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1 **Linking pollution induced community tolerance (PICT) and microbial community**
2 **structure in chronically metal polluted estuarine sediments.**

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Abstract

We tested the ability of Pollution Induced Community Tolerance (PICT) to detect the effects of chronic metal pollution on estuarine sediment microbial communities, along a gradient spanning two orders of magnitude in metal concentrations. In tandem, we investigated the associated microbial community structure using terminal restriction fragment length polymorphism (T-RFLP). Tolerance of microbes to Cu, measured as IC₅₀ (inhibitory concentration 50 %), was strongly correlated with pore water Cu concentration ($r^2=0.842$). No strong correlation existed for other metals tested, highlighting the ability of PICT to identify the pollutant causing a toxic effect. There was no correlation between microbial community structure and community tolerance to metals tested, but analysis of community structure did provide some information on reasons for observed PICT response. PICT methodology used here provided a greater strength and consistency of association with pollutant concentration compared to microbial community structure and can be recommended as a sensitive indicator of metal pollution on estuarine sediment microbial communities.

Keywords

PICT, heavy metals, T-RFLP, estuarine sediment, microbial community structure

1. Introduction

One of the main problems in pollution studies is to find a sensitive, reliable and consistent ecotoxicological tool that distinguishes the effect of natural variation from anthropogenic changes (Gray, 1979). Complex marine, terrestrial and estuarine ecosystems exhibit temporal and spatial variability which mask changes caused by pollution except in the most severe cases (see Warwick & Clarke, 1991; Grant & Millward, 1997). The ideal ecotoxicological tool needs to be sensitive enough to detect the effects of potential pollutants such as heavy metals well before gross impacts, such as large reductions in diversity occur (e.g. Gans et al., 2005). Pollution induced community tolerance (PICT) has been proposed as such a tool (Blanck et al., 1988). This is based on the principle that communities exposed to pollutants become tolerant due to individual acclimation, genetic or physiological adaptation and loss of sensitive species due to long-term exposure to a toxicant or mixture of toxicants (Luoma, 1977; Blanck et al., 1988; Millward and Grant, 2000). Whilst changes in community structure may be due to a number of environmental factors, tolerance to a specific toxicant at the community level can only be elicited by the toxicant itself, a chemically similar toxicant, or by compounds or conditions affecting its bioavailability. PICT provides strong evidence therefore that a chemical has altered a community. Quantification of pollutant effects on a community requires the detection of an end-point (an effect indicator), usually a metabolic or functional process (e.g. photosynthesis, respiration, and biosynthesis of nucleic acids and proteins) within a short-term dose response test (see Blanck, 2002; Boivin et al., 2002, for review).

Heavy metal contamination within coastal and estuarine environments is a recognized, worldwide problem (Clark, 2001). Estuarine sediments provide a sink for heavy metals (Salamons et al., 1987) which preferentially form complexes with sediment particles (Middleton and Grant, 1990) and so in turn, also present a potential contamination source (Bryan and

Langston, 1992). Microorganisms play a role in the distribution and fate of these metals (Ford and Ryan, 1995) alongside their importance to key ecosystem processes, such as biogeochemical cycling within sediments (Eismann and Montuelle, 1999). The known toxic effects of heavy metals on these functions (Giller et al., 1998), also gives ecological relevance to their use as ecotoxicological tools (Siciliano & Roy, 1999; van Beelen, 2003). Microbial communities have been used to demonstrate the long term consequences of heavy metal contamination, reporting positive correlations between environmental metal concentration and increased microbial community tolerance (Díaz-Raviña et al., 1994; Díaz-Raviña and Bååth, 1996; Pennanen et al., 1996; Blanck and Wängberg, 1988; Wängberg et al., 1990; Lock and Janssen, 2005). These increased tolerances were further correlated with changes in community structure e.g. Pennanen et al., (1996) found increased tolerances were accompanied by changes in phospholipid fatty acid (PLFA) patterns. More recently, developments in molecular fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) have allowed investigations of genetic diversity to distinguish between polluted and pristine environments (Clement et al., 1998; Tom-Peterson et al., 2003; Gillan et al., 2005). Using DGGE, Mertens et al., (2006) demonstrated soil ammonia oxidising community structure and Zn tolerance were correlated with soil Zn concentrations. These existing studies however, have focused mainly on soil and aquatic microbial communities with limited information existing on the response of estuarine sediment communities. Dahllöf et al., (2001) used functional diversity (Biolog™ plates) to indicate increased TBT tolerance in culturable microbial communities from estuarine sediment box-cosms. Millward and Grant., (2000), found estuarine sediment nematode communities to be a more sensitive indicator of metal impacts than nematode populations, but no information exists on the effects on the microbial communities to

which these nematode communities are inextricably linked. The aim of this study was therefore to a) examine whether field collected estuarine sediment microbial communities show increased tolerance to metals in response to concentrations that span two orders of magnitude and b) to determine if changes in tolerance are reflected in microbial community structure.

2. Materials and Methods

2.1 Collection, preparation and physico-chemical analysis of sediment samples

Estuarine sediment was collected in May 2002 from 11 sample sites, 10 within the Hayle Estuary [OS map reference SW 568381] (sites 10, 11; Figure 1A) and Fal Estuary [OS map reference SW 784388] (sites 1,2, 4-9; Figure 1B) plus one control site at Kingsbridge Estuary [OS map reference SX 736437] (site 3; see Figure 1). The sample sites located in Cornwall and Devon, South West England, U.K, represent a gradient of Cu and Zn pollution, spanning two orders of magnitude resulting from the production of up to 50 % of the world's supply of Cu, Sn and As (Bryan and Gibbs, 1983) from over 1000 mines during the 19th century. This 200 year history of mining activity has led to the deposition of large amounts of metals into surrounding streams and rivers, with high concentrations accumulating within the sediments of these receiving waters. At each site, duplicate sets of sediment samples 0.5m apart were collected for PICT analysis, pore water metal and salinity, pH, sediment particle size, total carbon and T-RFLP analysis. Samples for molecular analyses were collected in 3x 50 ml polypropylene sterile tubes (Corning), for all other analyses 2.5 l composite sediment samples (in airtight polypropylene buckets) from each site were collected. Separate polypropylene scoops were used for each site. On arrival back in the laboratory sediment samples were stored at -80 °C (for DNA

extraction), 15 °C (for PICT) and 4 °C (for physico-chemical measurements), respectively. Due to logistics of sample collection in Cornwall, time between first sample collection and being placed in appropriate storage conditions was up to 36 hours. Surface oxic sediment (0 -5 cm) was used for all analyses. The depth of the oxic layer was determined by visual inspection and varied between sites. Pore water was extracted from 3x 50 ml sediment samples from each field site by centrifugation at 4 °C for 10 minutes at 4200 rpm (Sorvall Microcentrifuge). Supernatants were filtered through a 0.45 µm syringe filter (Sartorius) and used to measure pH and salinity. 1 M HNO₃ was added to triplicate 10 ml pore water samples to give a concentration of 1 % and then stored in polypropylene containers in the dark at 4 °C until analysis of Cu, Zn, Cd and Cr using Inductively Coupled Plasma- Atomic Emission Spectrometry (ICP-AES) (Vista-Pro CCD Simultaneous ICP-AES). Procedural blanks (x3) using MilliQ™ ultrapure water were taken through each step of the extraction process. Sediment samples were dried to a constant weight and ground using a pestle and mortar. 10 mg (dry weight) aliquots of sediment were weighed accurately into tin capsules and sealed for elemental analysis (total carbon). Samples were analysed using the Carlo Erba C.H.N.S. EA 1108 Elemental Analyser.

2.2 Community tolerance measurements

Cu tolerance at the level of the community was tested at 11 samples sites. Community tolerance to Cd, Cr and Zn was measured in 4 sites (2, 3, 9, 11). These 4 sites were chosen as representative of the high and low range of metal contamination. PICT was measured using the thymidine and leucine microcentrifuge assay, as described below. At each site replicate 2 g sub samples were taken from a 2.5 l homogenized sediment sample. Each metal concentration was tested in duplicate or triplicate. 3-7 un-amended samples (controls) and zero-time controls

(blanks) for each sample site were used.

2.3 Thymidine and Leucine microcentrifuge assay (PICT measurement)

Metal tolerance of sediment microbial communities was determined by a microcentrifuge thymidine and leucine incorporation method essentially according to Bååth et al., (2001), with minor modifications. Briefly, microorganisms were extracted from sediment (2 g wet weight sediment /50 ml sterile 75 % artificial sea water) via sonication using an Ultrasonic II water bath for 3x 1minute then low speed centrifugation (1000 g for 10 minutes). Bacterial suspensions (1.35 ml) were placed in 2 ml vials and amended separately with Cu, Zn, Cd and Cr using metal salts (CuSO_4 , ZnSO_4 , CrSO_4 , CdCl_2) in 0.15 ml sterile 75 % ASW, at a range of concentrations (5-9 concentrations from 0-1000 mg l^{-1}) and incubated for 2 hrs at room temperature (23 – 26 °C). Metal concentrations were analysed in non-radioactive sample aliquots using ICP-AES (Vista-Pro CCD Simultaneous ICP-AES). To test for differences in exposure concentrations of metals during test procedure, metal concentrations in control bacterial extract and in metal spiked test samples were measured after 2 hours incubation. Triplicate samples at each metal concentration were filtered using a 0.45 μm syringe filter (Sartorius), after flushing with (3 x) 10 % HCl and MilliQ™. 1 M HNO_3 was added to give a concentration of 1 % and stored in the dark at 4 °C until analysis. Concentrations of metals in bacterial extracts were very low and there were no significant differences in metal concentrations between bacterial extracts from different sites spiked with the same metal concentration, thus allowing direct comparison of tolerances (data not shown).

2.4 Community structure analysis using T-RFLP

Microbial community DNA was extracted in duplicate from 2 composite sediment samples (each consisting of 3x 50ml sediment) 0.5m apart, at 8 samples sites (1-6, 9, 11), following a procedure described by Zhou et al., (1996) and modified by Osborn et al., (2000). Minor deviations from this protocol were as follows: sediment aliquots (0.5 g) were washed twice in pH 6 Milli Q and centrifuged at 4200 rpm for 1 minute before beginning protocol. The resultant DNA pellet was resuspended in 25 µl of sterile distilled water and left overnight at 4 °C to dissolve. DNA extractions were pooled and quality and quantity of resulting DNA was established by electrophoresis on a 1 % TBE (Tris-Borate-EDTA) gel. DNA samples were purified using the QIAEX II Gel extraction kit (Qiagen), quantified as described above, and stored at -20 °C until required. Reaction mixtures for polymerase chain reaction (PCR) contained template DNA (100 ng), 5 µl of 10x reaction buffer, 2.5 mM MgCl₂, 0.1 µM of each primer, 200 µM each of deoxynucleoside triphosphate and 1.25 units *Taq* DNA polymerase (Bioline) in a final volume of 50 µl. The primers used were 63 F (5'-CAGGCCTAACACATGCAAGTC-3') and 1389 R (5'-CGGCGGGTGTGTACAAG-3') (Osborn et al., 2000) labelled at the 5' end with the dyes 6-FAM and HEX (MWG UK), respectively. Cycling conditions were as follows: initial denaturation for 2 minutes at 94 °C; 30 cycles of; 1 min at 94 °C; 1.5 min at 55 °C; 1 min at 72 °C, followed by a final extension at 72°C for 10 min. Quality, size and yield of PCR product was determined by electrophoresis. Duplicate PCR products were produced and pooled to reduce amplification bias (Wintzingerode et al., 1997). After purification with Qiaquick® PCR Purification Kit (Qiagen), aliquots containing approximately 200 ng of PCR products were digested separately with 10 U of restriction endonucleases *Cfo* I, *Rsa* I (ROCHE) or *Alu* I (Invitrogen). The digestions were performed in a

total volume of 15 µl at 37 °C for 3 hours according to manufacturer's instructions. Aliquots (1 µl) of the digested samples were mixed with 1.5 µl deionised formamide, 0.5 µl of ROX-labelled GS2500 internal lane standard (PE Applied Biosystems) and 0.5 µl of loading buffer. Each sample was then denatured at 95 °C for 5 min and transferred to ice. Duplicates of each sample (1.5 µl) were electrophoresed on a 6 % polyacrylamide gel using an ABI 377 (Applied Biosystems Inc.) automated sequencer for 6 hours under the following conditions: 2875 V, 60 mA, 46 °C, filter set D. Fragment size was measured with reference to the internal lane standard and peak area of terminal restriction fragments using Genescan Analysis Software (Version 3.6, PE Applied Biosystem). Peak areas were scaled using Genotyper Software v3.7 (PE Applied Biosystems) and percentage abundances calculated. To reduce the influence of rare taxa and create a subset of reproducible peaks, only those peaks with a percentage abundance of ≥ 0.5 % in at least one sample were considered in the analysis.

2.5 Statistics

Biodiversity within estuarine sediment microbial communities was measured using Shannon-Weiner diversity (H'). Blackwood et al., (2007) have suggested diversity measures derived from T-RFLP data can be used to reflect differences in community composition. Similarity percentage breakdown (SIMPER analysis) was used to determine the Terminal Restriction Fragments (TRFs) typical of each site. To provide a measure of similarity between the profiles, multidimensional scaling (MDS), a multivariate ordination technique was used on square root transformed TRF abundance data using a Bray-Curtis similarity matrix (Clarke and Warwick, 1994). To select environmental variables 'best explaining' community structure the BIOENV procedure was used. BIOENV analysis maximizes rank correlations between a

similarity matrix for TRF data and a similarity matrix calculated on environmental variables. The variable or combination of variables giving the largest correlation co-efficient, are assumed to be the environmental factor(s) best explaining community structure. Significance of differences in community structure was tested for using a one way analysis of similarities (ANOSIM) using peak area abundances. Data resulting from the 6-FAM labeled primer (63F) only was used in analysis.

All statistical analysis was carried out using Primer v5 (Clarke, 1993). Microsoft® Excel (2002) was used for producing graphs, significance testing (t-test) and correlation analysis. All results were considered significant at $P < 0.05$.

2.6 Construction of a 16S rRNA gene clone library from sediment samples

A 16S rRNA gene clone library was constructed to aid identification of TRFs. DNA extracted from field collected sediments at Kingsbridge Estuary (OS map reference SX 736437) and Hayle Estuary (OS map reference SW 568381) was amplified using unlabelled universal primer set 27f and 1492r (Lane, 1991) with PCR conditions as before. 50 ng (insert:vector molar ratio of 3:1) of PCR product from sediment samples was cloned using the pGEM-T Easy vector System kit (Promega) following manufacturers instructions. Positive clones were selected and inserts amplified by whole cell PCR using T7 and SP6 plasmid primers under cycling conditions as previously described. To determine which clones to sequence, restriction fragment length polymorphism analysis (RFLP) of these amplification products (96 clones) was carried out using *Cfo* I (ROCHE) and *Alu* I (Invitrogene) restriction enzymes in separate 10 µl reactions containing 7.5 µl PCR product and 5 units of restriction enzyme. Restriction products were electrophoresed on a 3 % agarose gel (Metaphor) in TBE. From these RFLP patterns 80 clones

were chosen for sequencing of the 16S rRNA gene inserts. This was based on the presence of a RFLP fragment, unbeknown whether terminal or not, that corresponded to a T-RFLP fragment. Sequencing was carried out bi-directionally using T7 and SP6 primers at the John Innes Centre (Norwich U.K.) sequencing facility using an ABI 3700 (Applied Biosystems Inc.) after a TempliPhi amplification step of supplied colonies. A chimera check using the Bellerophon Server at <http://foo.maths.uq.edu.au/~huber/bellerophon.pl> was carried out on all sequences gained. Partial 16S rRNA gene sequences with TRFs of the same size as found in T-RFLP profiles gained from sample sites, were chosen and submitted to the BLAST search programme of the National Centre for Biotechnology Information (NCBI) website to find closely related sequences. Our cloned 16S rRNA gene sequences and closely related reference sequences were aligned using Clustal W multiple alignment tool (Thompson et al., 1994) and similarity values were obtained using the Pairwise Optimal Global alignment tool (BioEdit).

3. Results

3.1 Physico-chemical

The physico-chemical nature of the sample sites tested is given in Table 1. Site 10, within Hayle estuary, plus sites 9 and 6 within the Fal Estuary are most dissimilar from the rest of the sample sites. Sites 10 and 6 are distinct from other sites with coarser sediments and low salinity clearly distinguishes site 9, from the other sites. The rest of the sites have generally similar levels of total carbon, particle size, salinity and pH. Using ANOSIM no significant differences were found.

3.2 PICT

Incorporation into total macromolecules of microbial communities from sample sites ranged from 0.915 to 8.43pmol/l.h thy (mean value 3.04pmol/l.h thy) and 6.95 to 24.19pmol/l.h leu (mean value 10.56pmol/l.h leu) (n= 16). No significant differences in mean incorporation rate between sites were found. Incorporation rate of leucine was significantly greater than thymidine over all sites ($P<0.05$). There was a clear dose-response relationship between Cu concentration in sediment pore water and community tolerance to Cu as measured by thymidine and leucine incorporation (Figure 2). IC₅₀ (inhibitory concentration 50 %) values were strongly positively correlated with Cu in sediment pore water with correlation co-efficients (Spearman Rank) of, $r^2 = 0.842$ (thy & leu) for Cu ($P<0.001$). IC₅₀ values for Zn; ($r^2 = 0.080$ (thy) $r^2 = 0.006$ (leu), $P>0.05$), Cd; $r^2 = 0.16$ (thy), $r^2 = 0.090$ (leu), $P>0.05$ and Cr; $r^2=0.060$, (thy), $r^2=0.660$ (leu), $P>0.05$, were less strongly and non significantly correlated with pore water metal concentrations. Based on IC₅₀ values for leucine, the most tolerant microbial community from site 11 was over 2000 times less sensitive to Cu than microbial communities from the least tolerant at site 1. Pore water Cu concentrations were approximately 750 times greater in site 11 compared to site 1. Site 5 is up to 15 times less sensitive to Zn than the most sensitive site, site 6. Approximately 5 times more Zn was measured in site 5 pore waters than in site 6.

3.4 Microbial Community Structure Analysis

We hypothesised that changes in the PICT response may be reflected in community structure. Community structure was investigated within sediment samples approximately 0.5m apart to test whether natural variation within sites was greater than inter-site variation. MDS ordination was then carried out on T-RFLP profiles gained from spatially different sediment samples at every site (Figure 3). This revealed a high intra-site variability in community

structure at all sites. ANOSIM analysis was carried out to test whether between site differences in microbial community structure was greater than within site differences. No significant intra- or inter-site community structure differences were found. BIOENV analysis provided no strong correlation between one particular environmental characteristic and microbial community structure, with a highest ρ value of 0.238 gained for pore water metals (Zn, Cu and Cd). No significant differences in diversity were found between sites. The lowest diversity was at site 11 ($H' = 2.4$) and the highest at site 2 ($H' = 4.1$). Diversity was not significantly correlated with pore water metal concentrations or other environmental characteristic measured ($P > 0.05$). SIMPER analysis was used to identify typical TRFs of each site. From this analysis, TRFs common to and dominant at sample sites were identified. Table 2 lists these common and dominant TRFs with phylogenetic association. TRFs of size 60 bp, 63 bp (*Cfo I* digested) were found to be common to all sites. A 175 bp TRF was common to all sites, except site 9 and a TRF of 535 bp appeared in all except site 11. *Alu I* digested fragments of size 204 bp, 208 bp, 215 bp and 783 bp were common to all, except site 1 (minus 204 bp), site 3 (minus 208 bp and 783 bp TRFs) and 9 (minus 783bp). *Rsa I* digested fragments of size 275 bp and 457 bp were common to all sites except site 3 and 9. TRFs of 841 bp and 849 bp were common to all sites except site 5. Many of these common TRFs were also found to be dominant at field sites e.g. TRFs of size 215 bp (*Alu I*) and 175 bp (*Cfo I*), were most abundant in 5 out of the 8 sites studied (sites 1,6,8,9,11 and sites 1,4,6,8,11, respectively). These TRFs (215 bp and 175 bp) were closely related to a Marine bacterium (unclassified) and an uncultured bacterium clone (unclassified), respectively. Dominant ribotypes were similar at all sites with at least one of the most dominant ribotypes shared between all sites. Clones from 3 groups were detected; δ

Proteobacteria (including one clone of the *Desulfosarcina-Desulfococcus* group), γ *Proteobacteria* and *Flavobacteria*. Clones were typical of those found in other estuarine and marine environments in e.g. Arctic pack ice (Brunkmeyer et al., 2003), Antarctic sediments (Purdy et al., 2003), hydrothermal sediments (Huber et al., 2003) and in sediments above a gas hydrate (Knittel et al., 2003). Sites with the highest Cu tolerances and Cu concentrations (i.e. 6, 9, 11), were found in general to share more TRFs than sites with low concentrations of Cu and Cu tolerance (i.e. 1, 4) with between 27-75 % of TRFs in common compared to between 9-50 % of TRFs shared, respectively.

4. Discussion

Can PICT and microbial community structure be used to detect the effects of heavy metal pollution within estuarine sediments? In our study, PICT was a consistent and powerful indicator of metal pollution, discriminating between sites with low, intermediate and gross levels of pollution. A strong positive and statistically significant concentration-response relationship occurred between Cu concentration in pore waters at field sites and degree of sensitivity to Cu within estuarine sediment microbial communities. Indeed, Cu tolerances were at least 2000 fold different between the least and most impacted sites. Interestingly the PICT response for the other metals tested (Zn, Cd and Cr) was not significantly correlated with pore water metal concentrations measured. This indicates that Cu is exerting the greatest biological effect of all metals tested. These results are strikingly similar to the results from the small number of comparable studies carried out on terrestrial microbial communities. Pennanen et al., (1996), found that Cu tolerance of soil communities measured as IC50 values spanned a similar range. In addition PICT was detected within soil microbial communities at soil Cu concentrations of

greater than 100-200 $\mu\text{g g}^{-1}$. Similarly, Rutgers et al., (1998), reported EC50 (effect concentration 50 %) values (for Zn) within Zn contaminated field soil plots ranging from 50-2000 mg Zn l^{-1} . At our study sites, increased tolerance of microbial communities to Cu occurred at Cu pore water concentrations in the region of 15 $\mu\text{g l}^{-1}$ at sites where total Cu concentrations in sediments are approximately, 200 $\mu\text{g g}^{-1}$ (Millward and Grant, 2000). Nematode communities from the same study area also begin to show increased tolerance to Cu at the same total Cu concentration in sediment. Millward and Grant, (1995, 2000), have found a PICT threshold within nematode communities at Cu sediment concentrations of approximately 200 $\mu\text{g g}^{-1}$ dry weight sediment. Although this study has not set out to compare trophic responses to heavy metal pollution, the results are in agreement with other studies that show similar sensitivities of microbial and meiofaunal communities (e.g. Ellis et al., 2001, 2002).

4.1 Possible factors confounding the PICT response

There may be factors that co-vary with Cu (as Zn does) and thus are responsible for PICT responses measured here. Other factors possibly contributing to the decreased sensitivity to Cu must be explored before a direct linkage between deleterious effect and heavy metals can be made. Fisher (1977) suggested the heightened resistance to a wide range of pollutants possessed by estuarine diatoms was not the result of previous exposure but due to evolution within physiological variable conditions, as is found in the estuarine environment of the study sites sampled. Within the present study however, high Cu tolerance was not always associated with tolerance to a wide variety of metals e.g. Hayle estuary was not significantly more tolerant to Cr and Cd than sites with lower Cu tolerance (data not shown). Indeed, the magnitude of PICT to other metals including the co-varying Zn, paled into insignificance in comparison with Cu. The

PICT response for Cu between the least and most sensitive site was over 130 times greater than that measured for Zn. This reinforces one of the strengths of PICT. The response is usually contaminant specific so it points more clearly at the pollutant responsible for effects. Bååth et al., (1998), Diaz-Raviña and Bååth (1996) and Pennanen et al., (1996) also suggest that the magnitude of response to the metal tested could provide an indication of the pollutant providing the toxic effect. Within our sample sites it seems that Cu is causing much more of a biological effect than the co-varying metal Zn. It is possible that the results of our assay could be affected by sediment particles present in the bacterial extracts either increasing or decreasing metal concentrations to which organisms are exposed. However, the site from which bacteria were extracted had no significant effect on actual metal concentrations in the assay, thus allowing direct comparison of IC50 values across sites. Salinity was found to affect IC50 values in a preliminary toxicity test using Hayle Estuary samples (sites 10,11), with toxicity increasing as salinity decreased (L.Ogilvie, unpublished data), however, correlation analysis of results from this study found no significant relationship between tolerance to Cu and field site salinity measured (data not shown). The use of artificial sea water of a standardised salinity aids comparison of results between sites of differing salinities. Another factor causing variability in response may be nutrient availability. Nutrients such as P, Si & N, may act to increase or decrease the uptake of metals into cells (Wang & Dei, 2001b). These effects however, are thought to be highly species specific (Wang & Dei, 2001a,b), therefore any nutrient status differences between sites may be ameliorated by analysis of tolerance at the community level. Paulsson et al., (2002) found Zn at low concentrations to interfere in phosphorus uptake and hence decrease biomass production. Nutrient concentrations were not specifically measured within this present study, however no significant differences in activity i.e. uptake of thymidine

and leucine, were recorded between the range of sites tested indicating there are unlikely to be large differences in microbial biomass. Finally, by extracting bacteria from sediments we are testing the tolerance response of perhaps only 10 – 20 % of the total microbial community (Díaz-Raviña et al., 1994). But there is no reason why metal tolerance of the extracted community is not representative of the whole community.

4.2 Microbial community structure analysis as an indicator of pollutant effect

The PICT paradigm suggests that the increased tolerances to Cu measured here may be a result of a restructured microbial community consisting of tolerant types. We expected that increased Cu tolerance would be reflected in altered T-RFLP patterns. However, we found no clear link between T-RFLP profiles, site diversity and sediment metal concentrations. Similar results were reported by Gillan et al., (2005), who found that diversity of estuarine sediment associated bacterial communities within Sør fjord, was not significantly affected by > 80 years of chronic metal pollution. It is possible that the broad range approach of 16S rRNA gene diversity analysis used in the present study may miss more subtle functional group specific effects (Mertens et al., 2006). Although no conclusive evidence was found to show bacterial community structure was affected by metal concentrations in sediments, analysis of the individual ribotypes making up the community does provide some interesting insights into the differences and similarities found and possible reasons for differences in tolerance level measured. Percentages of shared TRFs within sites with the highest concentrations of Cu and Cu tolerances were greater than a third, up to a maximum of 75 %. This is similar to the results of Konstantinidis et al., (2003) using chronically polluted lake sediments with Cu concentrations of between 800 ppm and 5500 ppm over vertical gradients. T-RFLP analysis was able to show that

at least 50 % of the community was conserved over the vertical gradients sampled and at least a third of ribotypes were shared between the two sites sampled. Ribotypes present within less polluted sites may have been lost from the more highly impacted sites. Within the present study, at highly impacted sites e.g. sites 9 and 11, ubiquitous ribotypes are dominant. This suggests ribotypes existing within high Cu sites have a decreased sensitivity to Cu via either physiological acclimation or genetic adaptation, compared to the same ribotypes existing within less impacted sites (see Cooksey, 1994; Nies, 2003 for reviews of bacterial heavy metal resistance mechanisms). Interestingly, these findings are in agreement with work carried out on macrofauna within the area (e.g. Millward and Grant, 1995, 2000; Somerfield et al., 1994; Bryan and Hummerstone, 1971, 1973; Bryan and Gibbs, 1983). Phylogenetic associations of TRFs help to provide further information on possible reasons for observed tolerance responses. Clone sequences associated with the *Cfo I* digested TRF of 535bp and the *Rsa I* digested TRF of 457 bp, which were typical of most sites (all sites within the Fal Estuary), although in relatively low abundance, were closely related to *Alcinovorax sp.*, a member of the γ *Proteobacteria* found within petroleum contaminated sediments (unpublished) and a member of the *Desulfosarcina-Desulfococcus* group of the δ *Proteobacteria* (Purdy et al., 2003), respectively. Members of the *Desulfosarcina-Desulfococcus* group have been found in variety of polluted environments e.g. heavy metal contaminated sediments (Gillan et al., 2005) and petroleum contaminated marine sediments (Ravenschlag et al., 2000). We also speculate that the presence of such clones could be related to metal pollution (Gillan et al., 2005). Interestingly, the phylogenetic associations of common and dominant TRFs found within our study sites were strikingly similar to those reported by Gillan et al., (2005) within heavy metal contaminated sediments.

Even if a significant correlation between pollutant concentration and community structure

existed, a direct link between cause and effect cannot be established as one does not know whether the presence or absence of a ribotype is a direct consequence of its sensitivity to amount of metals or due to some other, unmeasured environmental factor. The use of microbial community structure within the present study to detect the ecological effects of metals has been less successful than other studies within the area using macro- and meio- fauna e.g. Somerfield et al., (1994) were able to show a very strong correlation between Cu concentration and nematode community structure using the BIOENV procedure within PRIMER with a correlation coefficient of $\rho = 0.77$ for Cu compared to a p value of 0.238 gained here for pore water metals and bacterial community structure. Analysis of community structure provides poor strength and consistency of association; compared to the high strength association with Cu we have found using the PICT approach.

5. Conclusion

The present study has shown that the Cu sensitivity of sediment microbial communities was highly positively and significantly correlated with Cu concentrations in sediment pore waters, thus highlighting the strength of PICT to point to the pollutant causing a toxic effect. No direct correlation between microbial community structure and community tolerance to metals tested was found. The PICT methodology used here provided a greater strength and consistency of association with pollutant concentration compared to microbial community structure and can be recommended as a more sensitive indicator of metal pollution on estuarine sediment microbial communities than analysis of community structure. In tandem however, information concerning the biological relevance of an increased tolerance response can be gained.

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Figure Legends

Figure 1 Map of sampling sites within the (a) Hayle Estuary and (b) Fal Estuary, Cornwall, U.K. Inset shows location within South West England, U.K, of the Hayle, Fal and Kingsbridge estuaries. Sites are numbered from lowest to highest according to increasing sediment pore water Cu concentration.

Figure 2 Estuarine sediment microbial community tolerance to Cu at sample sites 1-11. Site pore water Cu concentrations are shown. Sites are numbered from lowest to highest according to increasing sediment pore water Cu concentration. IC50 values and metal concentrations are in $\mu\text{g/l}$. Error bars show std. dev. ($n=3$, IC50 & pore water concentrations).

Figure 3 Multidimensional scaling (MDS) of a Bray Curtis similarity matrix of ribotype abundances ($\sqrt{\text{transformed}}$) derived from sample site T-RFLP profile data. Sites 1-6, 9 and 11 are included in the analysis. Each site label represents pooled amplification products (x2). Sites are numbered from lowest to highest according to increasing sediment pore water Cu concentration.

677 Fig 1:

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687 Table 1: Metal pore water concentration in ($\mu\text{g/ml}$) determined by ICP-AES, pH, salinity (ppt),
 688 median particle size (μm) and total carbon (%) at sampling sites (mean values $\pm$ standard
 689 deviation; $n=3$). nd: metal concentration below detection limits.

Site	Cu	Zn	Cd	Cr	pH	Salinity (ppt)	Median Particle Size (μm)	Total C (%)
1	0.61 $\pm$ 0.12	13.79 $\pm$ 2.76	7.51 $\pm$ 1.24	2.92 $\pm$ 0.06	7.3	25.67 $\pm$ 0.23	15.25	2.75 $\pm$ 0.05
2	1.40 $\pm$ 2.15	14.01 $\pm$ 0.49	10.64 $\pm$ 8.65	nd	8.0	31.33 $\pm$ 0.31	18.96	4.21 $\pm$ 0.16
3	1.67 $\pm$ 10.56	15.86 $\pm$ 9.33	3.87 $\pm$ 1.23	18.29 $\pm$ 0.18	8.2	33.25 $\pm$ 0.32	17.90	4.02 $\pm$ 0.02
4	4.02 $\pm$ 1.50	46.92 $\pm$ 7.94	2.40 $\pm$ 0.37	nd	8.0	24.23 $\pm$ 0.00	20.10	2.23 $\pm$ 0.03
5	15.13 $\pm$ 1.50	66.40 $\pm$ 3.89	1.10 $\pm$ 0.09	nd	7.7	30.23 $\pm$ 0.11	22.91	3.60 $\pm$ 0.10
6	17.69 $\pm$ 3.13	35.34 $\pm$ 10.68	2.83 $\pm$ 0.76	nd	8.1	32.82 $\pm$ 0.46	39.24	4.73 $\pm$ 0.88
7	19.66 $\pm$ 7.89	79.64 $\pm$ 17.36	4.71 $\pm$ 0.13	nd	7.4	32.76 $\pm$ 0.67	20.98	3.56 $\pm$ 0.02
8	22.70 $\pm$ 6.72	32.90 $\pm$ 6.78	7.51 $\pm$ 0.23	nd	7.6	29.10 $\pm$ 0.00	16.93	2.44 $\pm$ 0.01
9	27.65 $\pm$ 9.52	25.92 $\pm$ 5.67	6.29 $\pm$ 2.38	nd	7.7	11.84 $\pm$ 0.19	20.40	3.88 $\pm$ 0.03
10	463 $\pm$ 9.67	163 $\pm$ 5.43	1.49 $\pm$ 0.56	nd	8.1	31.45 $\pm$ 0.22	40.22	5.85 $\pm$ 0.49
11	783 $\pm$ 10.56	179 $\pm$ 3.45	5.04 $\pm$ 1.21	nd	8.3	29.23 $\pm$ 0.14	25.94	1.60 $\pm$ 0.09

Table 2 Phylogenetic association of common and dominant TRFs across sample sites. Nearest neighbour identified using BLAST search and similarity values calculated using BioEdit after multiple alignments using Clustal W. GenBank accession numbers are given. Identity based on ≥ 500 bp. Approximate values are assigned to fragments above 800bp due to inconsistencies in peak size calling using the GS2500 internal lane standards and Genescan software. (1) Range of average abundances across all sites from SIMPER values.

TRF Size (bp)	Phylogenetic association & GenBank Accession number	Identity	Average Abundance (%) ¹
<i>Cfo I</i>			
60	Uncultured bacterium clone KM 66; AY216454	0.74	0.7 – 27.2
63	Uncultured δ -proteobacterium, clone Hyd24-20; AJ535244	0.95	2.9 – 30.1
175	Uncultured bacterium clone; AF469388	0.90	0 – 42.6
535	<i>Alcanivorax</i> sp.; AB053122	0.99	0 – 14.3
<i>Alu I</i>			
204	Uncultured bacterium clone; AF469388	0.90	0 – 20.4
208	Arctic sea ice associated bacterium ARK10007; AF468407	0.89	0 – 11.3
215	Marine bacterium ATAM407_58; AF359533	0.96	0.7 – 37.1
783	Unidentified γ -proteobacterium, strain 3G02-10; DQ431906	0.96	0 – 33.3
<i>Rsa I</i>			
275	Marine bacterium ATAM407_58; AF359533	0.96	0 – 9.8
457	<i>Desulfocarsina</i> sp. clone SB4-53; AY177791	0.98	0 – 3.8
841	unknown	-	0 – 12.3
849	unknown	-	0 – 46.2