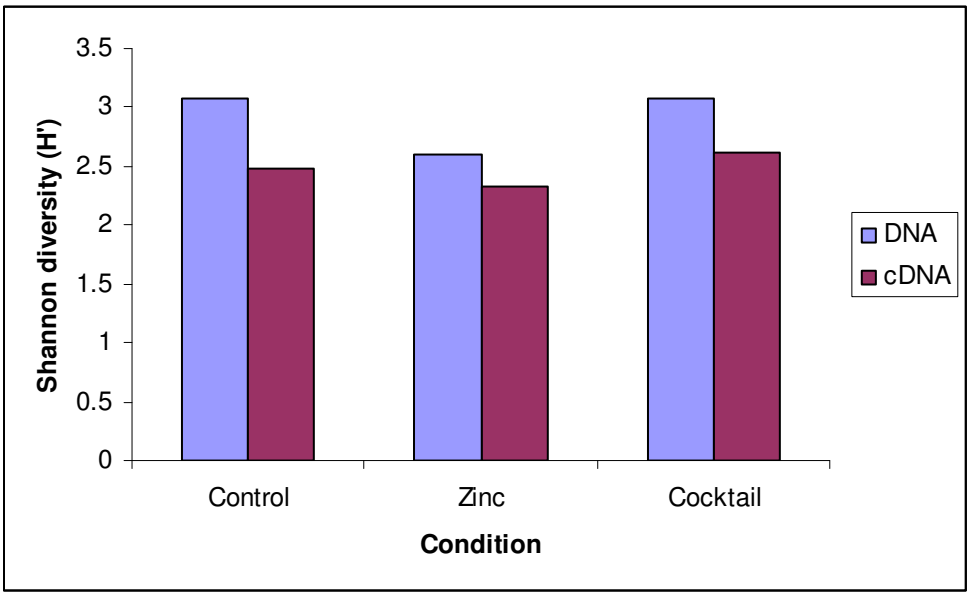


Figure 6.3 Diversity indices of T_0 samples. Samples were taken from the >0.2µm fraction following experimental additions and prior to initiation of incubation.

6.3.1.2 Analysis of DGGE derived from samples collected after 5 days of incubation

DGGE analysis of samples from T_5 showed that a small number of phylotypes were present in all conditions and all size fractions (Figure 6.4). As was observed in the T_0 samples, RNA derived fingerprints were generally less complex than their DNA derived counterparts. It was observed that bands present in the cDNA fingerprints were also present in the DNA derived fingerprints Both RNA and DNA derived fingerprints were more complex at T_5 than at the beginning of the incubation. Furthermore it was observed that 'new' bands i.e. those not present at T_0 were consistently present in both the DNA and RNA derived fingerprints (Figure 6.4). At T_5 differences between the size classes are beginning to become apparent. For example the regions between bands 6 and 8 (Figure 6.4) appear to be more populated in the >20µm fraction (Figure 6.4, A) than in either the 20µm>2µm (Figure 6.4, B) or 2µm - 0.2µm (Figure 6.4, C) fraction. A further difference is the comparative simplicity of the fingerprint in samples from the 2µm - 0.2µm (Figure 6.4, C) fraction of the 'cocktail' incubation when compared to the other conditions.

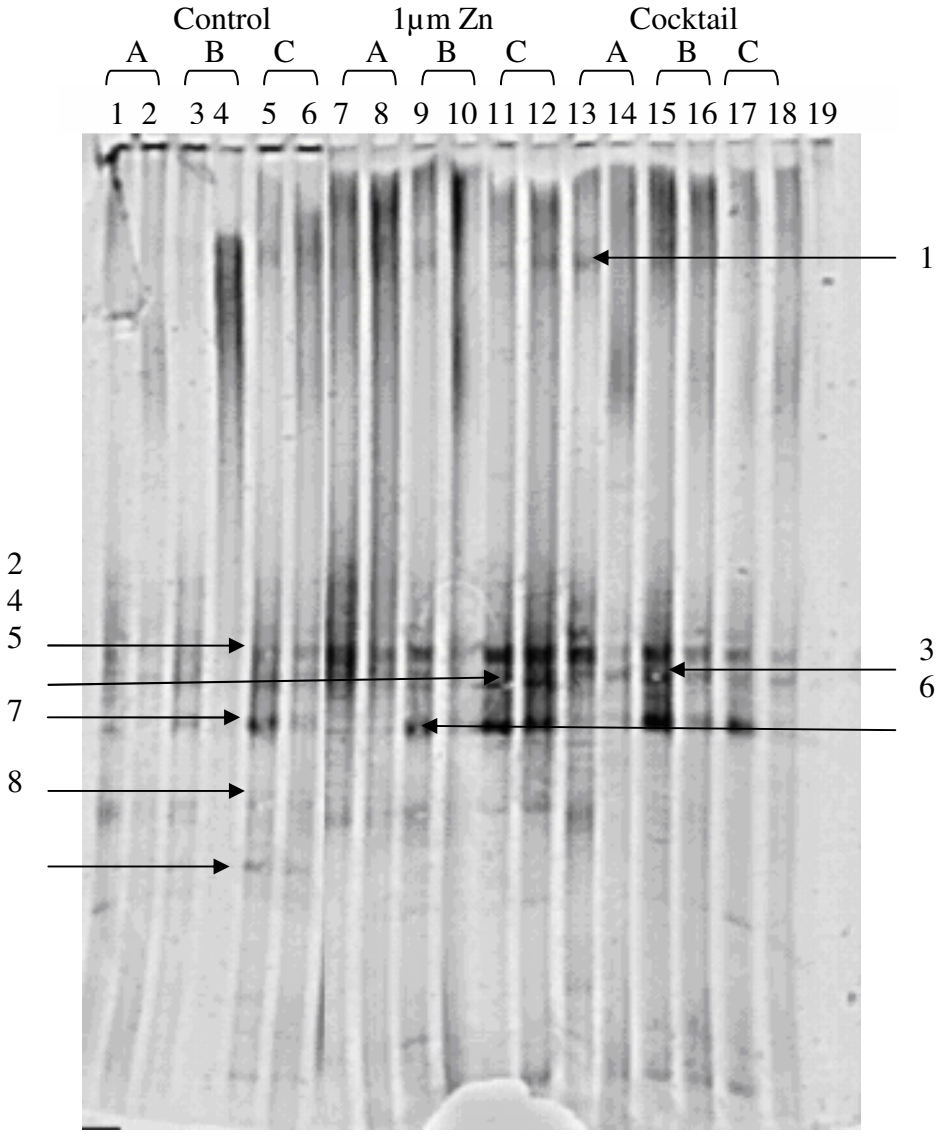


Figure 6.4 DGGE fingerprints of PCR products of samples taken from the Tamar estuary (T_5 days). Samples in lanes 1-6 were taken from the control, lanes 7-12 taken from the 1µm Zn condition and lanes 13-18 taken from the cocktail condition. Arrows indicate sequenced bands. Sample lanes with odd numbers were created with DNA as template (except for 19 which is the -ve reverse transcription (RT) control); sample lanes with even numbers were created with cDNA template. Letters indicate size fraction: A = >20µm, B = 20µm - 2µm, C = 2µm - 0.2µm.

6.3.1.3 MDS Analysis of DGGE derived from samples collected after 5 days of incubation

MDS analysis of DGGE fingerprints from each template between size fractions supported the visual assessment (Figure 6.5C) that the >20µm fraction was detectably different to the remaining fingerprints (although only in the DNA derived samples). Analysis of both the combined template (6.5A) and cDNA (6.5B) did not differentiate between the size fractions.

MDS analysis of DGGE fingerprints from each size fraction between incubation conditions showed that samples in the 2µm-0.2µm fraction clustered according to the incubation condition from which they were derived (Figure 6.6B). A high degree of similarity was calculated between DNA and cDNA derived fingerprints (Bray-Curtis similarity co-efficient values were: 73.0, 93.9, and 85.4 for the Control, 1µm Zn^{2+}, and Cocktail respectively). Conversely, both the 20µm-2µm and >20µm fraction derived fingerprints clustered according to whether they were control or amended samples (Figure 6.6C and D). When all size fractions were analysed together, whilst some groupings according to incubation condition were apparent, it was not possible to distinguish definitively between conditions (Figure 6.6A).

MDS analysis of DGGE fingerprints from each template between incubation conditions showed that products derived from cDNA clustered according to incubation conditions (Figure 6.7B). Analysis of DNA derived fingerprints however, only distinguished between control and amended samples (Figure 6.7C). As observed previously all templates together did cluster according to incubation conditions but without the required definition to definitively distinguish them.

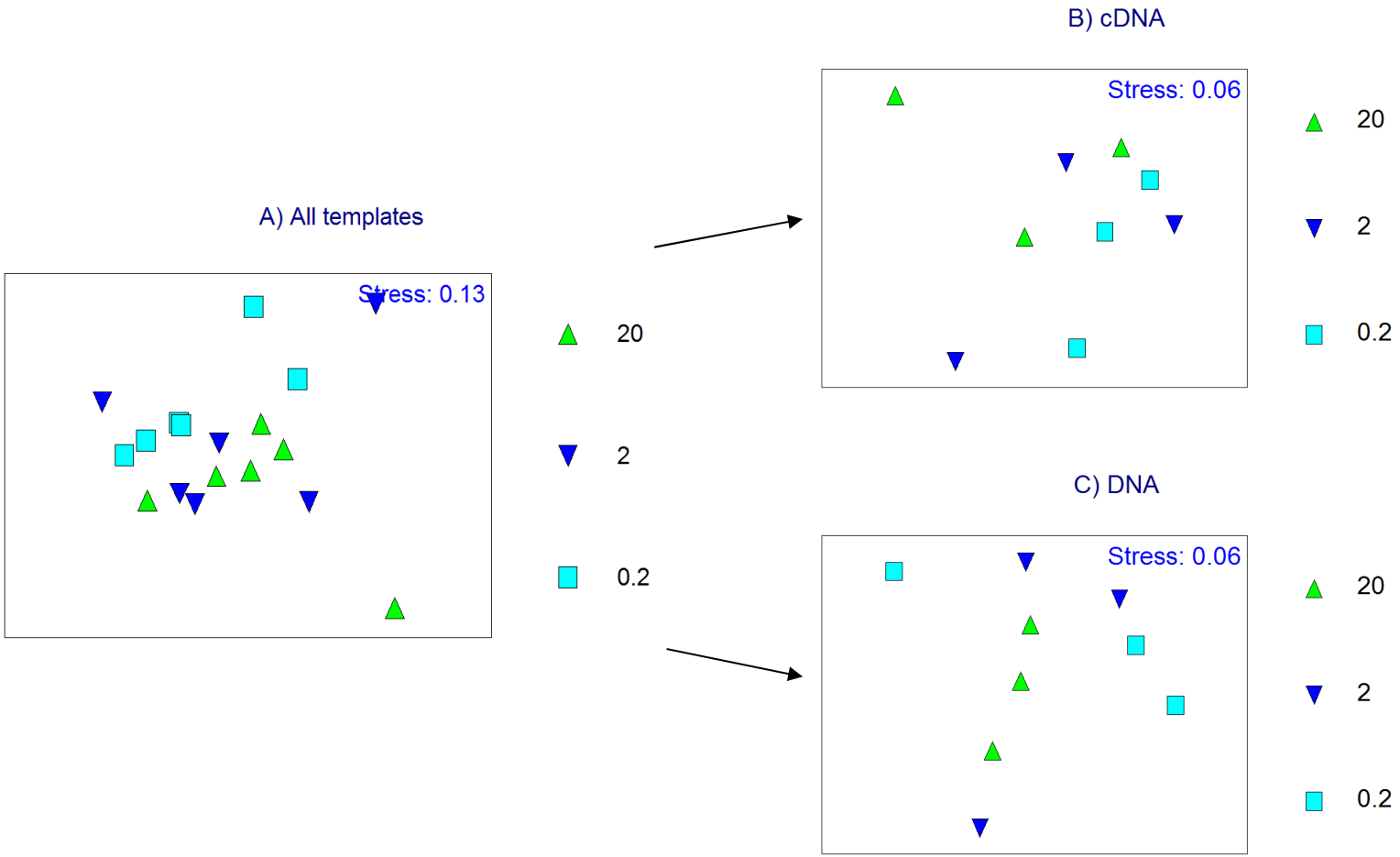


Figure 6.5 MDS plots showing similarity of DGGE fingerprints in each size fraction (T_5 days). Comparison of results derived from different PCR templates. A) All templates; B) cDNA; C) DNA. 20 = >20µm fraction; 2 = 2-20µm fraction; 0.2 = 0.2-2µm fraction.

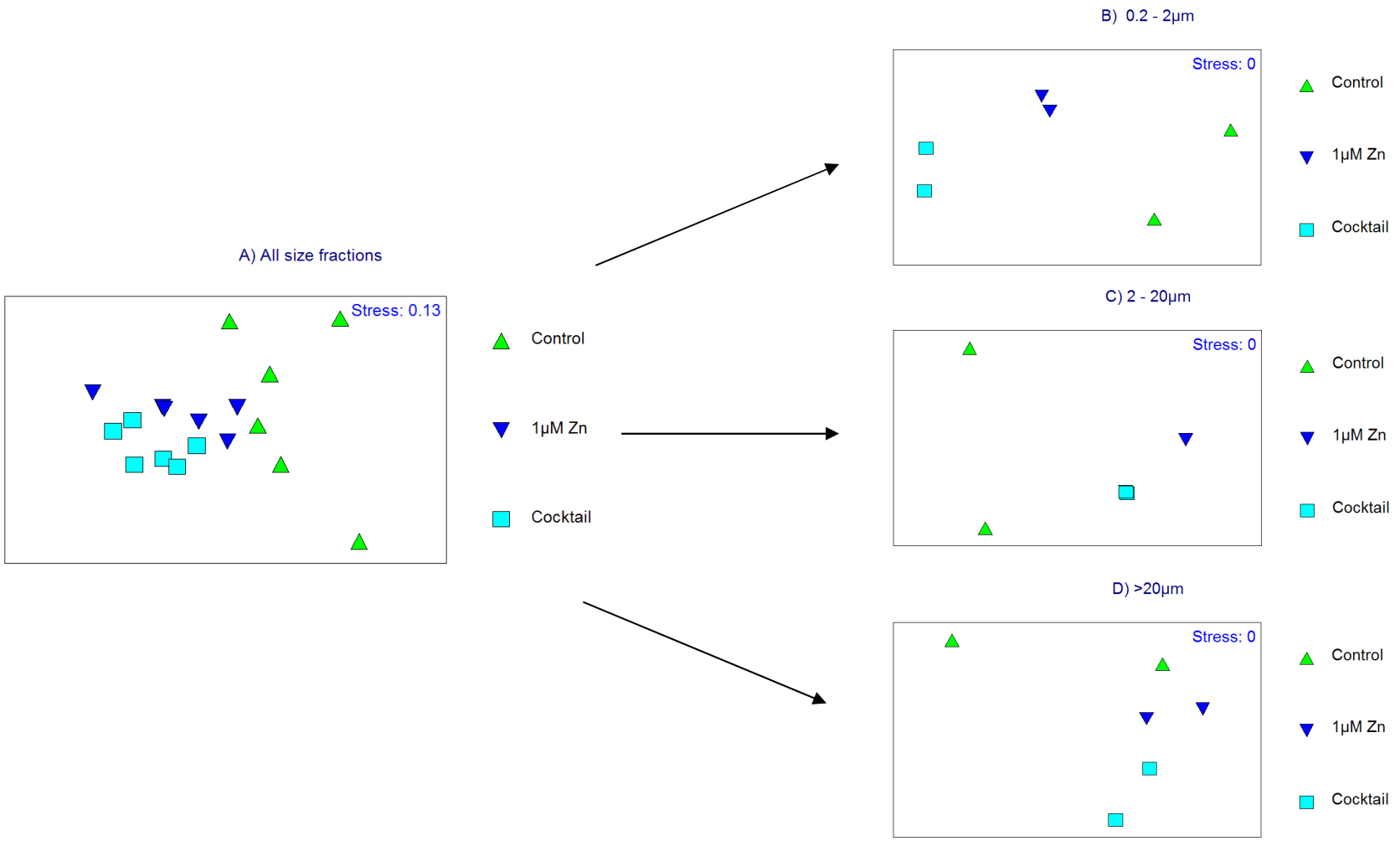


Figure 6.6 MDS plots showing similarity of DGGE fingerprints in each incubation condition (T_5 days). Comparison of results derived from each size fraction. A) All size fractions; B) 0.2-2µm size fraction; C) 2-20µm size fraction; D) >20µm size fraction.

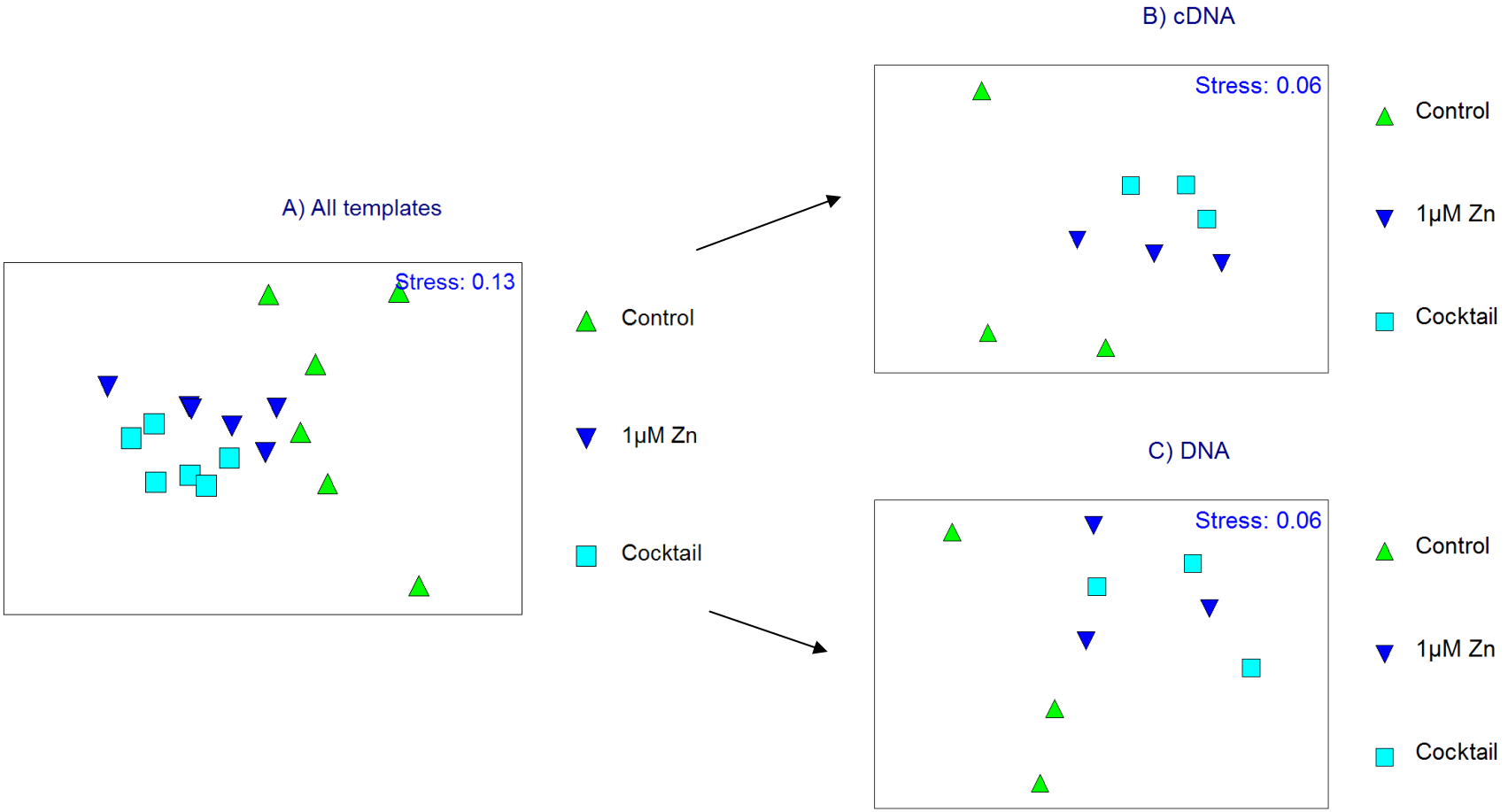


Figure 6.7 MDS plots showing similarity of DGGE fingerprints in each incubation condition (T_5 days). Comparison of results derived from different PCR templates. A) All templates; B) cDNA; C) DNA.

6.3.1.4 Analysis of DGGE derived from samples collected after 9 days of incubation

Visual analysis of DGGE fingerprints suggest that there was greater complexity at T_9 than was observed at either T_0 or T_5 in both the DNA and cDNA derived fingerprints (Figure 6.8). At T_9 it became apparent that there were greater differences between fingerprints from different size fractions than between fingerprints derived from different templates in both the control and 1µm Zn^{2+} conditions. However in the Cocktail condition differences between template types were more noticeable. In addition to the newly populated region observed in T_5 (tentatively suggested to be analogous to between bands 11 and 17, Figure 6.8), the region between band 1 and band 7 (Figure 6.8) showed a number of new bands. Again, these new bands were observed in both the DNA and cDNA derived fingerprints. As observed previously fingerprints derived from both DNA and cDNA were dominated by a small number of bands. However, the fingerprints derived from cDNA appeared to be more complex than previously observed.

6.3.1.5 MDS Analysis of DGGE derived from samples collected after 9 days of incubation

MDS analysis of DGGE fingerprints from each size fraction indicated that fingerprints from the 0.2-2µm fraction clustered separately (Figure 6.9B) according to incubation condition. This phenomenon was also observed at T_5. Furthermore, the trend observed in the similarity indices (Bray-Curtis co-efficient) at T_5 (i.e. Control < Cocktail < 1µm Zn^{2+}) was also apparent here although the actual similarity values were lower (54.4, 58.6 and 72.1 respectively). Fingerprints from the 2-20µm fraction were not sufficiently different to distinguish between conditions (Figure 6.9C). Similarly, significant overlap between conditions was observed in the >20µm size fraction (Figure 6.9D), the differences were however large enough to separate the control and cocktail conditions effectively. When all size fractions were analysed together (Figure 6.9A), although groupings according to incubation condition were apparent (in a similar manner to the corresponding T_5 analysis) it was not possible to distinguish definitively between conditions.

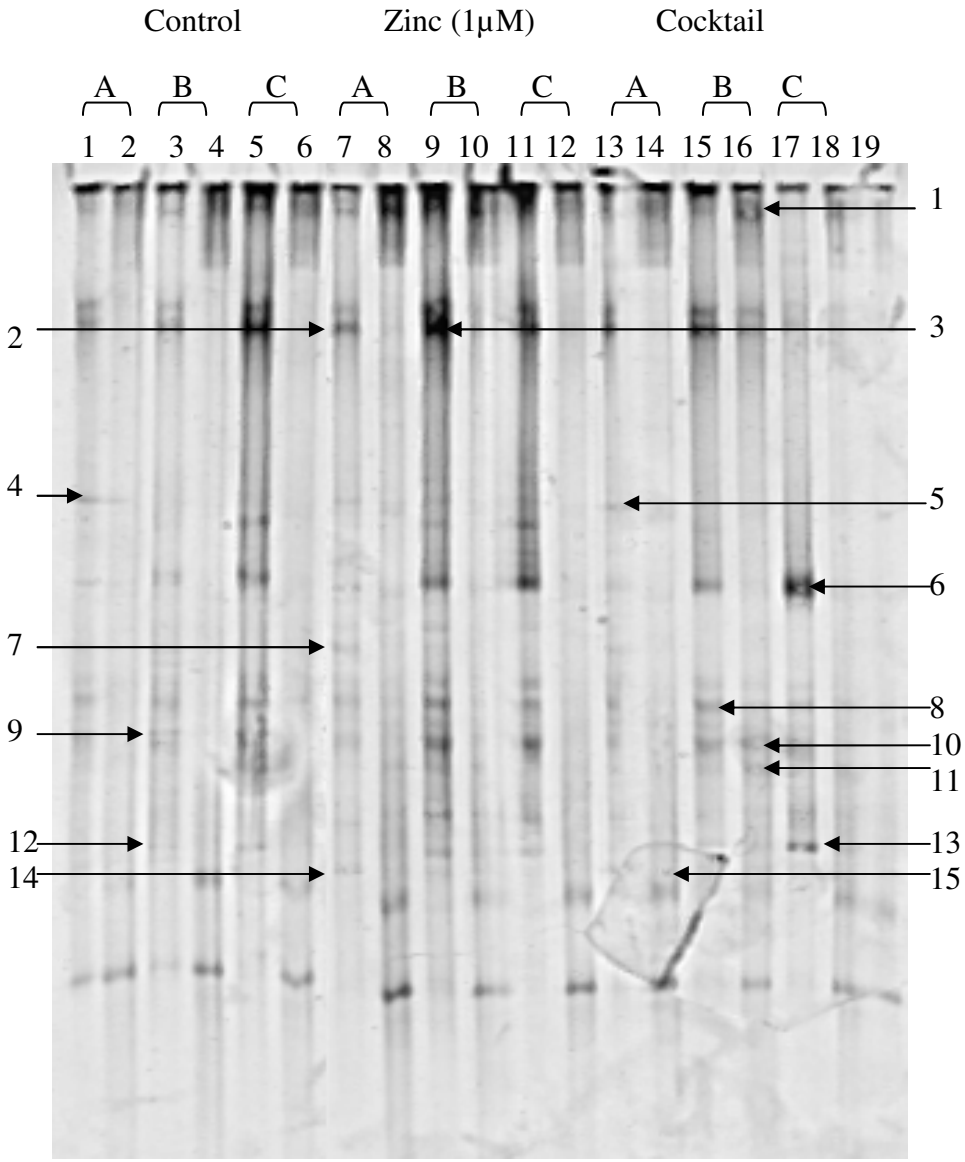


Figure 6.8 DGGE fingerprint of PCR products of samples taken from the Tamar estuary (T_9 days). Samples in lanes 1-6 were taken from the control, lanes 7-12 taken from the 1µm Zn condition and lanes 13-18 taken from the cocktail condition. Arrows indicate sequenced bands. Sample lanes with odd numbers were created with DNA as template (except for 19 which is the -ve reverse transcription (RT) control); sample lanes with even numbers were created with cDNA template. Letters indicate size fraction: A = >20µm, B = 20µm-2µm, C = 2µm-0.2µm. Bands present in the -ve RT control were excluded from further analyses.

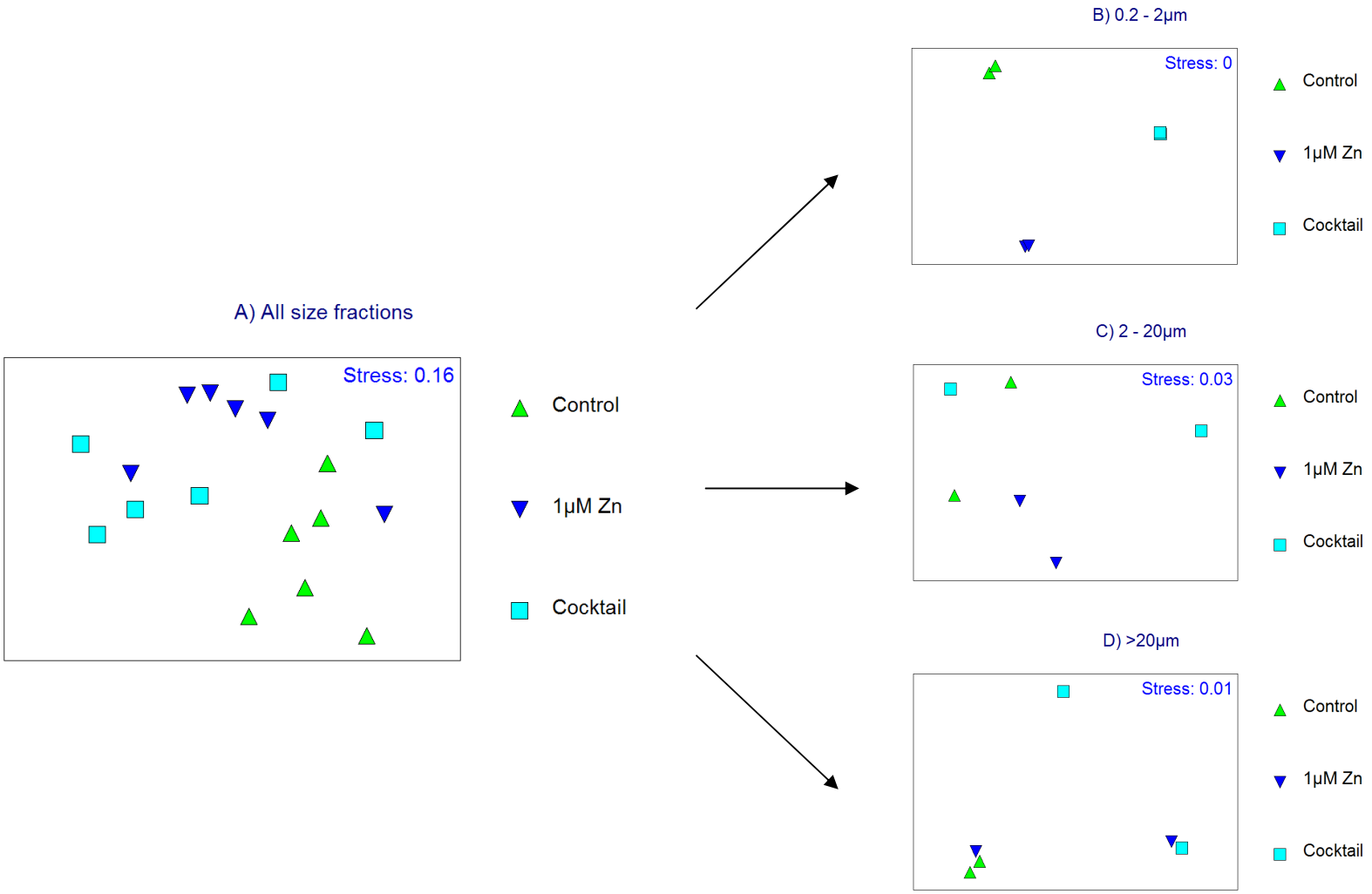


Figure 6.9 MDS plots showing similarity of DGGE fingerprints in each incubation condition (T_9 days). Comparison of results derived from different size fractions. A) All size fractions; B) 0.2-2µm size fraction; C) 2-20µm size fraction; D) >20µm size fraction

MDS analysis of DGGE fingerprints from each template between incubation conditions showed that fingerprints derived from cDNA clustered for the most part according to incubation condition (Figure 6.10B). The exception to this was the fingerprint from the 20µm 1µM Zn^{2+} sample which showed greater similarity to the fingerprints from the Cocktail incubation condition than the remaining Zn^{2+} fingerprints (Figure 6.10B). In contrast to the cDNA derived fingerprints, analogous DNA derived fingerprints were as similar between conditions as they were within conditions (Figure 6.10C). When all size fractions were analysed together (Figure 6.10A) groupings according to incubation condition were apparent with only two exceptions: >20µm Cocktail and 0.2-2µm Zn^{2+}, both of which were more similar to the Control fingerprints than those derived from their respective incubation conditions.

MDS analyses of DGGE fingerprints from each template between size fractions showed no discrete clusters according to size fractions in any of the analyses performed i.e. all templates (Figure 6.11A), cDNA (Figure 6.9B) or DNA (Figure 6.11C). However, cluster analyses (Figure 6.12) of individual conditions showed that in the Control and 1µM Zn^{2+} conditions the size fraction explained more of the similarity between fingerprints than did the template type (Figures 6.12A and B respectively). In the control the largest differences were observed between the >20µm and 2-20µm size fractions (Figure 6.12A). In the 1µM Zn^{2+} condition the greatest differences were observed between the fingerprints from the >20µm fraction and the remaining fingerprints (Figure 6.12B). Conversely in the Cocktail condition fingerprints were discretely separated into template type (Figure 6.12C).

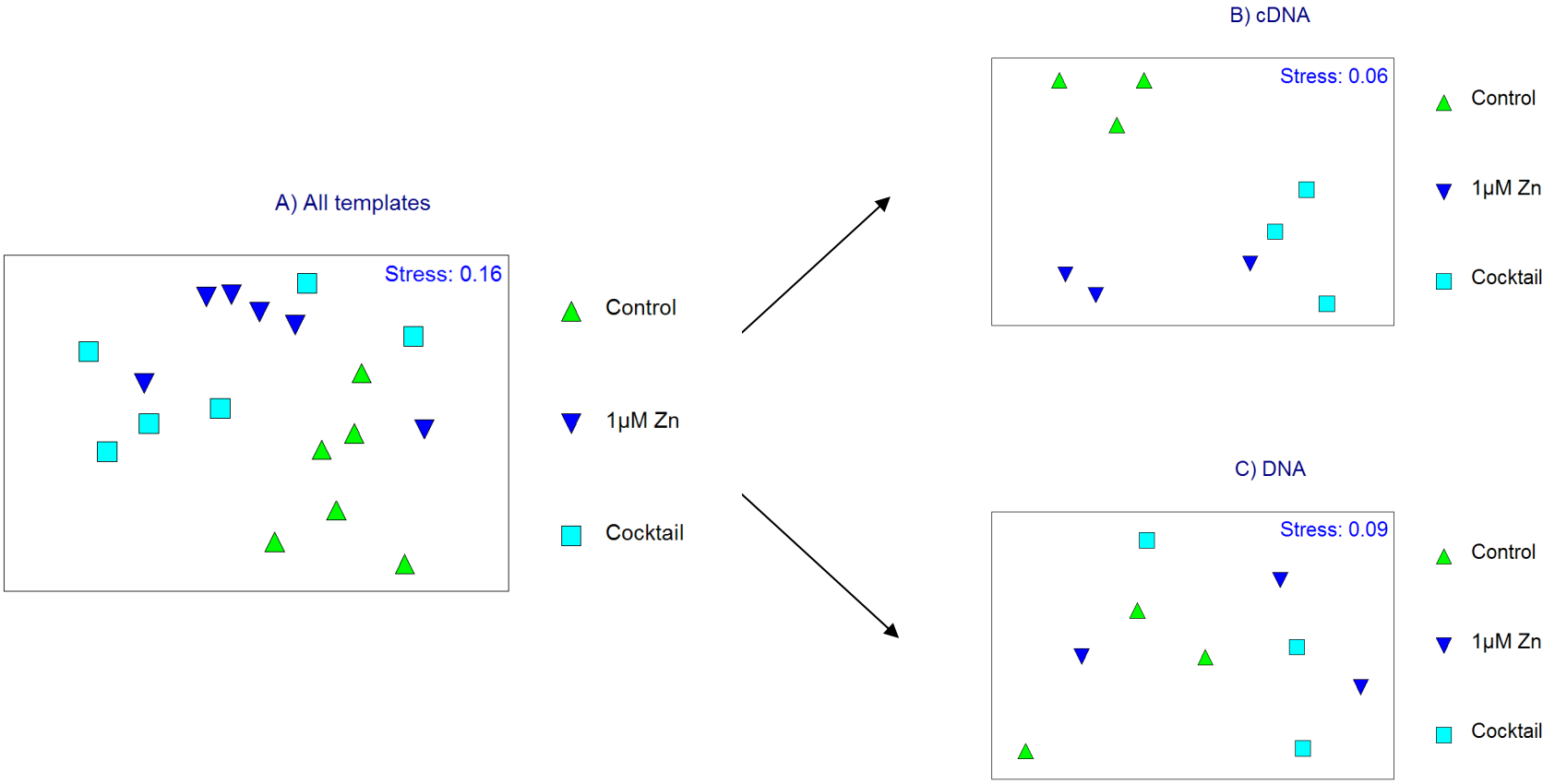


Figure 6.10 MDS plots showing similarity of DGGE fingerprints in each incubation condition (T_9 days). Comparison of different template types. A) All templates; B) cDNA; C) DNA

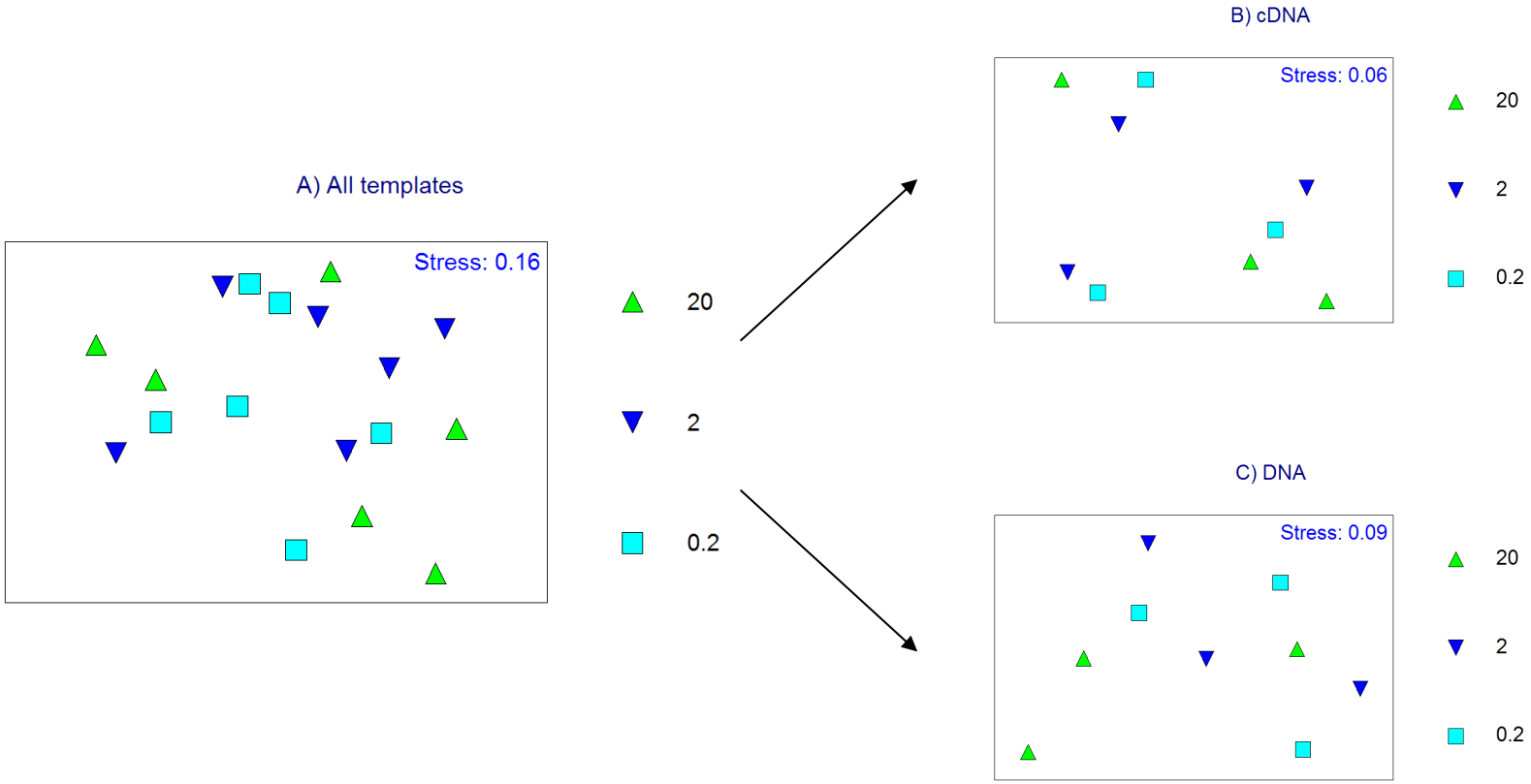


Figure 6.11 MDS plots showing similarity of DGGE fingerprints in each size fraction (T_9 days). Comparison of results derived from different template types. 20: >20µm size fraction; 2: 2-20µm size fraction; 0.2: 0.2-2µm size fraction.
A) All templates; B) cDNA; C) DNA

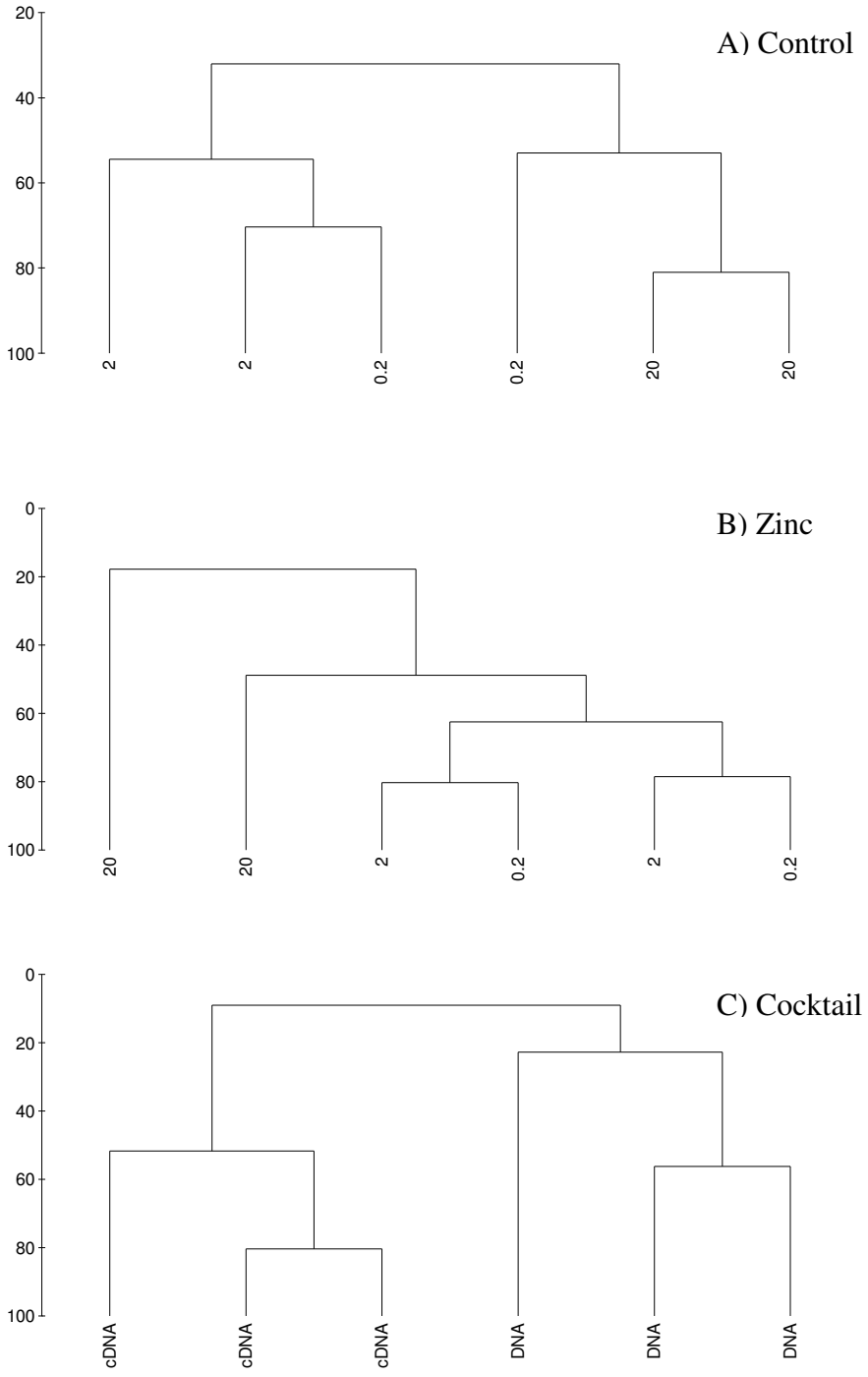


Figure 6.12 Cluster analysis for each condition displaying relationships between samples. Labels shown according to most influential factor (given in parentheses). A) Control (size fraction); B) Zinc (size fraction); C) Cocktail (PCR template type).

6.3.2 Diversity analyses

Diversity indices (Shannon diversity H') were calculated based on the DGGE fingerprints from Figure 6.4 and Figure 6.8. DNA derived fingerprints from T_5 (Figure 6.4) indicated that, with the exception of the > 20µm fraction from the Zn^{2+} incubation, bacterial diversity was higher than the control in both experimental conditions (Figure 6.13A). Conversely fingerprints derived from DNA for T_9 samples (Figure 6.8) showed that, with the same exceptional sample (> 20µm fraction, Zn^{2+} incubation) bacterial diversity was lower in the experimental samples than in the control (Figure 6.13C).

Fingerprints derived from cDNA template (T_5, Figure 6.4) showed a similar result to DNA derived fingerprints with two exceptions; firstly diversity in the 2 - 20 µm fraction of the Zn^{2+} incubation was lower than control; secondly, diversity in the 0.2 - 2 µm fraction of the cocktail incubation was the similar the control (Figure 6.13B). Conversely at T_9 fingerprints from all experimental samples (Figure 6.8) were noticeably less diverse than the control (Figure 6.13D).

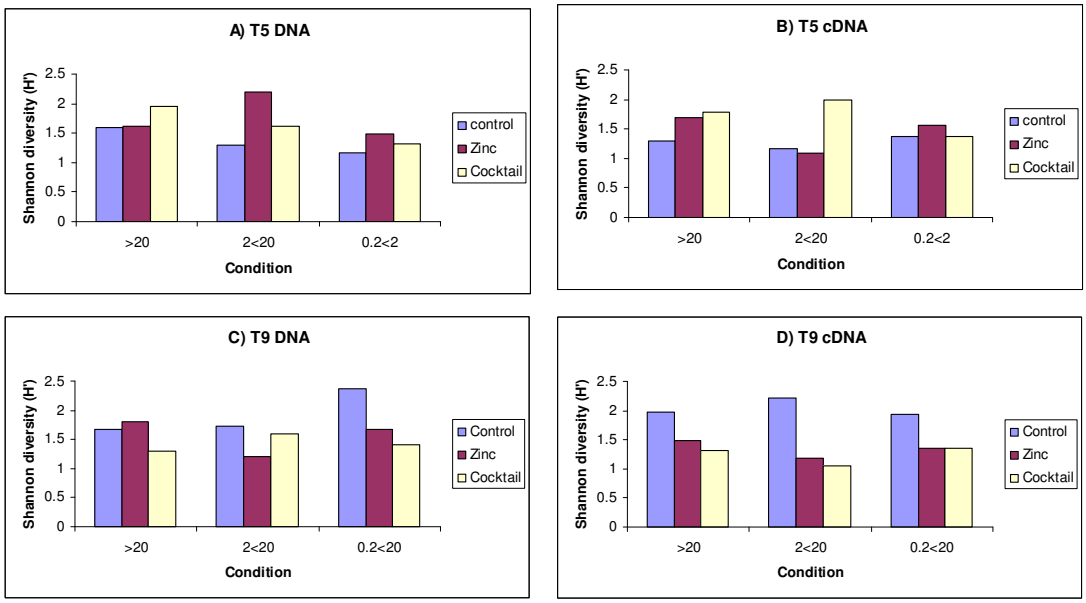


Figure 6.13 Shannon diversity indices (H') calculated for each incubation condition, template type and size fraction. A) T_5 DNA; B) T_5 cDNA; C) T_9 DNA; D) T_9 cDNA.

6.3.3 Sequence analysis

Sequence analysis of the bands extracted from the gel in Figure 6.1 showed that the bacterial community was dominated by bacteria from the α division of the proteobacteria. Closest known relatives are given in Table 6.1. All sequences had closest relatives previously detected in marine or estuarine environments. Band 5 (DNA) and Band 1(cDNA) which migrated similarly through the gel were both 99% similar to the same α proteobacterium. However, it should be noted that two other sequenced bands also showed this level of similarity with that particular α-proteobacterium despite differing patterns of migration. Sequence analysis of bands extracted from gels in figures 6.4 and 6.8 (T_5 and T_9) also showed dominance by the α proteobacteria. In all cases sequenced bands were observed at least once in all conditions within a given timepoint. From a total of 34 bands sequenced, only two sequences were represented in each of the three gels and both were α proteobacteria clones (specifically WB11-28 and Pl 4d7g). Three sequences were only detected in the T_0 gel (Figure 6.1); Roseobacter NAC11-7, α proteobacteria clone Pl-4m3h and an unidentified Rhodobacteraceae bacterium. Only one sequence was detected solely in the T_5 gel (Figure 6.4); a sulfitobacter KMM6006. Two sequences were represented in both the T_5 and the T_9 gels (Figures 6.4 and 6.8); Sulfitobacter SIMO-672 and Rhodobacteraceae SIMO-669. A total of five sequences unrepresented in the other time points were dominant in the T_9 gel (Figure 6.8). These were: sulfitobacter dubious strain KMM 3554T; bacterium clone Milano-WF2B-23; Sulfitobacter F2C84; Bacterium ARK10207 and Loktanella rosea.

6.3.4 Absorbance analysis

Measurements of absorbance showed that the absorbance of water taken from bottles after both 5 and 9 days of incubation were much higher in the Cocktail condition (Figure 6.14) than in either the Control or the 1µm Zn^{2+} condition. Conversely there was very little difference between the absorbance detected in the Control and 1µm Zn^{2+} condition. Furthermore, absorbance in the Cocktail condition was lower in the T_9 sample than in the T_5 sample.

Gel	Band	Template	Closest relative	% similarity	Reference	Accession number
A	1	DNA	α proteobacteria PI_4d7g	99	Acinas *et al.* 2004	AY580459
	2	DNA	Roseobacter NAC11-7	99	Gonzalez *et al.* 2000	AF245635
	3	DNA	α proteobacteria WB11-28	99	Selje and Simon 2004 (unpublished)	AF497867
	4	DNA	Sulfitobacter F4C85	100	Prabagaran and Shivaji (unpublished)	AY697924
	5	DNA	α proteobacteria PI_4d7g	99	Acinas *et al.* 2004	AY580459
	6	DNA	α proteobacteria PI_4m3h	99	Acinas *et al.* 2004	AY580440
	7	DNA	α proteobacteria PI_4d7g	99	Acinas *et al.* 2004	AY580459
	1	cDNA	α proteobacteria PI_4d7g	99	Acinas *et al.* 2004	AY580459
	2	cDNA	Rhodobacteraceae	99	Moran *et al.* (unpublished)	AY712206
B	1	DNA	α proteobacteria PI_4d7g	100	Acinas *et al.* 2004	AY580459
	2	DNA	α proteobacteria PI_4d7g	100	Acinas *et al.* 2004	AY580459
	3	DNA	Sulfitobacter Arctic-P49	99	Lee *et al.* (unpublished)	AY573043
	4	DNA	Sulfitobacter SIMO-672	100	Moran *et al.* (unpublished)	AY712209
	5	DNA	α proteobacteria WB11-28	100	Selje and Simon 2004 (unpublished)	AF497867
	6	DNA	Sulfitobacter KMM6006	99	Bowman and Ivanova 2004 (unpublished)	AY682196
	7	DNA	Rhodobacteraceae SIMO-669	100	Moran *et al.* (unpublished)	AY712206
	8	DNA	α proteobacteria WB11-28	100	Selje and Simon 2004 (unpublished)	AF497867

Gel	Band	Template	Closest relative	% similarity	Reference	Accession number
C	1	cDNA	Loktanella rosea	98	Bowman and Ivanova (unpublished)	AY682199
	2	DNA	Loktanella rosea	98	Bowman and Ivanova (unpublished)	AY682199
	3	DNA	Sulfitobacter SIMO-672	99	Moran *et al.* (unpublished)	AY712209
	4	DNA	Sulfitobacter F4C85	99	Prabagaran and Shivaji (unpublished)	AY697924
	5	DNA	Sulfitobacter Arctic-P49	99	Lee *et al.* (unpublished)	AY573043
	6	DNA	Sulfitobacter F4C85	99	Prabagaran and Shivaji (unpublished)	AY697924
	7	DNA	Sulfitobacter dubious strain KMM 3554T	98	Ivanova *et al.* 2004	AY180102
	8	DNA	bacterium clone Milano-WF2B-23	100	Heijs *et al.* (unpublished)	AY592911
	9	DNA	α proteobacteria WB11-28	99	Selje and Simon 2004 (unpublished)	AF497867
	10	DNA	Rhodobacteraceae SIMO-669	100	Moran *et al.* (unpublished)	AY712206
	11	cDNA	Sulfitobacter Arctic-P49	98	Lee *et al.* (unpublished)	AY573043
	12	cDNA	Sulfitobacter F2C84	100	Prabagaran and Shivaji (unpublished)	AY697885
	13	DNA	Loktanella rosea	98	Bowman and Ivanova (unpublished)	AY682199
	14	DNA	α proteobacteria PI_4d7g	99	Acinas *et al.* 2004	AY580459
	15	DNA	Bacterium ARK10207	98	Brinkmeyer *et al.* 2003	AF468373

Table 6.1 Closest known relatives of sequenced DGGE bands obtained from BLAST search.

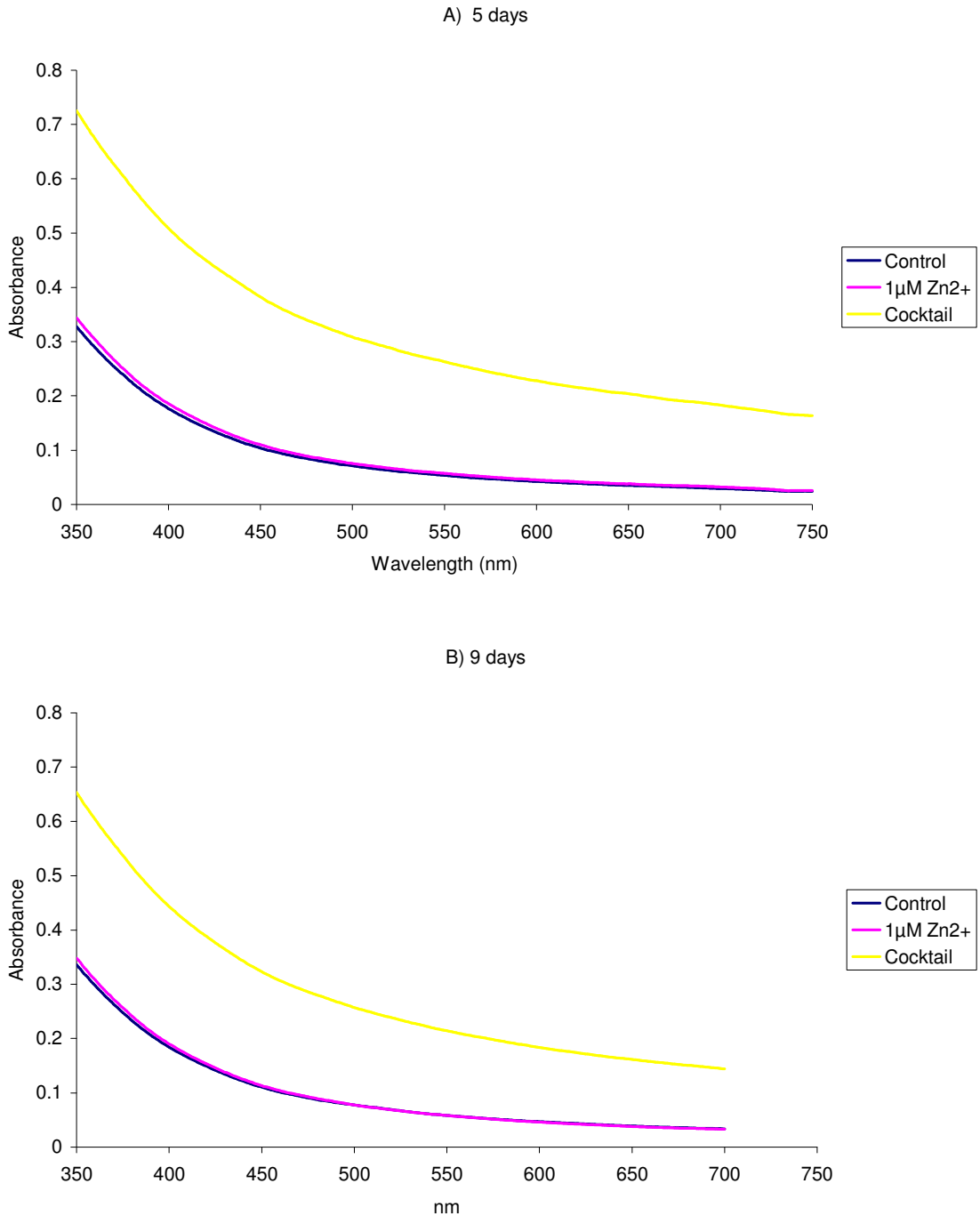


Figure 6.14 Absorbance curves for samples taken from the Tamar Estuary at T_5 days and T_9 days. Absorbance determined across the visible spectrum (350-700 nm). A) Measured after 5 days of incubation; B) Measured after 9 days of incubation.

6.4 Discussion

The effect of metal addition on bacterial community diversity has been analysed using PCR-DGGE of different size fractions. The results obtained using rRNA and rDNA and the different inferences drawn from each set of results are discussed. Multi-dimensional scaling (MDS) has been used to assess the similarity between samples based on their DGGE fingerprints, that is, the closer the samples are to one another on the ordination plot the more similar their DGGE fingerprints are.

6.4.1 Influence of aggregate association on changes in bacterial community in response to trace metals

6.4.1.1 Short term incubation effects: T_5

Bacterial communities amended with either 1µM Zn^{2+} or a cocktail of trace metals were shown to be different to the control after 5 days of incubation (MDS) (Figure 6.6 B-D). The differences were most apparent in the 0.2 - 2 µm size fractions after 5 days of incubation in both DNA and cDNA derived fingerprints (Figure 6.6B) which successfully separated incubation conditions. The analysis of the diversity of bacterial communities from this size fraction support this observation, indeed in the experimental condition variations between cDNA and DNA derived diversity were 0.08 and 0.07 for Zn^{2+} and the cocktail respectively (Figure 6.13 A and B). Furthermore this was the only size fraction in which the pattern of diversity was consistent between template types (Zn> Cocktail ≥ Control). Analysis (MDS) of the 2 - 20 µm and > 20 µm size fractions successfully distinguished between control and experimental conditions (Figure 6.6 C and D). One possible reason for these differences in resolution between the free-living and particle associated fractions is a mediating effect of particulate matter on metal toxicity. Particles have been shown to reduce the toxicity of metals by providing binding sites for free metals (Hatje *et al.* 2003). Furthermore the extracellular enzymes produced by bacteria associated with particles may act as ligands, again reducing toxic effects (Chen *et al.* 1999). Thus, attached bacteria may be less impacted by trace metals than free-living bacteria.

Diversity analyses also showed that at T_5 bacterial diversity was generally higher in experimental conditions than in the control (Figure 6.13 A and B). One possible explanation is that the introduction of metals resulted in mortality of

previously dominant bacterial phylotypes, allowing bacteria which were previously at a competitive disadvantage to become more dominant. A second possibility, and one which has been hypothesized for the other estuaries studied here (Chapters 4 and 5), is that the addition of metals resulted in a pulse of nutrients due to phytoplankton mortality or toxic effects. Diatom cultures exposed to metals have previously been shown to produce excess carbohydrates, presumably as a chelating agent, as a response to copper toxicity (e.g. Pistocchi *et al.*, 1997). The release of carbohydrates would alleviate nutrient limitation. A third possibility is that the introduction of metals would favour bacteria with metal tolerance capabilities and thus enable them to constitute a detectable fraction of the community following a period of incubation.

6.4.1.1 Longer term incubation effects: T_9

In contrast to the results discussed above, after 9 days of incubation bacterial diversity was, with a single exception, lower in experimental conditions than in the Control condition. This suggests that, following an initial increase in bacterial diversity attributable to the factors listed above, bacterial diversity is negatively affected by trace metals both in single and mixture form. The effect was more pronounced in the cocktail of metals than with the addition of zinc alone (with two exceptions). This indication by diversity analysis (H') is strongly supported by the ordination of samples by MDS which showed that the bacterial community in the 1µM Zn^{2+} condition was more similar to the Control than the community from the Cocktail condition after 9 days. The reduction in diversity reflects work performed by other authors who state that perturbation of bacterial communities may reduce diversity (Øvreas *et al.,*1998; Beaulieu *et al.*, 2000). As was noted previously the fingerprints from communities in the 0.2 - 2 µm size fractions showed the greatest differentiation between conditions. This may be due to more recent formation and colonisation by bacteria directly exposed to the influence of metals. Furthermore, a larger surface area to volume ratio in this size class would result in greater diffusion of metals into the immediate area of the bacterial community *c.f.* larger aggregates. In addition, the nature of aggregation would result in the formation of communities within the matrix of the aggregate. This would protect the bacteria from the effects of metal toxicity by enhancing the diffusion gradient relative to that experienced by bacteria associated with small / young aggregates. Accordingly, the 2 - 20 µm size fractions showed no distinct groupings according to incubation condition. Analysis of the > 20 µm fraction did, however,

differentiate between the control and cocktail conditions. This was reflected by a large difference between the diversity indices of these two conditions. One explanation for the difference between the aggregate associated fractions is the presumed age of the aggregates therein. As an aggregate ages bacterial production has been shown to decrease (Grossart and Ploug 2000; Ploug 2001), this includes the production of extracellular enzymes and other biopolymers i.e. ligands which would reduce metal toxicity (Chen *et al.* 1999; Guibaud *et al.* 2005; Iyer *et al.* 2005). As a result bacteria associated with older aggregates would be less protected from metal toxicity than those associated with younger aggregates.

6.4.2 Determination of suitability of DNA and cDNA derived DGGE fingerprints for the analysis of bacterial response to metals.

At T_0 comparison of the resolution of DNA and cDNA derived fingerprints suggested that the DNA derived fingerprints gave a more consistent result. However, to confer a degree of environmental relevance to results it is also necessary to determine the response of the active fraction of the bacterial community. The active part of the bacterial community was defined as the portion of the community actively synthesising rRNA. Bacterial rRNA is typically broken down within minutes-hours of synthesis (*c.f.* hours-days depending on environmental conditions for DNA; Paul *et al*., 1987) thus the use of rRNA to create cDNA enables the investigation of which of the phylotypes present are active under the conditions described above. This was achieved by comparison of DNA and cDNA derived community fingerprints.

6.4.2.1 Comparison of short term response: T_5

Comparison of MDS ordination plots to investigate the effect of incubation conditions at T_5 suggested that cDNA was the more appropriate template i.e gave greatest resolution between incubation conditions. DNA was able to distinguish between the control conditions and incubations which had been treated with metals, whereas cDNA derived fingerprints clustered according to incubation condition. The cDNA (rRNA) derived fingerprint has been suggested to represent the active fraction of the bacterial community (Schafer *et al.*, 2001; Koizumi *et al.*, 2003), thus it is likely that the differences observed are a result of the effect of incubation conditions on the bacterial community. Furthermore it is likely that these effects would impact directly on bacterial production because they pertain to the active fraction of the community.

The increased resolution between conditions with cDNA as template was not reflected by analyses of diversity. The nature of the Shannon diversity index results in simplification of what is frequently complex data into a single number which could explain the loss of distinguishing power when compared to MDS analysis. A further reason could be that the Shannon diversity index also encompasses the evenness of members of a given community hence domination by a small number of phylotypes, as observed here can overpower the significance of changes in the minor fraction of the community, a phenomenon which is also shared by the DGGE technique. It is probable that more extreme, less variable effects would be identified more consistently by the Shannon diversity index and as such it may be applicable in other studies if interpreted with caution.

6.4.2.2 Comparison of longer term response T_9

At T_9 it was also apparent from MDS analysis that cDNA derived DGGE fingerprints differentiated between conditions with the exception of the >20 µm fraction of the Zn^{2+} incubation which showed greater similarity to the Cocktail condition than the remaining Zn^{2+} conditions. Conversely the DNA derived fingerprints did not cluster according to incubation condition, indeed the >20 µm fraction of the Cocktail and 0.2 - 2 µm fraction of the Zn^{2+} condition were more similar to the Control fingerprints than fingerprints from the same condition. Diversity analyses confirmed that cDNA derived fingerprints provided more evidence for differences between conditions than did those derived from DNA. In all size fractions the diversity calculated from cDNA fingerprints followed the same general trend i.e. Control > Zn^{2+} > Cocktail. This suggests that under these conditions increased incubation time fulfils the pre-requisites for successful application of the Shannon diversity index (H'). In addition this result supports the possibility that the addition of a cocktail of trace metals has a more profound affect on bacterial community structure than addition of zinc alone. This conclusion has been discussed previously (Chapter 5) with supporting bacterial production and abundance data and appears to hold true in the moderately contaminated estuary studied here.

6.4.3 Is the effect of aggregate association on bacterial community structure more important than effects conferred by metal addition?

6.4.3.1 Comparison of influence of trace metals and aggregate association on bacterial community structure; T_5

Comparison (MDS) of fingerprints from different size fractions at T_5 showed as much variation within size fractions as was observed between size fractions, although there did appear to be greater similarity between samples from the > 20µm size fraction. This suggests that the influence of metal toxicity on bacterial community structure was greater than effects conferred by size fraction. It is possible that at this early stage of aggregate formation, the aggregate (and micro-aggregate) associated bacterial community is still undergoing exchange with the surrounding water column, furthermore the formation of aggregates is an ongoing process by which micro-aggregates are assimilated into larger aggregates and as such numerous overlaps between communities would be apparent. This could also account for the apparent similarity observed in the > 20µm fraction. The theory that there would be exchange between the two smaller size fractions is supported by the similarity of the dominant bands observed in the 2 - 20 µm and 0.2 - 2 µm size fractions within conditions. Previous studies of differences between diversity on and off estuarine particles (Acinas *et al.* 1999; Bidle and Fletcher 1995; Crump *et al.* 1999; Delong *et al.* 1993; Rath *et al.* 1998) have shown distinct differences between aggregate associated and free-living bacteria. They have also shown that some overlap exists between the two distinct communities, attributable to attachment and detachment processes. However, these studies were performed without any additional influencing factors (e.g. metal addition) and thus are not directly comparable with the results presented here. Differences observed between size fractions within experimental conditions in this study are supported by evidence presented in such papers.

6.4.3.2Comparison of influence of trace metals and aggregate association on bacterial community structure; T_9

Comparison of MDS between size fractions at T_9 showed no size specific grouping in either the cDNA or DNA derived fingerprints. Groupings were observed according to incubation condition suggesting that the effects of metals on bacterial community structure were greater than those conferred by aggregate association. When

analyses were performed on each condition it was apparent that the effects of aggregate association were more important than the effects of template type in both the Control and Zn^{2+} condition. This suggests that whilst the dominant factor controlling bacterial community structure was incubation condition, aggregate association also played a role. This pattern does not hold true for the Cocktail condition in which template type had a greater effect on bacterial community structure than aggregate association. This supports the suggestion that the influence of incubation condition is more important than aggregate association in highly contaminated conditions.

6.4.4 Affect of incubation time on dominant bacterial phylotypes

6.4.4.1 Short-term changes in dominant phylotypes due to incubation conditions

Sequence analysis of dominant phylotypes shows that, whilst the community is still dominated by a number of the same phylotypes as observed in the T_0 samples, a single phylotype appears which is not apparent in either the T_0 or T_9 samples and four more phylotypes appear which remain after 9 days of incubation. These phylotypes are closely related to two uncultured salt marsh bacteria, a protease producing arctic bacterium and a bacterium associated with particles in the Weser Estuary. It is suggested that the incubation technique used here provided a suitable environment for these phylotypes to become dominant. Their presence in all conditions implies that it is the formation of aggregates which lead to their dominance, however, their ubiquity at this time point also suggests a certain level of metal tolerance.

6.4.4.2 Longer term changes in dominant bacterial phylotypes as a result of incubation conditions

Analysis of sequence data from bands taken from the gel shown in Figure 6.8 (T_9) shows that a number of bacterial phylotypes, which were previously present as minor parts of the community, and as such were not sequenced, became dominant after 9 days of incubation. These phylotypes were most closely related to a marine biofilm bacterium (*Loktanella rosea*), Antarctic rRNA derived sequences (*Sulfitobacter* strains F4C85 and F2C84), a seagrass associated bacterium (*Sulfitobacter dubious* KMM3554T), a microbial mat bacterium (Milano-WF2B-23) and an Arctic sea ice associated *α-proteobacteria* clone (ARK 10207), the majority of which are associated with surfaces. This observation supports the theory that these bacteria have become dominant through the process of aggregate formation. As mentioned previously their

presence in experimental conditions also suggests a level of metal tolerance. This could be a result of biofilm formation which would create a diffusive barrier thus providing protection against the toxic effects of metals. It has been shown that the formation of biofilms by *Pseudomonas aeruginosa* (Teitzel *et al.* 2003) confers greater metal tolerance when compared to free-swimming *Pseudomonas aeruginosa* supporting this theory. Furthermore, the internal surface of the bottle may be a suitable surface for biofilm formation suggesting that formation of aggregates is not the sole reason for the development of these communities.

6.5 Conclusions

The results presented here clearly indicate that change in bacterial communities is detected more readily through the use of rRNA derived DGGE than rDNA derived DGGE. This conclusion is supported by the different nature and longevity of the nucleic acids in question. It was also apparent that the effects of metals on bacterial communities are more profound in the bacteria associated with the smaller (0.2-2 µm) fraction of the suspended particulate matter and the free-living community. This implies that the formation of and association with both micro- and macro-aggregates can reduce the negative impacts on bacterial communities associated with metal toxicity. Studies regarding the distribution of metals and bacterial communities within aggregates would significantly enhance our understanding of this phenomenon.

7 Conclusions and future work

The response of bacterioplankton communities to trace metals has been investigated using a combination of molecular and microbiological methods in a number of aquatic environments, encompassing a range of background trace metal concentrations with different compositions and concentrations of organic matter. This chapter summarises these responses, describes the wider implications thereof and outlines recommendations for future research.

7.1 Response of bacteria from a diverse range of environments to trace metal enrichment; controlling factors and key observations

The results obtained in chapters 3, 5 and 6 have demonstrated that it is difficult to generalise regarding the effects of trace metals on the bacterial communities from a wide range of environments such as those studied here. However, it is possible to reach the following conclusions. The addition of metals (concentrations one or two orders of magnitude higher than expected under normal *in situ* conditions) invariably resulted in a change in the diversity / species richness and community dynamics of the bacterial community (chapters 3, 5 and 6). The degree and nature of the change was dependant on the environmental conditions, bacterial community present, concentration of metal added (chapters 3 and 5), and mediation of effects through association with suspended particulate matter (Chapter 6). Furthermore, the work presented here (Chapter 5) showed that the metals added could be split into two broad types (Type 1 and Type 2) based on bacterial response in terms of production and abundance.

Bacterial communities from coastal and oceanic environments appeared to have different zinc tolerance regimes (Chapter 3). In the coastal environment bacteria were either tolerant or intolerant, regardless of concentration. In contrast, bacteria from the oceanic environment had a range of threshold levels above which they were intolerant.

The bacterial community from a pristine estuary was remarkably resilient to the effect of trace metal contamination (Chapter 5) in terms of maintaining productivity levels comparable to the control. Bacteria in the cocktail condition were the most inhibited with regards to both production and numbers both pristine and moderately impacted estuarine environments. Nevertheless, in all environments and conditions studied, bacteria were present with ribosomal activity (Chapter 6) or with the ability to

incorporate leucine into cellular protein (chapters 3, 4 and 5), even with the addition of a cocktail of trace metals (chapters 5 and 6).

The addition of trace metals was shown to result in decreased diversity of bacterial communities from a moderately contaminated estuary (Chapter 6) when compared to the control. In addition, sequence analysis of dominant bacterial phylotypes from estuarine samples (chapters 5 and 6) showed that metal impacted bacterial communities were dominated by members of the *Rhodobacteraceae* family of the alpha-proteobacteria.

The incubation of natural samples on aggregate rollers resulted in the formation of aggregate associated bacterial communities which were comparatively stable (in terms of dominant phlotypes) when compared to the free-living bacterial communities (chapters 4 and 6), even under trace metal stress (Chapter 6). Further investigation of bacterial communities associated with different size fractions of the SPM (Chapter 6) showed that free-living communities were more susceptible to trace metal toxicity, as demonstrated by reduced diversity in the 0.2-2 µm fraction (Chapter 6). It was also apparent that association with aggregates >2 µm in size conferred a measure of metal resistance on the bacterial community. Furthermore, this study demonstrated the increased sensitivity of an rRNA based approach (when compared to an rDNA approach) to following change in bacterial communities resulting from trace metal enrichment.

7.2 Implications of trace metal contamination in natural environments

7.2.1 Influence of metal contamination on cycling of major elements.

The changes in bacterial communities summarised above, namely reduced diversity and altered bacterial abundance and production is of critical importance in the cycling of major elements on a local scale. The likelihood of sufficient metal contamination to have a global affect is low, nevertheless, the effect of metal perturbation can have serious ramifications on a local scale due to bacterial roles in the cycling of both carbon and nitrogen. Decrease in bacterial productivity and diversity may have localised implications for the recycling of organic matter, transformation of greenhouse gases and transfer of carbon through food chains, out of estuaries and from the photic zone to the deep sea. Reduction in phylogenetic diversity can also reduce the metabolic diversity of a bacterial community which may have serious implications for

the removal of certain forms of organic matter from a system. Furthermore, reduced bacterial nitrogen fixation would affect the provision of nutrients in an available form to phytoplankton, thus reducing the capacity to fix carbon dioxide and produce oxygen.

7.2.2 Considerations for determination of metal toxicity in aquatic environments

The studies described here have clearly demonstrated that insufficient length of incubation can result in an overestimation of long term affects of trace metal enrichment on bacterial communities. Investigations performed here showed that in terms of bacterial numbers and production estuarine bacterial communities as a whole are remarkably resilient, even when they originate from pristine environments (Chapter 5). However, analysis of bacterial production and numbers observed in the early stages of incubation indicated serious inhibition of bacterial communities as a result of the addition of trace metals. The majority of single-species toxicity tests using bacteria (reviewed by Bitton and Koopman, 1992) are selected, aside from economic and practical considerations, based on fulfilment of the following criteria: Firstly, response time to toxicants, e.g. Microtox® is commonly performed over durations of up to 30 minutes (e.g. Kaiser and Palabrica, 1991), and thus enables high throughput of samples. The requirement for longer incubations to determine the toxicity of a number of trace metals (specifically zinc, copper and cadmium) by such methods has been highlighted previously (Preston *et al*., 2000). Secondly, sensitivity to the substance under observation, however, the diversity of the bacterial communities in most environments is likely to consist of bacteria with variable levels of resistance to toxic effects of any given contaminant. Single 'indicator species' give a good indication of the worst case scenario thus providing a useful starting point for the protection of ecosystems (Versteeg et al., 1999), however, detrimental effects may be overestimated and thus not necessarily environmentally relevant. The next best way to determine affects of contaminants on receiving systems is through ecosystem modelling, however, the lack of environmentally relevant data hampers the ability of modellers to create accurate predictive models of the effects of contaminants on aquatic ecosystems. Furthermore, single species tests do not take into account the affects of contaminants on the diversity of bacterial communities and the associated myriad different substrates utilised by bacteria under *in situ* conditions. This is arguably more important when assessing the impact of a contaminant on an ecosystem in terms of function.

A further consideration when assessing the effects of metal toxicity on bacterial communities is the apparent role played by aggregates in mediating community change as a result of metal enrichment. Analysis of the diversity of bacteria associated with aggregates demonstrated that this portion of the community was less variable, over the time scales investigated, than the free-living portion of the community. This suggests that the physical environment may ameliorate the diversity reducing affects of metal toxicity, and may imply that the effects of metals should be assessed separately for the attached and free-living communities. Furthermore, the relative proportions of the bacterial community associated with aggregates should be assessed to give a clearer indication of the likely impacts of metal toxicity on nutrient cycles in contaminated environments.

7.2.3 Relationship between metal resistance and antibiotic resistance

The response of bacterial communities to trace metal enrichment may also affect the proportion of the community which are able to resist the effects of antibiotic treatment. It has become more apparent in recent years that the ability of commonplace bacteria to withstand treatment by antibiotics has serious implications, particularly in the sick, very young or elderly people. As early as 1977, bacteria which were resistant to metals were discovered to also harbour resistance to antibiotics (Allen *et al.,* 1977, cited in Calomiris *et al.,* 1984; Timoney *et al*., 1978). The genes responsible for antibiotic resistance have been found to be located on a plasmid clustered with genes which conferred metal tolerance on the bacteria, thus introduction of metals into aquatic environment is suspected to increase selection pressure for bacteria with the ability to tolerate antibiotics (Baquero *et al*., 1998). This relationship between metal and antibiotic resistance should be taken into account when legislating contaminant inputs into aquatic environments, particularly those used for bathing and recreational purposes.

7.3 Future directions

7.3.1 Screening of isolates for determination of metal resistance

During the course of the studies presented here a number of putatively metal tolerant bacteria / consortia have been isolated (methods and data not presented here). Preliminary examination of randomly selected representatives indicated enhanced

growth under metal enriched conditions *c.f.* identical media without metal enrichment (Appendix A, Figure A.1). Identification and screening of these isolates / consortia for a) degree of metal tolerance, b) metal tolerance genes and c) metal binding ligands / proteins would be the next logical phase of this research.

To clarify the importance of the type (and amount) of organic matter present in determining the degree of bacterial response to trace metal enrichment, the isolates would provide a good starting point for laboratory studies. One possible study would involve the enrichment of actively growing bacterial cultures with controlled amounts of different types of organic matter, in concert with exposure to metal stress. This would indicate to what degree metal toxicity is mediated by different components of the environmental matrix and allow determination of the most influential substances.

7.3.2 Identification of key genes involved in metal resistance

A number of key metal tolerance genes have been identified in bacteria commonly associated with metal rich environments (reviewed by Nies, 1999), in addition the entire genome of a key metal tolerant species *Ralstonia metallidurans* has been sequenced and screened for metal resistance genes (Mergeay et al., 2003). These genes have been subjected to a proteomics approach to allow characterisation of the proteins involved in metal resistance. The use of data presented and bioinformatics approaches to create primers with appropriate degeneracy may enable the amplification and identification of genes present in the environment which confer metal tolerance on the native bacterial community. Use of DNA based approaches would facilitate the identification of metal tolerance genes and give an indication of the potential of bacteria in a given environment to tolerate trace metals. However the information that could be gleaned from this approach is limited by the longevity of DNA in the environment, an RNA based approach would provide greater insight into which functional genes are expressed and therefore likely to play a significant role in metal tolerance in a given environment / metal stress level.

The use of an RNA based approach introduces a new set of limitations, particularly when working with genes expressed in response to a narrow range of environmental stimuli. This is one of the factors which make the use of 16S rRNA, which is expressed whenever a cell is actively producing protein, so attractive. It is likely that a large volume of environmental sample or high density of cells would be required to obtain sufficient RNA to analyse which genes are being expressed and

when. Despite these limitations there is potential for the molecular approaches described here to lead to the identification of marker genes which would indicate metal stress in different functional (and possibly phylogenetic) groups of bacteria, thus providing a more environmentally relevant indication of the effects of trace metal contamination on bacteria.

An extension of the genomic approach described above would involve examination of the environmental metagenome via the creation of fosmid libraries. This approach involves the extraction of nucleic acids from the environment and insertion of fragments (usually around 40 kb) into appropriate fosmid vectors. There is potential for the same fragment to carry functional and phylogenetic information which would clarify which bacteria were the origins of which metal tolerance genes. This would then enable the researcher to identify which bacteria were responsible for the maintained function of the bacterial community in terms of metabolic activity (as observed in Chapter 5).

7.3.3 Investigations of effects of metals on cycling of major elements

Bacteria are clearly implicated in the cycling of major nutrients in aquatic systems, and are particularly involved in carbon and nitrogen cycles. The activity of bacteria in aquatic environments supports the productivity of phytoplankton and therefore the majority of the aquatic food web. Whilst it is acceptable to hypothesise about the likely effects of metal toxicity on bacterial function, there are a number of methodologies available which would clarify the effects of decreased bacterial diversity (in response to trace metal enrichment) on bacterial activities which influence the cycling of major nutrients / elements. One example is the use of labelled (fluorogenic / radiolablelled / stable isotope) substrates to determine change in the rate of substrate uptake by bacteria.

Stable isotope probing would also facilitate downstream applications such as identification of active bacteria using 16S rRNA. Alternatives would include the use of Biolog ® plates to determine substrate utilisation by bacteria subjected to trace metal stress. The investigation of such factors, ideally in concert with genomic analysis, would give a more informative picture of the potential effects of trace metal enrichment on the ability of bacteria to recycle major nutrients. A further possible mechanism for the analysis of the effects of trace metals on nutrient cycles would be via the analysis of expression of key genes (such as rubisco or nif genes involved in carbon and nitrogen

cycles). Determination of the change in expression, perhaps via RT-real time PCR, would demonstrate the degree of inhibition or enhancement of the bacterial contribution to relevant cycles.

7.3.4 Collaboration between disciplines

The interpretation of data presented here would have been greatly facilitated by the availability of data regarding changes in metal speciation / dissolved concentration, composition of aggregates, aggregate associated bacterial production and numbers of bacteria associated with particles. This highlights the importance of collaboration between disciplines when investigating systems of this level of complexity.

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Method	Advantages	Disadvantages	Environments	Selected publications
Automated ribosomal intergenic spacer analysis ARISA	Highly reproducible community profiles	Requires large quantities of DNA Resolution tends to be low PCR biases	Soils Fresh water Estuary	Ranjard *et al.* 2000 [a,b,c]; Borneman & Triplett 1997 Yannarell & Triplett 2004 ; Fisher & Triplett 1999 Hewson & Fuhrman 2004
Terminal restriction fragment length polymorphism T-RFLP	Simpler banding patterns than RFLP Can be automated High resolution	Dependent on extraction and lysing efficiency Requires very pure DNA Large PCR biases Type of Taq can increase variability Delicate, expensive equipment Choice of restriction enzymes will influence community fingerprint	Activated/bioreactor sludge, termite guts. Marine Contaminaged (Hg) soils Rhizosphere Agricultural soils	Liu *et al.* 1997 Moeseneder *et al.* 1999 Bruce 1997 Schmalenberger & Tebbe 2002 Toms-Petersen *et al.* 2003 Ovreas *et al.* 2003
Denaturing / Temperature gradient gel electrophoresis D/TGGE	Large number of samples can be analyzed simultaneously Reliable, reproducible and rapid Relatively inexpensive	Dependent on lysing and extraction efficiency PCR biases One band can represent more than one bacterial type (co-migration)	Marine Marine sediments Microbial mat/biofilm Soil	Moeseneder *et al.* 1999 Gillan 2004 Muyzer *et al.* 1993, Santegoeds *et al.* 1996 Nicol *et al.* 2003 ; Nakatsu *et al.* 2000; Sandaa *et al.* 2001

Method	Advantages	Disadvantages	Environments	Publications
DGGE continued	equipment	Only detects dominant species (>1%)	Hypersaline solar salterns Fresh water Review	Ovreas *et al.* 2003 Cummings *et al.* 2003; Massieux *et al.* 2004 Muyzer 1999
Single strand conformation polymorphism SSCP	Same as DGGE/TGGE No GC clamp No gradient	Same as DGGE/TGGE Reannealing during electrophoresis Some ssDNA can form more than one stable conformation Short DNA fragments (150-400bp)	Rhizosphere Activated soils Compost	Schweiger and Tebbe 1998 Beaulieu *et al.* 2000 Peters *et al.* 2000
Amplified ribosomal DNA restriction analysis ARDRA (also known as RFLP)	Detect structural changes in microbial community	Banding patterns often too complex Multiple bands from single bacterial type	Plant root microenvironments Agricultural soil Estuary	Moenne-loccoz *et a*l. 2001 Moffett *et al.* 2003 Crump *et al.* 1999 Liu *et al*. (1997)

Table A.1 Examples of molecular fingerprinting methods used to assess the effects of perturbation on microbial communities and the environments in which they have been successfully applied. Table adapted from Kirk *et al*. (2004).

Pigment	Algal Class
19’ Butanoyloxyxanthin	Some prymnesiophytes, one crysophyte, several dinoflagellates
19’ Hexanoyloxyxanthin	Prymnesiophytes, several dinoflagellates
Alloxanthin	Cryptophytes
Chlorophyll a	All photosynthetic microalgae except prochlorophytes
Diadinoxanthin	Diatoms, dinoflagellates, prymnesiophytes, chrysophytes, raphidophytes, euglenophytes
DV Chlorophyll a	Prochlorophytes
Fucoxanthin	Diatoms, prymnesiophytes, chrysophytes, raphidophytes, several dinoflagellates
Lutein	Green algae: chlorophytes, prasinophytes
Peridinin	Dinoflagellates
Violaxanthin	Green algae: chlorophytes, prasinophytes, eustigmatophytes
Zeaxanthin	Cyanophytes, prochlorophytes, rhodophytes, chlorophytes, eustigmatophytes

Table A.2 Signature pigments useful as indicators of algal class.

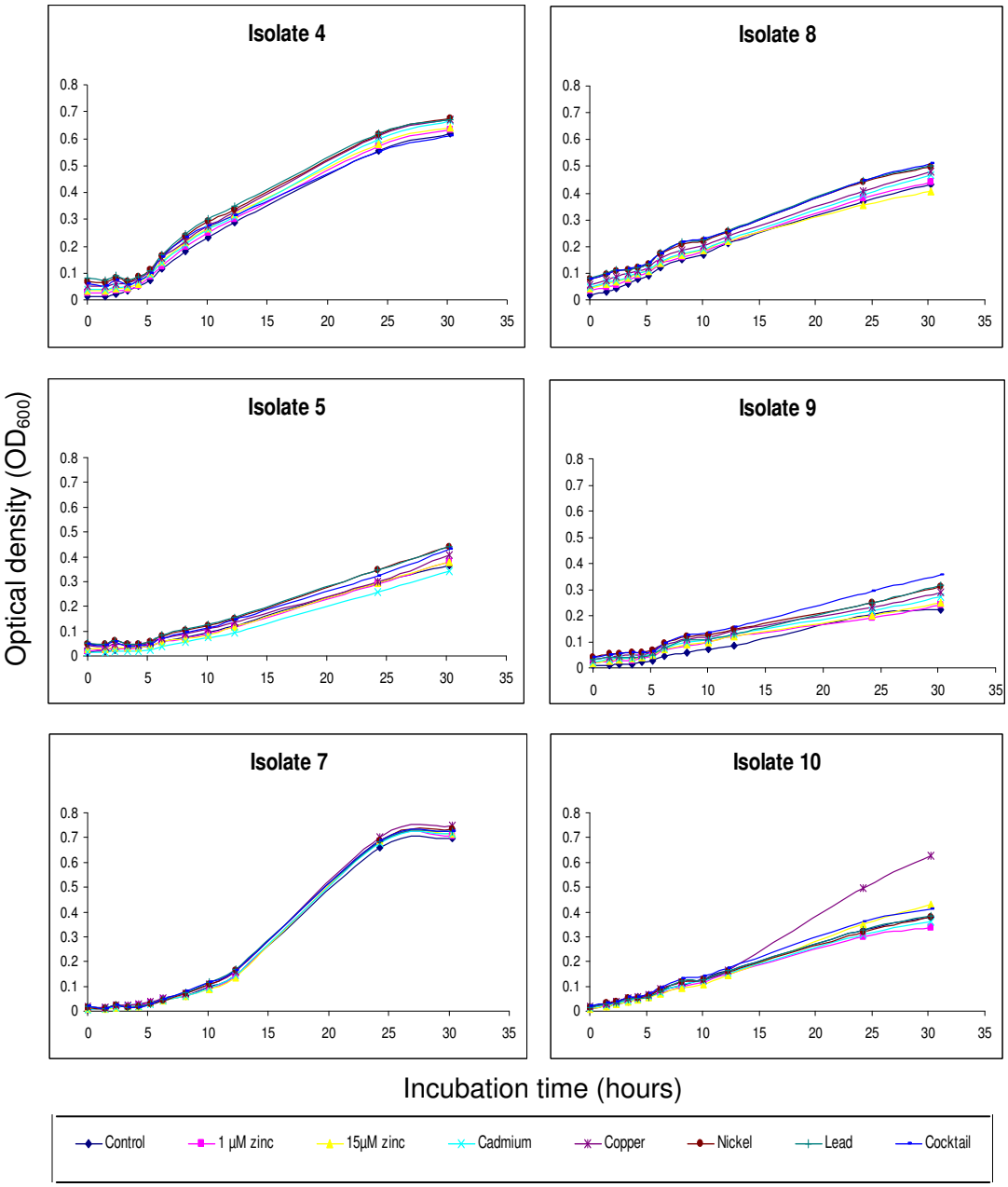

Figure A.1 Examples of response of bacterial isolates to different trace metal enrichments. Cultures grown over 3 days in 210 medium enriched with trace metals as indicated.