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Diversity patterns of benthic bacterial communities along the salinity continuum of the Humber estuary (UK)

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- 9 Illumina MiSeq sequencing
- 10 Abstract

11 Sediments from intertidal mudflats are fluctuating environments that support very diverse 12 microbial communities. The highly variable physicochemical conditions complicate the understanding 13 of the environmental controls on diversity patterns in estuarine systems. This study investigated 14 bacterial diversity and community composition in surface (0-1 cm) and subsurface (5-10 cm) sediments 15 along the salinity gradient of the Humber estuary (UK) using amplicon sequencing of the 16S rRNA gene, and it correlates variations with environmental variables. The sediment depths sampled were 16 17 selected based on the local remobilisation frequency patterns. In general, bacterial communities 18 showed similar composition at the different sites and depths, with Proteobacteria being the most 19 abundant phylum. Richness of operationally defined taxonomic units (OTUs) was uniform along the

20 salinity gradient. However, Hill numbers, as bacterial diversity measures, showed that the common 21 and dominant OTUs exhibited a decreasing trend from the inner towards the outer estuary sites. 22 Additionally, surface and subsurface bacterial communities were separated by NMDS analysis only in 23 the mid and outer estuary samples, where redox transitions with depth in the sediment profile were 24 more abrupt. Salinity, porewater ammonium concentrations and reduced iron concentrations were the 25 subset of environmental factors that best correlated with community dissimilarities. The analysis of the 26 regional diversity indicated that the dataset may include two potentially distinct communities. These 27 are a near surface community that is the product of regular mixing and transport which is subjected to 28 a wide range of salinity conditions, and thus contains decreasing numbers of common and dominant 29 OTUs seawards, and a bacterial community indigenous to the more reducing subsurface sediments of 30 the mid and outer mudflats of the Humber estuary.

31 1 Introduction

32 Estuaries are transitional environments where substantial physicochemical and biological 33 gradients from freshwater to marine environments develop (Attrill & Rundle, 2002; Crump et al., 2004; 34 Elliott & Whitfield, 2011; Lallias et al., 2015). The continuous mixing of water and sediments leads to 35 high variability in the local physicochemical characteristics (e.g. pH, temperature, salinity, particle 36 size, turbidity, sulphate concentration, organic matter, light exposure, river flow seasonal fluctuations, 37 etc.), which can affect the stability and composition of microbial communities along the estuarine 38 continuum (Crump et al., 1999; Liu et al., 2014; O'Sullivan et al., 2013; Wei et al., 2016). However, 39 no consensus on the factors controlling microbial abundance in estuarine systems has yet emerged 40 (Elliott & Whitfield, 2011; Telesh et al., 2013). Marine coastal sediments host very abundant and 41 diverse microbial communities, and, although these communities play a key role in estuarine 42 biogeochemical processes (Federle et al., 1983; Reed & Martiny, 2012; Zinger et al., 2011), the 43 relationship between microbial composition and ecosystem functioning remains unclear (Bertics &

Ziebis, 2009; Reed & Martiny, 2012). Quantifying the microbial community variations along estuarine
gradients will improve the understanding of their role in these ecosystems and their response to
environmental change (Bier *et al.*, 2015; Reed & Martiny, 2012).

47 Salinity is known to be a major abiotic factor controlling the patterns of benthic and pelagic 48 diversity in estuaries (Attrill, 2002; Campbell & Kirchman, 2013; Crump et al., 1999; Crump et al., 2004; Elliott & Whitfield, 2011; Herlemann et al., 2011; Lallias et al., 2015; Lozupone & Knight, 49 50 2007; Telesh et al., 2011; Zhang et al., 2014a). The variation of macrozoobenthos in estuaries has been 51 traditionally explained using the conceptual model known as Remane's concept (Remane, 1934) 52 (Figure 1), which was developed for the non-tidal Baltic Sea, and it models the species richness along 53 a salinity gradient. It concludes that there is a relationship between species diversity and salinity. 54 Species diversity reaches a minimum (Artenminimum) in the region of 5-8 psu salinity ('the critical 55 salinity zone', Khlebovich, 1968) because the number of brackish specialists does not compensate for 56 the decline of the marine and freshwater species richness (Elliott & Whitfield, 2011). However, despite 57 several modifications (Schubert et al., 2011; Telesh et al., 2011; Whitfield et al., 2012) and critiques 58 (Attrill, 2002; Attrill & Rundle, 2002; Barnes, 1989; Bulger et al., 1993), Remane's model has significant limitations as a description of diversity in estuarine systems. Telesh et al. (2011) conducted 59 60 a meta-analysis of large data sets from previous studies in the Baltic Sea and found that protists showed 61 a diversity maximum in the 'critical salinity zone' (Figure 1). Subsequently, Telesh et al. (2013) 62 proposed that the salinity stress may create niches in the brackish waters where there is less competition for resources, so these niches can be occupied by highly adaptable unicellular organisms (i.e. 63 64 planktonic organisms). However Herlemann et al. (2011) found that the diversity of pelagic bacteria exhibited a different pattern to protists and displayed a steady distribution in the Baltic Sea with no 65 66 trend with salinity (Figure 1) possibly due to the mixing of freshwater and marine communities.



67

68

Figure 1: Diversity variation patterns along a salinity gradient. Coloured areas represent the Remane's

69 conceptual model for the variation in macrobenthic biodiversity (after Whitfield *et al.* 2012). Variations

in the diversity of pelagic protists (Telesh *et al.*, 2011) and planktonic bacteria (Herlemann *et al.*, 2011)

71 are shown as dashed lines (dark red and black respectively). The dotted lines indicate boundaries for

the salinity zonation defined for the Humber estuary (see methods section).

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74 Although it is widely accepted that microbial communities are sensitive to environmental 75 change (Lozupone & Knight, 2007), no consensus on the factors controlling microbial abundance in 76 estuarine systems has yet emerged (Elliott & Whitfield, 2011; Telesh et al., 2013). In tidal estuaries, 77 the large salinity variations are expected to impact on bacterial community composition, activity and 78 diversity (Campbell & Kirchman, 2013; Feng et al., 2009; Liu et al., 2014; Wei et al., 2016). Benthic 79 microbial communities will experience different environmental stresses to pelagic organisms, and may 80 be expected to exhibit different diversity patterns. For example, vertical stratification of sediment geochemistry influences in the composition and function of benthic microbial communities (Canfield 81 82 & Thamdrup, 2009; Lavergne et al., 2017; Liu et al., 2014; Musat et al., 2006; O'Sullivan et al., 2013). 83 However, sediments in tidal estuaries are frequently disturbed and thus may not exhibit clear links 84 between geochemical zones and the bacterial communities present, particularly since geochemical 85 profiles tend to re-establish more quickly than diversity profiles within the sediments (O'Sullivan et 86 al., 2013). Moreover, sediment resuspension facilitates the interaction and mixing of microbial 87 assemblages between water and shallow sediments (Crump et al., 1999; Feng et al., 2009; Hewson et al., 2007). Consequently, sediment dynamics may also be an important environmental factor shaping 88 89 estuarine microbial diversity.

Lately high-throughput sequencing techniques have become widely available (Bier *et al.*, 2015; Buttigieg & Ramette, 2014; Liu *et al.*, 2014). These techniques offer an opportunity to investigate microbial communities in more depth. However, challenges remain as the very large data sets produced reveal the hyperdiverse nature of microbiota, which is difficult to evaluate rigorously with the traditional mathematical and statistical approaches to biodiversity estimation (Buttigieg & Ramette, 2014; Kang *et al.*, 2016; Oulas *et al.*, 2015). Hill numbers (D_q) are a unified and index-independent diversity concept; they were developed by Hill (1973) and were reintroduced to ecologists by Jost

97 (2006, 2007). They have been proposed as a unified framework for measuring bacterial diversity
98 measure given the sequencing depth, in order to control the variability associated with rare taxa,
99 sampling issues and other bias associated with experimental procedures (Chao *et al.*, 2014; Kang *et al.*, 2016).

101 The aims of this study were: 1) to describe the bacterial communities in estuarine sediments at 102 centimetre scale resolution, 2) to identify microbial diversity trends along the salinity gradient, and 3) 103 to investigate how the environmental variables control such trends. This work has focused on intertidal 104 sediments of the Humber estuary (UK) which were sampled during the same tidal cycle at low tide in 105 summer conditions. The authors have extensively sampled the Humber Estuary in the past, observing 106 that tidal resuspension moved just the few top mm of sediment, and during this intensive sampling, the 107 entire top 10 cm of sediment were only removed during a powerful storm (Mortimer et al., 1998; 108 Mortimer et al., 1999). The sampling strategy was based in this observed remobilisation patterns, and 109 thus samples were collected at two depths; surface sediments that are frequency mobilised on the tidal 110 cycle; and subsurface sediments that are only mobilised during medium/moderate resuspension events 111 caused by seasonal storms that occur once or twice a year in the Humber. Sequencing data from amplicon sequences of the V4 hyper-variable region of the 16S rRNA gene, were processed and the 112 113 benthic community composition was correlated with geochemical data using multivariate statistics to 114 identify the environmental drivers controlling microbial diversity patterns and test whether sediment 115 depth has an impact on microbial diversity.

116 2 Material and Methods

117 2.1 Field sites and sample collection

118 The Humber estuary (UK) is a highly turbid and shallow well-mixed macrotidal estuary situated 119 on the east coast of northern England and drains an urbanised catchment with an industrial and mining

120 heritage (Figure 2). Its catchment area is 24,240 km² (20% of the area of England), it has 150 km² of 121 mudflats, and the region of freshwater-saltwater mixing stretches from Naburn Weir on the Ouse, and 122 Cromwell weir on the Trent, to the mouth of the estuary at Spurn Head. The Humber represents the 123 main UK freshwater input to the North Sea. Generally the estuarine turbidity maximum (ETM) is 124 situated at the inner estuary although it moves seasonally with the river flow (Uncles et al., 1998a). 125 Water column salinity records from 14 locations on the Humber over a period of ~25 years have been 126 collated to better delimit the salinity variation along the estuary and to provide a proxy for the salinity range experienced by surficial sediments (Figure 3). Three salinity zones can be empirically identified. 127 128 Firstly, the inner estuary extends from 0 to 60 km below Naburn weir (the tidal limit of the Ouse 129 system) where the water column salinity is always ≤ 5 psu (from freshwater to oligohaline water) (blue 130 area in Figure 2 and 3, see also annotation in Figure 1). Secondly, the mid estuary extends from 60 to 131 100 km downstream of Naburn weir, and in this zone the water column salinity ranges between 0 to \sim 25 psu (purple area in Figure 2 and 3, see annotation in Figure 1), which includes oligonaline, 132 133 mesohaline and polyhaline waters. Finally, the outer estuary extends from 100 km below Naburn weir 134 to open coastal waters. Here the water column salinity typically varies from ~18 psu to seawater (35 135 psu) (pink area in Figure 2 and 3, see annotation in Figure 1), which includes polyhaline to euhaline 136 waters.



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Figure 2: Map of the Humber Estuary (UK) with the sampling locations (Boothferry (S1), Blacktoft
(S2), Paull (S3), and Skeffling (S4)) and the salinity variation zones (blue for ≤5psu; purple for 0-25
psu; and pink for 18-35 psu).



141

142 Figure 3: Salinity zonation based on salinity records of different sites along the Humber estuary (x) 143 (ABP Research 2000; Barnes & Owens, 1998; Burke et al., 2005; Freestone, 1987; Fujii & Raffaelli, 144 2008; Garcia-Alonso et al., 2011; Millward et al., 2002; Mitchell, 1998; Mortimer et al., 1999; NRA, 145 1995, 1996; Prastka & Malcolm, 1994; Sanders et al., 1997; Uncles et al., 1998b; Uncles et al., 2006; Williams & Millward, 1999). Salinity ≤5 psu (blue area); 0-25 psu salinity range (purple area); and 18-146 35 psu salinity range (pink area). The triangle markers indicate the porewater salinity measurements of 147 148 this study (S1-S4) (empty and coloured markers for surface and subsurface porewater salinity 149 respectively).

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Sediment samples were collected at low tide from the intertidal mudflats along a 65 km transect in the north bank of the Humber estuary during the same tidal cycle on 15th July 2014. The four sites were at Boothferry (S1), Blacktoft (S2), Paull (S3), and Skeffling (S4), and they were selected to span the salinity range. A sample of surface (s) (0-1 cm) and subsurface (d) (5-10 cm) sediment was recovered from each location in 1L containers, transported back in the dark to the laboratory. Subsamples of the homogenised sediment were stored in 2 mL microcentrifuge tubes at -20°C for subsequent DNA extraction.

158

2.2 Physical and chemical analysis of water and sediments

159 Water pH, conductivity and temperature were determined *in situ* using a Myron Ultrameter 160 PsiII handheld multimeter. Water samples from each site were collected in 2L polythene containers. 161 Porewater was recovered from sediment subsamples by centrifugation (30 min, 6000 g) in the 162 laboratory. All water and porewater samples were filtered (0.2 μ m Minisart ®) and stored at 4 or -20°C, 163 as appropriate, for further analysis. Nutrient concentrations were determined by ion chromatography 164 (nitrate, nitrite, sulphate, and chloride) on a Dionex CD20, and colorimetrically (ammonium) on a 165 continuous segmented flow analyser (SEAL AutoAnalyser 3 HR). Dissolved Mn and Fe were determined after acidification with 1% AnalaR HNO3 (VWR) using ion coupled plasma-mass 166 spectroscopy (Thermo Scientific ™ ICP-MS). Wet sediments were analysed for: particle size by laser 167 168 diffraction on a Malvern Mastersizer 2000E and 0.5 N HCl extractable iron (Lovley & Phillips, 1987; 169 Viollier, 2000). Acid volatile sulphide (AVS) (Canfield et al., 1986) and pyrite (Fossing & Jørgensen, 170 1989) were extracted from freeze-dried sediments and quantified by weight. Finally, subsamples of 171 ground and oven- dried sediments (60°C) were acid washed with HCl 10% (v/v) prior to the total 172 organic carbon (TOC) analysis by combustion with non-dispersive infrared detection on a LECO SC-173 144DR Sulphur and Carbon Analyser. All these physicochemical analysis of sediments and water 174 samples were carried out in triplicates.

175 2.2 DNA extraction, amplicon sequencing and sequence analyses

176 DNA was extracted from environmental samples (~0.5 g of wet sediment) using a FastDNA™ 177 SPIN Kit for Soil DNA Extraction (MP Biomedicals, USA). To purify and isolate the DNA fragments larger than 3 kb, an agarose gel electrophoresis was run. The 1% agarose "1x" Tris-borate-EDTA 178 179 (TBE) gel was stained with ethidium bromide for viewing under UV light (10x TBE solution supplied 180 by Invitrogen Ltd., UK). DNA was extracted from the gel using the QIAquick gel extraction kit 181 (QIAGEN Ltd, UK); final elution was by 1/10th strength elution buffer. DNA concentration was 182 quantified fluorometrically using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific Inc., 183 USA). The manufacturer's protocols supplied with the above kits were all followed precisely.

184 DNA samples $(1ng/\mu L \text{ in } 20 \ \mu L \text{ aqueous solution})$ were sent for sequencing at the Centre for Genomic Research, University of Liverpool, where Illumina adapters and barcodes were attached to 185 186 DNA fragments in a two-step PCR amplification that targets hyper-variable V4 region of the 16S rRNA 187 gene. The protocol was based on Caporaso et al. (2011) which uses the forward target specific primer 5°-188 5'-GTGCCAGCMGCCGCGGTAA-3' and the reverse target specific primer 189 GGACTACHVGGGTWTCTAAT-3'. Pooled amplicons were paired-end sequenced on the Illumina 190 MiSeq platform (2x250 bp) generating ~12M paired-end reads. Illumina adapter sequences were 191 removed, and the trimmed reads were processed on a command-line using the UPARSE pipeline 192 (Edgar, 2013) within the USEARCH software package (version 8.1.1861) (Edgar, 2010) installed on 193 Linux OS platform. First of all, overlapping paired-end reads were assembled using the 194 fastq_mergepairs command. Then, the reads from each sample were quality-filtered using the fastq_filter command (expected error cutoff was set at 1.0 and length truncation was not applied), 195 196 relabelled, and de-replicated before they were randomly subsampled (500,000 paired-end reads with 197 an average length of 296 bp) to produce a manageable sample size for combined analysis (~4M reads). 198 After further de-replication of the combined pool of reads, clustering and chimera filtering was 10

199 performed simultaneously within the pipeline by using the *cluster_otus* command (with the *-minsize 2* 200 option to specify a minimum abundance of 2 to discard singletons). The sequence identity threshold 201 was fixed at 97% to define OTUs. The utax command was applied for taxonomic assignment using the 202 RDP 16S rRNA training database (RDP15) and a confidence value of 0.7 to give a reasonable trade-203 off between sensitivity and error rate in the taxonomy prediction. The entire dataset (~6M paired-end 204 reads) was then allocated to the OTUs using the usearch_global command and the results were reported 205 in an OTU-table. For the diversity and statistical analyses, OTUs which were not classified to the 206 Bacteria phylum level with a confidence >0.7 or classified as Archaea, were not included. Sequence 207 reads were submitted to the National Center for Biotechnology Information (NCBI) under the 208 Sequence Read Archive (SRA) accession number SRP105158.

209 2.3 Statistical analyses

Hill numbers, D_q , (Hill, 1973) were used to evaluate the bacterial diversity. D_q are a unified family of diversity indices that compensate for the disproportionate impact of rare taxa by weighting taxa based on abundance. Hence, they are more suitable for working with the large datasets produced by amplicon sequencing technologies (Kang *et al.*, 2016). The basic expression for the Hill number is represented in Equation 1.

215
$$D_q = \left(\sum_{i=1}^{S} p_i^q\right)^{\frac{1}{1-q}}$$
 (Equation 1)

216 Where S is total number of species (OTUs in this study) and p_i is the proportion of individuals 217 belonging to the *i*th species in the dataset. The degree of weighting is controlled by the index *q* 218 (increasing *q* places progressively more weight on the high-abundance species in a population and 219 discounts rare species) (Chao *et al.*, 2014; Hill, 1973; Jost, 2006, 2007; Kang *et al.*, 2016). Three Hill 220 numbers were used to evaluate the alpha-diversity of each individual sample; D_0^a , (the species 221 richness), D_1^a (common species) and D_2^a (dominant species) (Jost, 2006, 2007). Traditional diversity

222	indices, such as Shannon entropy or Gini-Simpson concentrations, can be converted to D_1^{α} and D_2^{α} by
223	simple algebraic transformations (Supplementary Information, Table S6). The regional OTU diversity
224	(gamma-diversity, D_I^{γ}) was calculated using the combined dataset. The beta-diversity, D_I^{β} , which
225	reflects the proportion of regional diversity contained in a single average community, was calculated
226	from the gamma diversity and the statistically weighed alpha-diversity , using Whittaker multiplicative
227	law (* $D_I^{\alpha} \ge D_I^{\beta} = D_I^{\gamma}$) (Whittaker, 1972). * D_I^{α} compensates for unequal sample sizes, so is not the
228	arithmetic average of the alpha diversities of the individual samples (see Supplementary Information).
229	All the statistical analyses were performed with RStudio software (v 0.99.486) (RStudioTeam,
230	2015) using the package'vegan' (Oksanen et al., 2013). The microbial community data were input as
231	a matrix of the relative abundance of each OTU in each of the eight samples. Non-metric Multi-
232	Dimensional Scaling (NMDS) analysis (distances based on Bray Curtis dissimilarity index) was used
233	to graphically represent the similarity between bacterial assemblages in a two-dimensional space. Non-
234	parametric multivariate analysis of variance (PERMANOVA) (Anderson, 2001) was used to assess the
235	similarity in the microbial abundance among samples. BIOENV ('biota-environment') analysis (Clarke
236	& Ainsworth, 1993) was also performed to further investigate the relationship between the microbial
237	populations and the environmental variables using Spearman's rank correlation coefficient and Bray
238	Curtis dissimilarities. This test finds the combination of environmental variables that best explain the
239	patterns in the biological data. The Mantel test was also performed to study the significance of the
240	BIOENV results. The environmental data used the BIOENV analysis included: salinity; ammonium,
241	nitrate, sulphate, iron and manganese porewater concentrations; TOC content; pyrite and total iron in
242	solids; particle size; percentage of acid extractable iron (II) in solids; and iron associated with pyrite.

243 3 Results

244 **3.1 Environmental characterisation of the samples**

245 The environmental characterisation of the water, porewater, and sediment samples is shown in Table 1. The water column salinity at the sampling locations spanned from very low salinity at the 246 247 freshwater end (0.4 psu at S1) to high salinity water at the sea end of the estuary (26.1 psu at S4). 248 Porewater salinity was slightly lower than the water column salinity in all sites with the exception of 249 S4. Nitrate concentration in the water column decreased along the estuary, while ammonium 250 concentration increased slightly. With the exception of S4s, nitrate concentrations in the porewater 251 were lower than those in the water column, whereas ammonium concentrations were higher, especially 252 in the sites where more reducing sediments were found. Sulphate concentrations increased with salinity 253 from 1 to 22 mM in the water column, and from 2 to 40 mM in the porewater (there was no trend with 254 sediment depth). The total amount of iron in solids did not vary with sediment depth but increased 255 along the estuary. The proportion of the acid extractable iron that was Fe(II) was constant in the surface 256 sediment, however in the subsurface sediments it increased along the estuary. Sediments of the mid 257 and outer estuary mudflats were also finer and contained slightly more TOC than sediments from the 258 inner estuary sites.

Table 1: Physicochemical properties of the water column, sediment porewater and sediments at the study sites (S1-S4). Suffixes *s* and *d* refer to surface and subsurface sediments respectively. Particle size is expressed as the upper bound diameter of 50% of cumulative percentage of particles by volume (D_{50}) .

Water column												
	<u>S1 S2 S3 S4</u>											
Salinity (psu)	0.4	3.5	21.6	26.1								
рН	7.87	7.52	7.90	8.02								
Conductivity (mS/cm)	0.7383	5.731	30.48	36.42								
NO ₃ [•] (μM)	266	250	248	24								
$NO_2(\mu M)$	1.6	1.6	0.4	0.7								
NH4 ⁺ (μM)	7	7	12	23								
$SO_4^{2-}(mM)$	1	3	16	22								
Cl [•] (mM)	2	38	306	443								

Sediment porewater												
	S1s S1d S2s S2d S3s S3d S4s S4d											
Porewater salinity (psu)	0.3	0.2	3.1	1.8	17.0	17.7	28.0	32.1				
NO ₃ ⁻ (μM)	36	37	17	26	66	17	78	7				
$NO_2(\mu M)$	0.2	0.4	0.1	0.3	0.9	<dl< th=""><th>1.0</th><th><dl< th=""></dl<></th></dl<>	1.0	<dl< th=""></dl<>				
$NH_{4}^{+}(\mu M)$	12	67	12	25	73	934	166	126				
SO_4^{2-} (mM)	2	2	6	3	33	33	32	40				
Cl ⁻ (mM)	4	3	49	28	265	276	347	501				
Fe (aq) (µM)	0.4	4.9	0.1	0.3	1.6	3.6	0.9	3.3				
Mn²⁺ (aq) (μM)	3.4	82.3	5.1	49	60	0	15	62				
		Se	ediment									
	S1s	S1d	S2s	S2d	S3s	S3d	S4s	S4d				
(%) Acid extractable	52	61	53	53	39	84	57	96				
$Fe^{2+}(s)$												
Total Fe (wt %)	2.1	2.7	2.7	2.4	3.5	4.0	4.3	3.9				
%TOC	1.3	2.3	2.5	1.8	2.1	2.6	2.2	2.7				
%TS	0.16	0.18	0.18	0.14	0.22	0.35	0.31	0.52				
Grain size (µm) (D50)	57	51	52	49	14	17	14	17				

²⁶³

264 **3.2** Bacterial community composition and bacterial diversity along the salinity gradient

The Illumina MiSeq run yielded >500,000 paired-end reads per sample after quality control 265 (see Supplementary Information; Table S7). This dataset was randomly sampled to give exactly 266 267 500,000 reads per sample. The combined pool of 4 million reads was used to identify the characteristic 268 OTUs in the regional dataset. A total of 3,596,003 reads in the combined pool passed the chimera check, and these were clustered into OTUs (>97% sequence identity), and assigned to taxonomic 269 270 groups. Then, the entire dataset of 6,179,119 reads were allocated to these OTUs. The OTUs classified 271 as Archaea (4% of non-chimeric reads), and the OTUs which were not classified to the Bacteria phylum 272 level with a confidence >0.7 (14% of non-chimeric reads) were excluded from further analysis. This 273 resulted in 5,064,424 reads that were allocated to 7,656 OTUs that were classified to the Bacteria 274 phylum level with a confidence level >0.7.

There were 20 phyla that individually represented more than 0.1% on average of the total reads (Figure 4), the most abundant of which were Proteobacteria (51% on average of the total reads),

Acidobacteria (11%), Bacteroidetes (10%) and Chloroflexi (9%). At this taxonomic level, the community structure of all the samples had a similar composition, with the exception of the sample of subsurface sediment from Paull (S3d). In this sample Proteobacteria were dominant, accounting for 92% of the OTUs present versus the 45% (on average) that Proteobacteria represented in the other sites. Further information about the classification of each bacterial community to the class level can be found in the Supplementary Information.







Figure 4: Taxonomical composition of the microbial community at Bacteria phylum level. Phyla with

relative abundance below 0.1% are grouped as "Other phyla".

287

288 More detailed analysis of the phylum Proteobacteria reveals changes in composition along the 289 estuary. The class Gammaproteobacteria was the most numerous, and increased from 18% of total 290 reads in the inner estuary to 25% of total reads in the outer estuary (sample S3d is thought to be atypical 291 so, unless explicitly stated, it was omitted from the reported averages). This increase in abundance 292 along the estuary was associated with an increase in the number of reads currently with uncertain 293 placement (order incertae sedis; see Supplementary Information Table S5). Betaproteobacteria was the 294 next most numerous class in the inner estuary samples with 9% of total reads, but were <3% of total 295 reads in the outer estuary. On the other hand, it was notable that the abundance of Deltaproteobacteria 296 was similar in all the inner estuary samples and the outer estuary surface samples (~7% of total reads), 297 but they represented ~17% of S4d. This was mainly the result of an increase in the order Desulfobacterales from ~2% of total reads in the inner estuary to ~13% of total reads in S4d. 298

299 Acidobacteria was the second most abundant bacterial phylum representing ~15% of the total 300 reads in the inner estuary, but ~8% of reads in the outer estuary samples. Within the Acidobacteria, the 301 subdivision 6 (Class Acidobacteriia) was most numerous in the inner estuary (~6% of total reads), but 302 was 1% of total reads in the outer estuary. Bacteroidetes was the third most abundant Bacterial phylum 303 representing ~9% of total reads in the inner estuary, but ~16% of total reads in the outer estuary. Within 304 the Bacteroidetes, the class Flavobacteriia was the most abundant in all the samples. Flavobacteriacaea 305 was the dominant family in this class. Chloroflexi was the fourth most abundant Bacterial phylum, and 306 it exhibited very little systematic change along the estuary. The two most abundant classes within the 307 Chloroflexi were Caldilineae and Anaerolineae (~3% and 2% respectively of total reads from the whole 308 estuary).

The OTU richness, D_0^{a} , in each sample is shown in Figure 5a. The average richness at the 309 310 different sites and sediment depths was ~5,000 OTUs; although sites towards the outer estuary showed 311 slightly lower D_0^{α} . Diversity measures that indicate the number of common OTUs (D_1^{α}) and dominant 312 OTUs (D_2^{α}) both showed a stronger pattern of decreasing OTU diversity along the salinity gradient (Figures 5b and 5c). These differences in OTU relative abundance between the inner and outer zones 313 314 of the estuary were significant (PERMANOVA analysis indicated p < 0.05). Between the innermost 315 and outermost estuary samples (S1 and S4) there was a drop in both D_1^{α} and D_2^{α} for the surface and the subsurface sediments by 60-70%. To further illustrate the diversity trends, the values of D_I^{α} and 316 317 D_2^{α} have been used to estimate the percentage of reads within the common and dominant OTUs. 318 Common OTUs accounted for >80% of total sequence reads in all samples, and dominant OTUs 319 accounted for 54-73% of total sequence reads in all samples. Therefore, the decrease observed in the 320 number of common and dominant OTUs along the estuary represented a shift towards fewer but more abundant OTUs towards the sea. The statistically weighted alpha-diversity (${}^{*}D_{I}{}^{\alpha}$) was 438; the regional 321 diversity $(D_I)^{\gamma}$ was 934; which following Whittaker's multiplicative law, $(D_I^{\beta} = D_I^{\gamma/*} D_I^{\alpha})$, gave a beta 322 323 component (D_I^{β}) of 2.

324

Microbial Diversity of the Humber Estuary



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Figure : Alpha-diversity D_q^{α} values for each location (Hill numbers of order 0, 1, and 2): (a) D_{θ}^{α} or OTUs richness; (b) D_1^{α} ; and (c) D_2^{α} . The colour of the bars follows the colour code for the inner (blue), mid (purple) and outer (pink) estuary defined by salinity variation range<u>, and colour darkens as q</u> increases (from D_{θ}^{α} to D_2^{α}).

330

331 NMDS analysis indicates that the variation of species frequencies in the samples is well 332 represented in two-dimensions (Figure 6, stress value < 0.05). The NMDS ordination showed the split 333 between the inner estuary samples, that were ordinated in a relatively close group, and the outer estuary

samples which were progressively more distant from the inner estuary group. The mid and outer estuary samples were also separated by depth, but there are too few samples to determine whether is significant (p > 0.05).



Figure 6: NNMDS ordination for dissimilarities in the bacterial community distribution among samples based on Bray-Curtis distances. Samples are colour-coded according to the salinity variation zones (inner (blue), mid (purple) and outer (pink) estuary). Surface sediment samples (circle markers) are coloured lighter than the corresponding subsurface sediment samples (squared markers). Dashed ellipse has been added to indicate the inner estuary samples.

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337

The BIOENV analysis showed that salinity, ammonium concentration in porewater and reduced
 iron in solids were the subset of environmental variables that best correlated (0.94) with the community

composition of the different sites along the Humber estuary (Mantel statistic based on Pearson correlation, R = 0.72, p < 0.05) (see Supplementary Information).

348 4 Discussion

349 The Humber estuary is a shallow well-mixed estuary where water mixing is strongly driven by 350 tidal forcing. Surface and subsurface sediments in the Humber are both subjected to reoxidation 351 processes due to resuspension, albeit at different frequencies (Mortimer et al., 1998; Mortimer et al., 352 1999). Additionally, the spatial heterogeneity of nutrient concentrations and the patterns of movement 353 of the ETM within the Humber are influenced by seasonal variations of river flow (Mitchell, 1998; 354 Sanders et al., 1997; Uncles et al., 1998a). Intertidal fine-grained sediments support highly diverse microbial communities (Reed & Martiny, 2012; Zinger et al., 2011) and environmental gradients are 355 356 likely to be shaping the spatial distribution of the communities in these estuarine systems (Campbell 357 & Kirchman, 2013; Findlay et al., 1990; Liu et al., 2014; O'Sullivan et al., 2013; Wei et al., 2016; 358 Zhang et al., 2014b).

359 The large scale spatial gradients in salinity and nutrient concentrations observed in this study 360 are reflective of natural environmental gradients expected within estuarine systems (Crump et al., 361 2004; Jeffries et al., 2016; Liu et al., 2014). Overall, the mid estuary experiences the widest salinity 362 variation in the Humber; although sediment porewater salinity is expected to vary more slowly than river water salinity in muddy fine-grained sediments, and it probably remains close to the long term 363 364 average of river water salinities. Concentrations of nitrate decreased in the water column towards the 365 outer estuary, while sulphate became a more important chemical species as seawater had more 366 influence on the water column composition. Other than that, the main differences between the inner 367 and the mid/outer estuary were the more reducing nature of the later. The sediments recovered from 368 the mudflats of the mid and outer estuarine showed some iron enrichment compared to the sites from

369 the inner estuary. Iron and ammonium concentrations in the porewater increased also toward the marine 370 end of the system, as well as the proportion of reduced iron from solids found in subsurface sediments. 371 Field observations of the sediment colour at the mid and outer estuary sites (reddish-brown at the 372 surface but dark grey-black in the subsurface) evidenced an abrupt redoxcline at these sites. Although 373 H₂S concentrations were not measured and AVS concentrations were relatively low, others reported 374 that the subsurface sediments of the outer estuary Humber mudflats can be sulfidic (Andrews et al., 375 2000; Mortimer et al., 1998). Such an abrupt redox change with depth was probably not developed at 376 the inner estuary sites, where the subsurface sediments appeared to be poised between nitrate and iron 377 reducing conditions. Sediment was finer in the samples from the mid and outer estuary, which may 378 have further implications in the temperature gradients, organic matter turnover, and the erodibility of 379 the sediments (Blanchard et al., 2000; Bühring et al., 2005; Harrison & Phizacklea, 1987; Musat et al., 380 2006).

381 4.1 Bacterial community composition along the estuarine gradient

382 Taxonomically, all samples except for S3d had a similar composition. Proteobacteria was the 383 most represented phylum in all the bacterial communities, followed by Acidobacteria, Bacteroidetes 384 and Chloroflexi. This distribution of phyla was consistent with other studies in coastal and estuarine 385 sediments (Halliday et al., 2014; Jeffries et al., 2016; Liu et al., 2014; Wang et al., 2012; Wei et al., 386 2016). The increase in abundance of *Proteobacteria* along the estuary was, mainly the result of an 387 increase in abundance of Gammaproteobacteria incertae sedis. The detailed phylogenetic relationships 388 in this taxonomic group are currently unknown, but it contains many aerobic and facultative anaerobic 389 genera recovered from brackish and saline environments (Distel et al., 2002; Lin & Shieh, 2006; 390 Romanenko et al., 2004; Spring et al., 2009), so this increased abundance may be related with 391 increasing salinity. However, the increase in abundance of reads from the order Desulfobacterales of 392 the Deltaproteobacteria in sample S4d, could be a response to the salinity and redox conditions in the

393 outer estuary subsurface sediments, as this order contains strictly anaerobic sulphate-reducing bacteria 394 that are most frequently found in brackish and marine habitats (Kuever, 2014a, b, c). There was also 395 an increase in the abundance of *Bacteroidetes* along the estuary, and particularly of species in the 396 family Flavobacteriacaea. The marine genera of Flavobacteriaceae are a major component of the 397 oceanic microbial biomass in the pelagic zone (Kirchman, 2002; McBride, 2014). A decrease in the 398 abundance of Acidobacteria along the estuary was observed, which was principally the result of the 399 decrease in abundance of the subdivision 6. Subdivision 6 (Class Acidobacteriia) is widespread in 400 terrestrial and marine environments, and tend to be highly abundant in nutrient-rich environments 401 (Janssen, 2006; Kielak et al., 2016).

402 The taxonomic composition of sample S3d differed markedly from the other samples. Here the 403 bacterial community was dominated by Epsilonproteobacteria. This taxonomic group has been found 404 in other estuarine and coastal sediments and pelagic redoxclines (Bruckner et al., 2013; Campbell et 405 al., 2006; Grote et al., 2008; Jeffries et al., 2016; Labrenz et al., 2005), and is occasionally abundant 406 (Wang et al., 2012). Epsilonproteobacteria has been suggested to be one of the dominant 407 microorganisms involved in the coupling of C, N and S cycles (Campbell et al., 2006). Many 408 Epsilonproteobacteria within the order of Campylobacterales (the most important in sample S3d) are 409 microaerophilic chemolitotrophs that can couple the oxidation of sulphur compounds or hydrogen to 410 the reduction of oxygen or nitrate (Bruckner et al., 2013; Campbell et al., 2006; Grote et al., 2008; 411 Labrenz et al., 2005). This taxonomic group has also been associated with shellfish (as a reservoir of food-borne and waterborne pathogens) and faecal pollution (Levican et al., 2014). The dominance of 412 413 Campylobacterales in the subsurface sediments from S3 and the low bacterial diversity measured could be due to the sampling of a specialist niche in S-reducing geochemical conditions. However other 414 415 causes of these anomalous results (i.e. sampling or sequencing technology biases, or the proximity of 416 shellfish to the sample) cannot be discarded.

417

7 4.2 Trends and environmental drivers of microbial diversity

418 Ever since publication of Remane's model, there has been substantial interest in the role of 419 salinity stress in shaping estuarine biodiversity (Attrill, 2002; Whitfield et al., 2012). In this study we 420 found that the OTU richness of benthic bacteria (as measured by D_0^{α}) was relatively uniform along the 421 Humber estuary, which appears to confirmed with previous reports of uniform bacterial richness along 422 a salinity gradient (Herlemann et al., 2011; Hewson et al., 2007; Zhang et al., 2014b). However, due 423 to the hyperdiverse nature of microorganisms in many ecosystems, richness can give a distorted view 424 of microbial diversity because it gives equal weight to common and rare taxa (i.e. richness takes no 425 account of OTU relative abundance). Also it is rarely possible to evaluate richness accurately, as it is 426 extremely difficult adequately sample rare taxa even with high-throughput sequencing technologies 427 (Kang et al., 2016). Therefore Hill numbers of higher order (q = 1 or 2) are considered to be a more 428 suitable mathematical approach to microbial diversity that give consistent measures of the prominence 429 of common or dominant species in a community since they are not sensitive to sequencing depth (Kang 430 et al., 2016).

431 The analysis of the microbial diversity in the Humber mudflats using D_1^{α} and D_2^{α} (Figure 5b and 5c) revealed a decreasing trend of microbial diversity in terms of common and dominant OTUs 432 433 with increasing salinity. The numbers of common and dominant OTUs in the mid and outer estuary 434 samples were only about 40% and 35% of the average number in the inner estuary. This indicated a 435 change towards a community structure with a smaller number of more abundant OTUs along the 436 estuarine salinity gradient. Other studies also reported a similar decreasing trend in pelagic and benthic 437 bacterial diversity along the salinity gradient (Campbell & Kirchman, 2013; Liu et al., 2014; Wang et 438 al., 2015; Zhang et al., 2014a), which may be in part be explained by the influence of the riverine 439 inputs on the inner estuary communities (Crump et al., 1999; Monard et al., 2016; Rappé et al., 2000; 440 Zhang et al., 2014a). Generally Site 3 fitted this trend, despite being in the area of highest salinity

441 variation. The surface sample (S3s) showed D_1^{α} and D_2^{α} measurements that were intermediate between 442 the inner and outer estuary, which was not surprising given the regular resuspension and mixing 443 processes of surface sediments by tidal forces. However, as mentioned above, the subsurface sample 444 (S3d) showed lower D_{1}^{α} and D_{2}^{α} values than any other sample analysed. This could be associated with 445 salinity stress, or possibly sampling or sequencing bias, but it is more likely that some other environmental pressure had produced a specialist niche that favoured just a few bacterial species at this 446 447 location. Microbial DNA was extracted from <0.5 g of sediment, and thus very local geochemical 448 effects could affect the bacterial community within individual samples.

449 NMDS ordination showed differences in the bacterial community associated with progression 450 toward the outer estuary. Also, the NMDS analysis clustered all the inner estuary samples together, 451 suggesting that the bacterial populations of the inner estuary mudflats were not significantly different 452 between depths. The colour pattern in the heat map (see Supplementary Information) also showed these 453 samples as being similar in their composition. The effects of the mixing at the ETM and the presence 454 of more coarse sediments could enhance the homogenisation of surface and subsurface bacterial 455 communities (Bühring et al., 2005; Crump et al., 1999; Feng et al., 2009; Lavergne et al., 2017; Musat 456 et al., 2006). The NMDS analysis also separated the subsurface mid and outer estuary samples from 457 their surface counterparts, but insufficient samples were used to determine whether this trend was 458 significant. Nevertheless, field observations and geochemical measurements indicated that subsurface 459 mid/outer estuarine sediments were more reducing than the inner estuarine sediments. Other studies in 460 similar environmental conditions suggested that such vertical stratification in the microbial 461 communities should be expected in the presence of strong redox stratification in estuarine mudflats (Bertics & Ziebis, 2009; Lavergne et al., 2017; Liu et al., 2014; Musat et al., 2006; O'Sullivan et al., 462 463 2013).

464 Overall, salinity, ammonium in porewater and reduced iron in solids were the set of environmental variables that best explained the variability of our dataset. Although the significance of 465 466 salinity determining microbial compositions has been well documented; the importance of other 467 environmental variables may be hidden as they co-vary with salinity along the gradient. For example, 468 Liu et al. (2014) found that sulphate concentration might be hidden by salinity as a driver for the 469 distinct distribution of methanogens and sulphate-reducing bacteria between fresh and seawater 470 sediments. Stronger redox stratification would be expected in the less-frequently disturbed subsurface 471 sediments, which in the more sulphidic mid and outer Humber mudflats, may provide the geochemical 472 conditions for more specialist communities to develop (Bertics & Ziebis, 2009; Hewson & Fuhrman, 473 2004). We hypothesise that the weaker redox stratification in the inner Humber estuary is likely the 474 reason of the similarity of the microbial populations between depths, although the coarser (i.e. more 475 permeable) nature of the inner mudflats and the position of the ETM (i.e. more intense mixing) could also be enhancing the uniformity of the microbial populations in the freshwater end of the Humber. 476 477 Apart from the resuspension, other external parameters (temperature, wind, tidal cycle, light exposure, 478 organic matter, benthic fauna and microphytobentic activity) will strongly influence the distribution of 479 bacterial communities, especially in the surface sediment layer. These could cause important seasonal 480 differences in microbial metabolism in different zones, as observed by different authors (Hubas et al., 481 2007; Lavergne et al., 2017; Orvain et al., 2014).

The regional microbial diversity of the Humber estuary ($D_I^{\gamma} = 934$) indicated that many of the OTUs that were common in individual samples were common within regional dataset. Further, the beta-diversity calculated for common species ($D_I^{\beta} \sim 2$) indicated that the regional diversity could be explained by there being two distinct compositional groups dispersed amongst the various local communities. We suggest that the first of these compositional units may be a community that is subjected to remobilisation and is regularly mixed and transported along the estuary, but is stressed by

the varying salinity conditions (there will be less of a direct link between the geochemistry and the bacterial community in frequently disturbed estuarine sediments (O'Sullivan *et al.*, 2013)). The second compositional unit may develop in the more strongly reducing and less frequently disturbed subsurface sediments of the mid and outer estuary mudflats which is in agreement with the multivariate analysis results..

493 4.3 Conclusions

494 To conclude, this study has provided the insight to the microbial diversity of the Humber 495 estuary. The large amount of data produced by using high throughput sequencing technologies resulted 496 in a deep coverage of the individual samples. A taxonomic approach to the community data did not 497 show clear differences between sampling sites. Similarly, OTU richness, D_0^{α} , was relatively uniform 498 for benthic bacteria in the estuary. However, Hill numbers of higher order $(D_1^{\alpha} \text{ and } D_2^{\alpha})$ decreased 499 towards the sea, which indicates a change towards communities where a smaller number of OTUs 500 represent a larger proportion of the population. The discovery of this trend along the salinity gradient 501 illustrated the importance of using a rigorous and consistent mathematically approach to characterise 502 bacterial diversity, particularly when working with amplicon sequencing data. Beyond salinity 503 variation, there was some evidence that redox transitions with depth may apply further selective 504 pressure on the microbial populations of the mid and outer mudflats, but other spatiotemporal 505 fluctuations in the physicochemical conditions (redox gradients and sediment remobilisation and 506 mixing) may have also an impact on the bacterial community composition. Further studies will be 507 needed to explore more deeply the effects of these and other biotic and abiotic variables on microbial 508 diversity and activity through different seasons.

509 Conflict of Interest

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