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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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13 Abstract

14

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

28

29

30 Keywords

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

32

33

34

35 1. Introduction

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

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

59 Langston, 1992). Microorganisms play a role in the distribution and fate of these metals (Ford
60 and Ryan, 1995) alongside their importance to key ecosystem processes, such as biogeochemical
61 cycling within sediments (Eismann and Montuelle, 1999). The known toxic effects of heavy
62 metals on these functions (Giller et al., 1998), also gives ecological relevance to their use as
63 ecotoxicological tools (Siciliano & Roy, 1999; van Beelen, 2003). Microbial communities have
64 been used to demonstrate the long term consequences of heavy metal contamination, reporting
65 positive correlations between environmental metal concentration and increased microbial
66 community tolerance (Díaz-Raviña et al., 1994; Díaz-Raviña and Bååth, 1996; Pennanen et al.,
67 1996; Blanck and Wängberg, 1988; Wängberg et al., 1990; Lock and Janssen, 2005). These
68 increased tolerances were further correlated with changes in community structure e.g. Pennanen
69 et al., (1996) found increased tolerances were accompanied by changes in phospholipid fatty acid
70 (PLFA) patterns. More recently, developments in molecular fingerprinting techniques such as
71 denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length
72 polymorphism (T-RFLP) have allowed investigations of genetic diversity to distinguish between
73 polluted and pristine environments (Clement et al., 1998; Tom-Peterson et al., 2003; Gillan et al.,
74 2005). Using DGGE, Mertens et al., (2006) demonstrated soil ammonia oxidising community
75 structure and Zn tolerance were correlated with soil Zn concentrations. These existing studies
76 however, have focused mainly on soil and aquatic microbial communities with limited
77 information existing on the response of estuarine sediment communities. Dahllöf et al., (2001)
78 used functional diversity (Biolog™ plates) to indicate increased TBT tolerance in culturable
79 microbial communities from estuarine sediment box-cosms. Millward and Grant., (2000), found
80 estuarine sediment nematode communities to be a more sensitive indicator of metal impacts than
81 nematode populations, but no information exists on the effects on the microbial communities to

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

86

87 2. Materials and Methods

88

89 *2.1 Collection, preparation and physico-chemical analysis of sediment samples*

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

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

120

121 2.2 Community tolerance measurements

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

128 (blanks) for each sample site were used.

129

130 2.3 Thymidine and Leucine microcentrifuge assay (PICT measurement)

131 Metal tolerance of sediment microbial communities was determined by a microcentrifuge
132 thymidine and leucine incorporation method essentially according to Bååth et al., (2001), with
133 minor modifications. Briefly, microorganisms were extracted from sediment (2 g wet weight
134 sediment /50 ml sterile 75 % artificial sea water) via sonication using an Ultrasonic II water bath
135 for 3x 1minute then low speed centrifugation (1000 g for 10 minutes). Bacterial suspensions
136 (1.35 ml) were placed in 2 ml vials and amended separately with Cu, Zn, Cd and Cr using metal
137 salts (CuSO_4 , ZnSO_4 , CrSO_4 , CdCl_2) in 0.15 ml sterile 75 % ASW, at a range of concentrations
138 (5-9 concentrations from 0-1000 mg l^{-1}) and incubated for 2 hrs at room temperature (23 – 26
139 °C). Metal concentrations were analysed in non-radioactive sample aliquots using ICP-AES
140 (Vista-Pro CCD Simultaneous ICP-AES). To test for differences in exposure concentrations of
141 metals during test procedure, metal concentrations in control bacterial extract and in metal spiked
142 test samples were measured after 2 hours incubation. Triplicate samples at each metal
143 concentration were filtered using a 0.45 μm syringe filter (Sartorius), after flushing with (3 x) 10
144 % HCl and MilliQ™. 1 M HNO_3 was added to give a concentration of 1 % and stored in the dark
145 at 4 °C until analysis. Concentrations of metals in bacterial extracts were very low and there
146 were no significant differences in metal concentrations between bacterial extracts from different
147 sites spiked with the same metal concentration, thus allowing direct comparison of tolerances
148 (data not shown).

149

150 2.4 Community structure analysis using T-RFLP

151 Microbial community DNA was extracted in duplicate from 2 composite sediment
152 samples (each consisting of 3x 50ml sediment) 0.5m apart, at 8 samples sites (1-6, 9, 11),
153 following a procedure described by Zhou et al., (1996) and modified by Osborn et al., (2000).
154 Minor deviations from this protocol were as follows: sediment aliquots (0.5 g) were washed
155 twice in pH 6 Milli Q and centrifuged at 4200 rpm for 1 minute before beginning protocol. The
156 resultant DNA pellet was resuspended in 25 μ l of sterile distilled water and left overnight at 4 °C
157 to dissolve. DNA extractions were pooled and quality and quantity of resulting DNA was
158 established by electrophoresis on a 1 % TBE (Tris-Borate-EDTA) gel. DNA samples were
159 purified using the QIAEX II Gel extraction kit (Qiagen), quantified as described above, and
160 stored at -20 °C until required. Reaction mixtures for polymerase chain reaction (PCR)
161 contained template DNA (100 ng), 5 μ l of 10x reaction buffer, 2.5 mM MgCl₂, 0.1 μ M of each
162 primer, 200 μ M each of deoxynucleoside triphosphate and 1.25 units *Taq* DNA polymerase
163 (Bioline) in a final volume of 50 μ l. The primers used were 63 F (5'-
164 CAGGCCTAACACATGCAAGTC-3') and 1389 R (5'-CGGCGGGTGTGTACAAG-3')
165 (Osborn et al., 2000) labelled at the 5' end with the dyes 6-FAM and HEX (MWG UK),
166 respectively. Cycling conditions were as follows: initial denaturation for 2 minutes at 94 °C; 30
167 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
168 for 10 min. Quality, size and yield of PCR product was determined by electrophoresis. Duplicate
169 PCR products were produced and pooled to reduce amplification bias (Wintzingerode et al.,
170 1997). After purification with Qiaquick® PCR Purification Kit (Qiagen), aliquots containing
171 approximately 200 ng of PCR products were digested separately with 10 U of restriction
172 endonucleases *Cfo* I, *Rsa* I (ROCHE) or *Alu* I (Invitrogen). The digestions were performed in a

173 total volume of 15 μl at 37 °C for 3 hours according to manufacturer's instructions. Aliquots (1
174 μl) of the digested samples were mixed with 1.5 μl deionised formamide, 0.5 μl of ROX-labelled
175 GS2500 internal lane standard (PE Applied Biosystems) and 0.5 μl of loading buffer. Each
176 sample was then denatured at 95 °C for 5 min and transferred to ice. Duplicates of each sample
177 (1.5 μl) were electrophoresed on a 6 % polyacrylamide gel using an ABI 377 (Applied
178 Biosystems Inc.) automated sequencer for 6 hours under the following conditions: 2875 V, 60
179 mA, 46 °C, filter set D. Fragment size was measured with reference to the internal lane standard
180 and peak area of terminal restriction fragments using Genescan Analysis Software (Version 3.6,
181 PE Applied Biosystem). Peak areas were scaled using Genotyper Software v3.7 (PE Applied
182 Biosystems) and percentage abundances calculated. To reduce the influence of rare taxa and
183 create a subset of reproducible peaks, only those peaks with a percentage abundance of $\geq 0.5\%$ in
184 at least one sample were considered in the analysis.

185

186 2.5 Statistics

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

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

202 All statistical analysis was carried out using Primer v5 (Clarke, 1993). Microsoft® Excel
203 (2002) was used for producing graphs, significance testing (t-test) and correlation analysis. All
204 results were considered significant at $P < 0.05$.

206 2.6 Construction of a 16S rRNA gene clone library from sediment samples

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

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

231

232 3. Results

233 3.1 *Physico-chemical*

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

240

241 3.2 *PICT*

242 Incorporation into total macromolecules of microbial communities from sample sites
243 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
244 leu (mean value 10.56pmol/l.h leu) (n= 16). No significant differences in mean incorporation
245 rate between sites were found. Incorporation rate of leucine was significantly greater than
246 thymidine over all sites ($P<0.05$). There was a clear dose-response relationship between Cu
247 concentration in sediment pore water and community tolerance to Cu as measured by thymidine
248 and leucine incorporation (Figure 2). IC50 (inhibitory concentration 50 %) values were strongly
249 positively correlated with Cu in sediment pore water with correlation co-efficients (Spearman
250 Rank) of, $r^2 = 0.842$ (thy & leu) for Cu ($P<0.001$). IC50 values for Zn; ($r^2 = 0.080$ (thy) $r^2 =$
251 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), $r^2=$
252 0.660 (leu), $P>0.05$, were less strongly and non significantly correlated with pore water metal
253 concentrations. Based on IC50 values for leucine, the most tolerant microbial community from
254 site 11 was over 2000 times less sensitive to Cu than microbial communities from the least
255 tolerant at site 1. Pore water Cu concentrations were approximately 750 times greater in site 11
256 compared to site 1. Site 5 is up to 15 times less sensitive to Zn than the most sensitive site, site
257 6. Approximately 5 times more Zn was measured in site 5 pore waters than in site 6.

258

259 *3.4 Microbial Community Structure Analysis*

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

265 structure at all sites. ANOSIM analysis was carried out to test whether between site differences
266 in microbial community structure was greater than within site differences. No significant intra-
267 or inter-site community structure differences were found. BIOENV analysis provided no strong
268 correlation between one particular environmental characteristic and microbial community
269 structure, with a highest ρ value of 0.238 gained for pore water metals (Zn, Cu and Cd). No
270 significant differences in diversity were found between sites. The lowest diversity was at site 11
271 ($H' = 2.4$) and the highest at site 2 ($H' = 4.1$). Diversity was not significantly correlated with
272 pore water metal concentrations or other environmental characteristic measured ($P > 0.05$).
273 SIMPER analysis was used to identify typical TRFs of each site. From this analysis, TRFs
274 common to and dominant at sample sites were identified. Table 2 lists these common and
275 dominant TRFs with phylogenetic association. TRFs of size 60 bp, 63 bp (*Cfo I* digested) were
276 found to be common to all sites. A 175 bp TRF was common to all sites, except site 9 and a
277 TRF of 535 bp appeared in all except site 11. *Alu I* digested fragments of size 204 bp, 208 bp,
278 215 bp and 783 bp were common to all, except site 1 (minus 204 bp), site 3 (minus 208 bp and
279 783 bp TRFs) and 9 (minus 783bp). *Rsa I* digested fragments of size 275 bp and 457 bp were
280 common to all sites except site 3 and 9. TRFs of 841 bp and 849 bp were common to all sites
281 except site 5. Many of these common TRFs were also found to be dominant at field sites e.g.
282 TRFs of size 215 bp (*Alu I*) and 175 bp (*Cfo I*), were most abundant in 5 out of the 8 sites
283 studied (sites 1,6,8,9,11 and sites 1,4,6,8,11, respectively). These TRFs (215 bp and 175 bp)
284 were closely related to a Marine bacterium (unclassified) and an uncultured bacterium clone
285 (unclassified), respectively. Dominant ribotypes were similar at all sites with at least one of the
286 most dominant ribotypes shared between all sites. Clones from 3 groups were detected; δ

287 *Proteobacteria* (including one clone of the *Desulfosarcina-Desulfococcus* group), γ
288 *Proteobacteria* and *Flavobacteria*. Clones were typical of those found in other estuarine and
289 marine environments in e.g. Arctic pack ice (Brunkmeyer et al., 2003), Antarctic sediments
290 (Purdy et al., 2003), hydrothermal sediments (Huber et al., 2003) and in sediments above a gas
291 hydrate (Knittel et al., 2003). Sites with the highest Cu tolerances and Cu concentrations (i.e. 6,
292 9, 11), were found in general to share more TRFs than sites with low concentrations of Cu and
293 Cu tolerance (i.e. 1, 4) with between 27-75 % of TRFs in common compared to between 9-50 %
294 of TRFs shared, respectively.

295

296 4. Discussion

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

310 greater than 100-200 $\mu\text{g g}^{-1}$. Similarly, Rutgers et al., (1998), reported EC50 (effect
311 concentration 50 %) values (for Zn) within Zn contaminated field soil plots ranging from 50-
312 2000 mg Zn l^{-1} . At our study sites, increased tolerance of microbial communities to Cu occurred
313 at Cu pore water concentrations in the region of 15 $\mu\text{g l}^{-1}$ at sites where total Cu concentrations
314 in sediments are approximately, 200 $\mu\text{g g}^{-1}$ (Millward and Grant, 2000). Nematode communities
315 from the same study area also begin to show increased tolerance to Cu at the same total Cu
316 concentration in sediment. Millward and Grant, (1995, 2000), have found a PICT threshold
317 within nematode communities at Cu sediment concentrations of approximately 200 $\mu\text{g g}^{-1}$ dry
318 weight sediment. Although this study has not set out to compare trophic responses to heavy
319 metal pollution, the results are in agreement with other studies that show similar sensitivities of
320 microbial and meiofaunal communities (e.g. Ellis et al., 2001, 2002).

321

322 *4.1 Possible factors confounding the PICT response*

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

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

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

361

362 *4.2 Microbial community structure analysis as an indicator of pollutant effect*

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

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

401 Even if a significant correlation between pollutant concentration and community structure

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

413

414 5. Conclusion

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

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660 Figure Legends

661

662 Figure 1 Map of sampling sites within the (a) Hayle Estuary and (b) Fal Estuary, Cornwall, U.K.

663 Inset shows location within South West England, U.K, of the Hayle, Fal and Kingsbridge

664 estuaries. Sites are numbered from lowest to highest according to increasing sediment pore

665 water Cu concentration.

666

667 Figure 2 Estuarine sediment microbial community tolerance to Cu at sample sites 1-11. Site pore

668 water Cu concentrations are shown. Sites are numbered from lowest to highest according to

669 increasing sediment pore water Cu concentration. IC50 values and metal concentrations are in

670 $\mu\text{g/l}$. Error bars show std. dev. ($n=3$, IC50 & pore water concentrations).

671

672 Figure 3 Multidimensional scaling (MDS) of a Bray Curtis similarity matrix of ribotype

673 abundances ($\sqrt{}$ transformed) derived from sample site T-RFLP profile data. Sites 1-6, 9 and 11

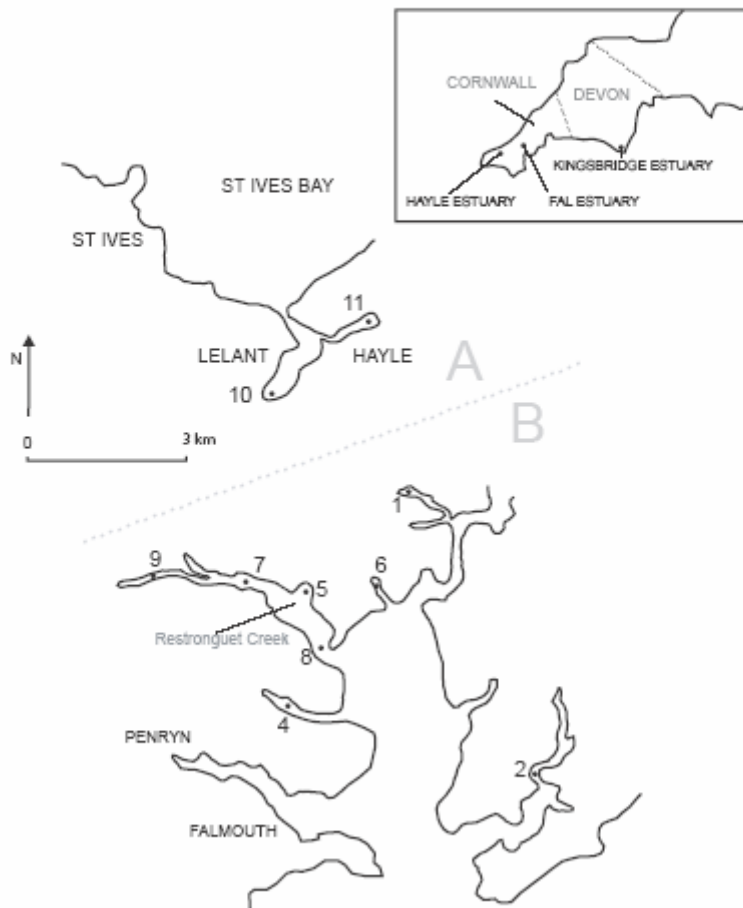
674 are included in the analysis. Each site label represents pooled amplification products (x2). Sites

675 are numbered from lowest to highest according to increasing sediment pore water Cu

676 concentration.

677 Fig 1:

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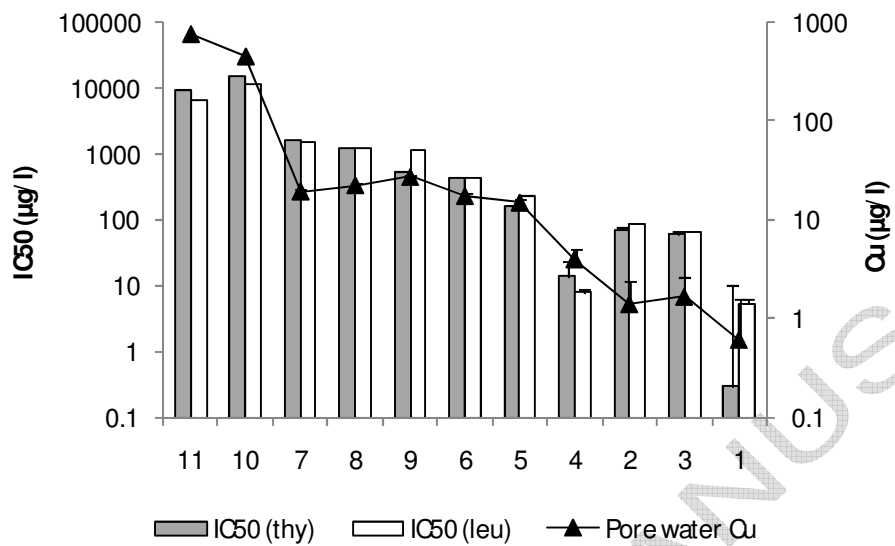


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680 Fig 2:

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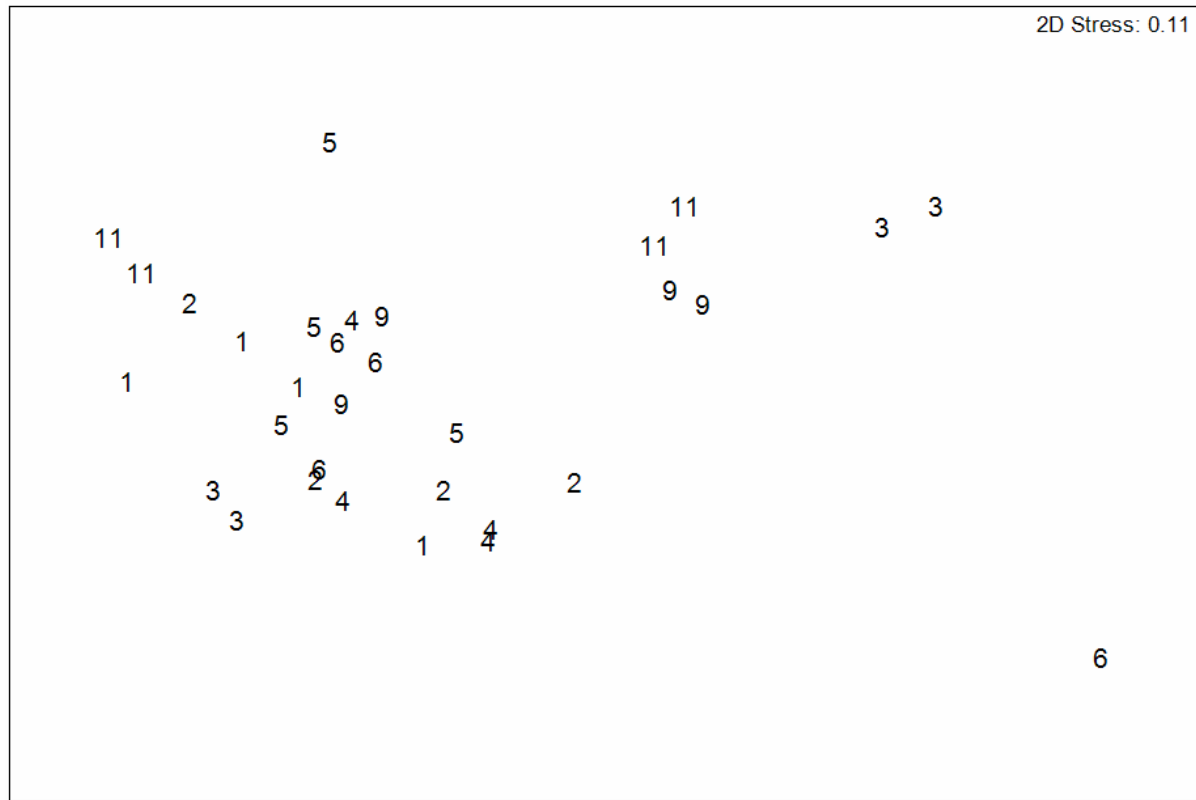
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684 Fig 3:

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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.

<i>Site</i>	<i>Cu</i>	<i>Zn</i>	<i>Cd</i>	<i>Cr</i>	<i>pH</i>	<i>Salinity (ppt)</i>	<i>Median Particle Size (μm)</i>	<i>Total C (%)</i>
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

690

691 Table 2 Phylogenetic association of common and dominant TRFs across sample sites. Nearest
 692 neighbour identified using BLAST search and similarity values calculated using BioEdit after
 693 multiple alignments using Clustal W. GenBank accession numbers are given. Identity based on
 694 ≥ 500 bp. Approximate values are assigned to fragments above 800bp due to inconsistencies in
 695 peak size calling using the GS2500 internal lane standards and Genescan software. (1) Range of
 696 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 sp.</i> ; AB053122	0.99	0 – 14.3
<i>Alu I</i>			
<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>			
<i>Rsa I</i> 275	Marine bacterium ATAM407_58; AF359533	0.96	0 – 9.8
457	<i>Desulfocarsina sp.</i> clone SB4-53; AY177791	0.98	0 – 3.8
841	<i>unknown</i>	-	0– 12.3
849	<i>unknown</i>	-	0 - 46.2

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