

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

1 Andrea Vidal-Durà^{1*}, Ian T. Burke¹, Robert J.G. Mortimer³ and Douglas I. Stewart²

2 ¹School of Earth and Environment, University of Leeds, Leeds, UK.

3 ²School of Civil Engineering, University of Leeds, Leeds, UK.

4 ³School of Animal Rural & Environmental Sciences, Nottingham Trent University, Brackenhurst
5 Campus, Southwell, Nottinghamshire, UK

6 ***Correspondence:**

7 Andrea Vidal-Durà: andreavidaldura@gmail.com

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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
16 gene, and it correlates variations with environmental variables. The sediment depths sampled were
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

Microbial Diversity of the Humber Estuary

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**

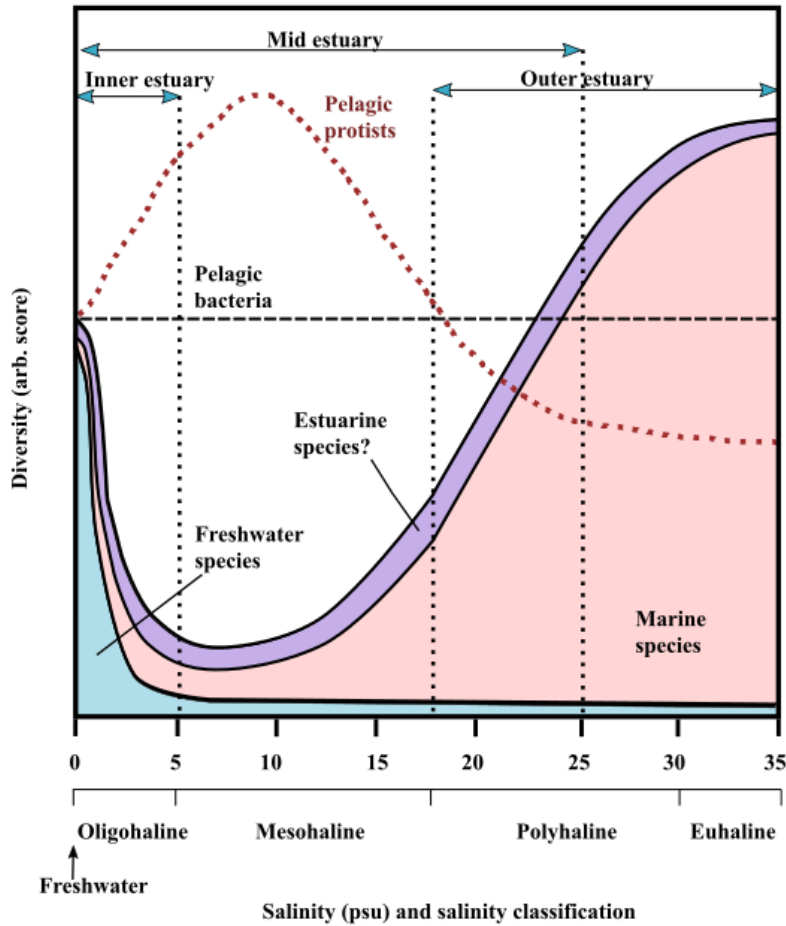
32 Estuaries are transitional environments where substantial physicochemical and biological
33 gradients from freshwater to marine environments develop (Attrill & Rundle, 2002; Crump *et al.*, 2004;
34 Elliott & Whitfield, 2011; Lallias *et al.*, 2015). The continuous mixing of water and sediments leads 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 &

Microbial Diversity of the Humber Estuary

44 Ziebis, 2009; Reed & Martiny, 2012). Quantifying the microbial community variations along estuarine
45 gradients will improve the understanding of their role in these ecosystems and their response to
46 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.*,
49 2004; Elliott & Whitfield, 2011; Herlemann *et al.*, 2011; Lallias *et al.*, 2015; Lozupone & Knight,
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
59 significant limitations as a description of diversity in estuarine systems. Telesh *et al.* (2011) conducted
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
63 for resources, so these niches can be occupied by highly adaptable unicellular organisms (i.e.
64 planktonic organisms). However Herlemann *et al.* (2011) found that the diversity of pelagic bacteria
65 exhibited a different pattern to protists and displayed a steady distribution in the Baltic Sea with no
66 trend with salinity (Figure 1) possibly due to the mixing of freshwater and marine communities.

Microbial Diversity of the Humber Estuary



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
 70 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
 72 the salinity zonation defined for the Humber estuary (see methods section).

Commented [AVD1]: I may remove labels of outer, inner and mid estuary

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Microbial Diversity of the Humber Estuary

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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
81 geochemistry influences in the composition and function of benthic microbial communities (Canfield
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 al.*,
86 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.*,
88 2007). Consequently, sediment dynamics may also be an important environmental factor shaping
89 estuarine microbial diversity.

90 Lately high-throughput sequencing techniques have become widely available (Bier *et al.*, 2015;
91 Buttigieg & Ramette, 2014; Liu *et al.*, 2014). These techniques offer an opportunity to investigate
92 microbial communities in more depth. However, challenges remain as the very large data sets produced
93 reveal the hyperdiverse nature of microbiota, which is difficult to evaluate rigorously with the
94 traditional mathematical and statistical approaches to biodiversity estimation (Buttigieg & Ramette,
95 2014; Kang *et al.*, 2016; Oulas *et al.*, 2015). Hill numbers (D_q) are a unified and index-independent
96 diversity concept; they were developed by Hill (1973) and were reintroduced to ecologists by Jost

Microbial Diversity of the Humber Estuary

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*
100 *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
112 amplicon sequences of the V4 hyper-variable region of the 16S rRNA gene, were processed and the
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

Microbial Diversity of the Humber Estuary

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
127 range experienced by surficial sediments (Figure 3). Three salinity zones can be empirically identified.
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
132 ~25 psu (purple area in Figure 2 and 3, see annotation in Figure 1), which includes oligohaline,
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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One of the reviewers wanted this figure in the Introduction or elsewhere, not in Discussion.

I proposed to move this figure to Supporting Information. Originally we put a lot of effort on this since we were focused on Paull as a representative site of the zone which experienced the highest salinity variation. However, I think now this has become a secondary point in the argument

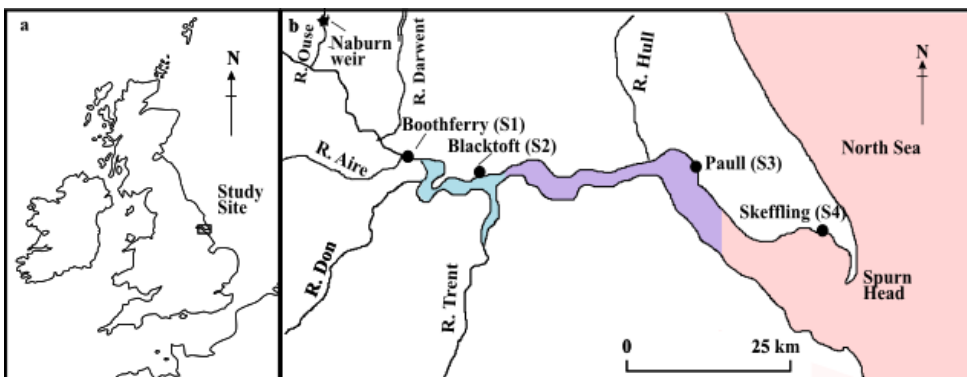
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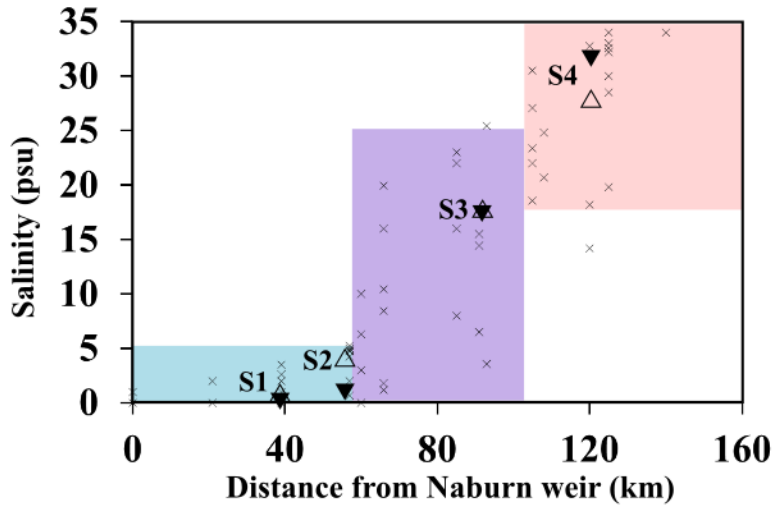
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Microbial Diversity of the Humber Estuary

138 **Figure 2:** Map of the Humber Estuary (UK) with the sampling locations (Boothferry (S1), Blacktoft
 139 (S2), Paull (S3), and Skeffling (S4)) and the salinity variation zones (blue for ≤ 5 psu; purple for 0-25
 140 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;
 146 Williams & Millward, 1999). Salinity ≤ 5 psu (blue area); 0-25 psu salinity range (purple area); and 18-
 147 35 psu salinity range (pink area). The triangle markers indicate the porewater salinity measurements of
 148 this study (S1-S4) (empty and coloured markers for surface and subsurface porewater salinity
 149 respectively).

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 Following reviewer's comments, it belongs to introduction, and therefore I am not sure if our data points (results) should be in the figure. If it is moved to introduction or to methods, it will be Figure 2 or 3 (depending if it goes before or after the map)
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Microbial Diversity of the Humber Estuary

151 Sediment samples were collected at low tide from the intertidal mudflats along a 65 km transect
152 in the north bank of the Humber estuary during the same tidal cycle on 15th July 2014. The four sites
153 were at Boothferry (S1), Blacktoft (S2), Paull (S3), and Skeffling (S4), and they were selected to span
154 the salinity range. A sample of surface (s) (0-1 cm) and subsurface (d) (5-10 cm) sediment was
155 recovered from each location in 1L containers, transported back in the dark to the laboratory.
156 Subsamples of the homogenised sediment were stored in 2 mL microcentrifuge tubes at -20°C for
157 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 (0.2µm 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
166 determined after acidification with 1% AnalaR HNO₃ (VWR) using ion coupled plasma-mass
167 spectroscopy (Thermo Scientific™ ICP-MS). Wet sediments were analysed for: particle size by laser
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.

Microbial Diversity of the Humber Estuary

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
178 larger than 3 kb, an agarose gel electrophoresis was run. The 1% agarose “1x” Tris-borate-EDTA
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/μL in 20 μL aqueous solution) were sent for sequencing at the Centre for
185 Genomic Research, University of Liverpool, where Illumina adapters and barcodes were attached to
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
188 5'-GTGCCAGCMGCCGCGTAA-3' and the reverse target specific primer 5'-
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
195 *fastq_filter* command (expected error cutoff was set at 1.0 and length truncation was not applied),
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

Microbial Diversity of the Humber Estuary

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

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

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

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^{α} , (the species
221 richness), D_1^{α} (common species) and D_2^{α} (dominant species) (Jost, 2006, 2007). Traditional diversity

Microbial Diversity of the Humber Estuary

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_1^γ) was calculated using the combined dataset. The beta-diversity, D_1^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 ($*D_1^\alpha \times D_1^\beta = D_1^\gamma$) (Whittaker, 1972). $*D_1^\alpha$ 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

Microbial Diversity of the Humber Estuary

245 The environmental characterisation of the water, porewater, and sediment samples is shown in
 246 Table 1. The water column salinity at the sampling locations spanned from very low salinity at the
 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.

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

	Water column			
	S1	S2	S3	S4
Salinity (psu)	0.4	3.5	21.6	26.1
pH	7.87	7.52	7.90	8.02
Conductivity (mS/cm)	0.7383	5.731	30.48	36.42
NO₃⁻ (μM)	266	250	248	24
NO₂⁻ (μM)	1.6	1.6	0.4	0.7
NH₄⁺ (μM)	7	7	12	23
SO₄²⁻ (mM)	1	3	16	22
Cl⁻ (mM)	2	38	306	443

Microbial Diversity of the Humber Estuary

Sediment porewater								
	<i>S1s</i>	<i>S1d</i>	<i>S2s</i>	<i>S2d</i>	<i>S3s</i>	<i>S3d</i>	<i>S4s</i>	<i>S4d</i>
Porewater salinity (psu)	0.3	0.2	3.1	1.8	17.0	17.7	28.0	32.1
NO₃⁻ (μM)	36	37	17	26	66	17	78	7
NO₂⁻ (μM)	0.2	0.4	0.1	0.3	0.9	<DL	1.0	<DL
NH₄⁺ (μM)	12	67	12	25	73	934	166	126
SO₄²⁻ (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
Sediment								
	<i>S1s</i>	<i>S1d</i>	<i>S2s</i>	<i>S2d</i>	<i>S3s</i>	<i>S3d</i>	<i>S4s</i>	<i>S4d</i>
(%) Acid extractable Fe²⁺_(s)	52	61	53	53	39	84	57	96
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

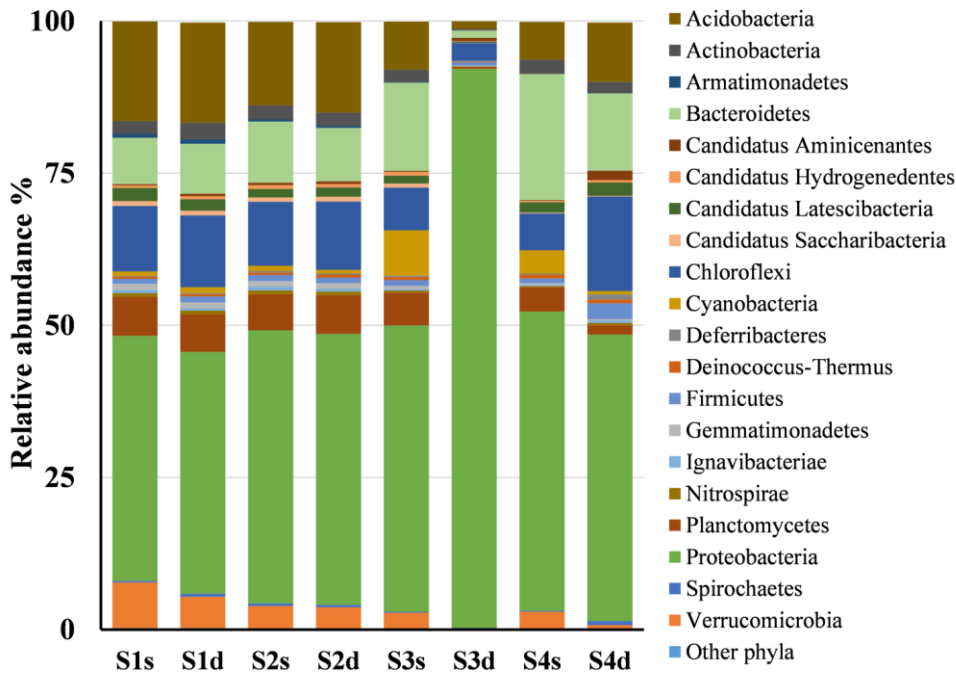
265 The Illumina MiSeq run yielded >500,000 paired-end reads per sample after quality control
 266 (see Supplementary Information; Table S7). This dataset was randomly sampled to give exactly
 267 500,000 reads per sample. The combined pool of 4 million reads was used to identify the characteristic
 268 OTUs in the regional dataset. A total of 3,596,003 reads in the combined pool passed the chimera
 269 check, and these were clustered into OTUs (>97% sequence identity), and assigned to taxonomic
 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.

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

Microbial Diversity of the Humber Estuary

277 Acidobacteria (11%), Bacteroidetes (10%) and Chloroflexi (9%). At this taxonomic level, the
 278 community structure of all the samples had a similar composition, with the exception of the sample of
 279 subsurface sediment from Paull (S3d). In this sample Proteobacteria were dominant, accounting for
 280 92% of the OTUs present versus the 45% (on average) that Proteobacteria represented in the other
 281 sites. Further information about the classification of each bacterial community to the class level can be
 282 found in the Supplementary Information.

283



284

285 **Figure 4:** Taxonomical composition of the microbial community at Bacteria phylum level. Phyla with
 286 relative abundance below 0.1% are grouped as “Other phyla”.

Microbial Diversity of the Humber Estuary

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
298 Desulfobacterales from ~2% of total reads in the inner estuary to ~13% of total reads in S4d.

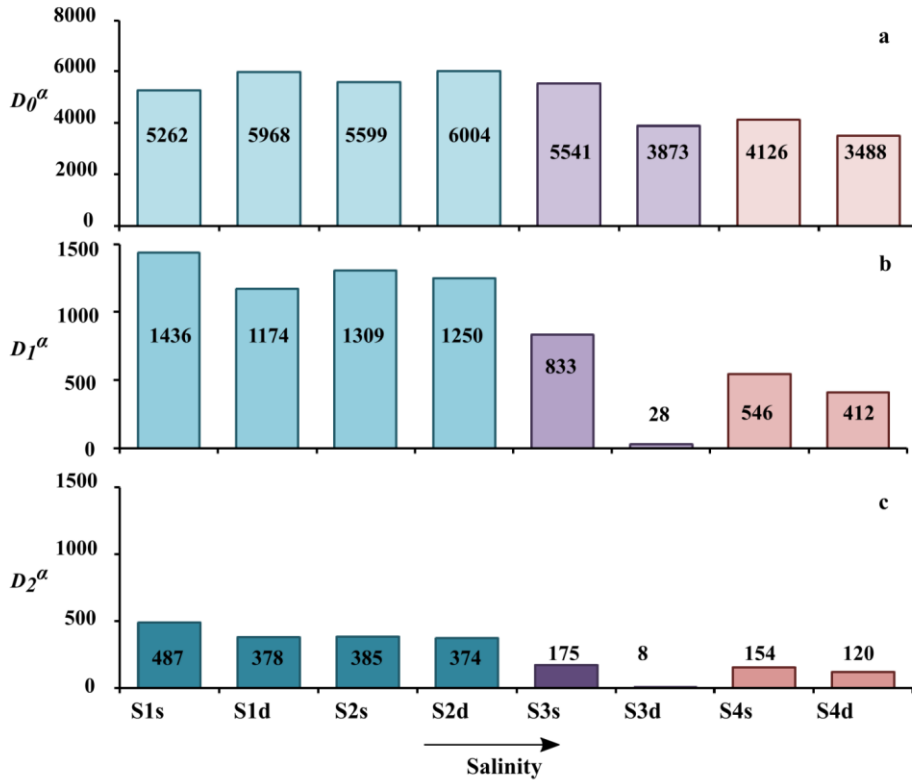
299 Acidobacteria was the second most abundant bacterial phylum representing ~15% of the total
300 reads in the inner estuary, but ~8% of reads in the outer estuary samples. Within the Acidobacteria, the
301 subdivision 6 (Class Acidobacteriia) was most numerous in the inner estuary (~6% of total reads), 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. *Flavobacteriaceae*
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).

Microbial Diversity of the Humber Estuary

309 The OTU richness, D_0^α , in each sample is shown in Figure 5a. The average richness at the
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
313 (Figures 5b and 5c). These differences in OTU relative abundance between the inner and outer zones
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
316 the subsurface sediments by 60-70%. To further illustrate the diversity trends, the values of D_1^α and
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
321 abundant OTUs towards the sea. The statistically weighted alpha-diversity ($^*D_1^\alpha$) was 438; the regional
322 diversity (D_1^β) was 934; which following Whittaker's multiplicative law, ($D_1^\beta = D_1^\beta / ^*D_1^\alpha$), gave a beta
323 component (D_1^β) of 2.

324

Microbial Diversity of the Humber Estuary

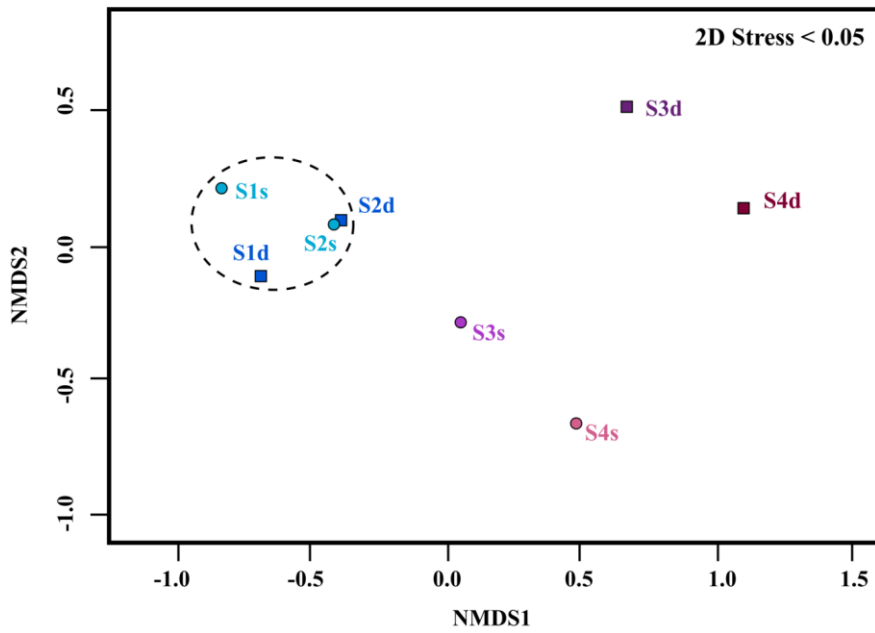


325
 326 **Figure** : Alpha-diversity D_q^α values for each location (Hill numbers of order 0, 1, and 2): (a) D_0^α or
 327 OTUs richness; (b) D_1^α ; and (c) D_2^α . The colour of the bars follows the colour code for the inner (blue),
 328 mid (purple) and outer (pink) estuary defined by salinity variation range, and colour darkens as q
 329 increases (from D_0^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

Microbial Diversity of the Humber Estuary

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



337
338 **Figure 6:** NNMDS ordination for dissimilarities in the bacterial community distribution among
339 samples based on Bray-Curtis distances. Samples are colour-coded according to the salinity variation
340 zones (inner (blue), mid (purple) and outer (pink) estuary). Surface sediment samples (circle markers)
341 are coloured lighter than the corresponding subsurface sediment samples (squared markers). Dashed
342 ellipse has been added to indicate the inner estuary samples.

343
344 The BIOENV analysis showed that salinity, ammonium concentration in porewater and reduced
345 iron in solids were the subset of environmental variables that best correlated (0.94) with the community

Microbial Diversity of the Humber Estuary

346 composition of the different sites along the Humber estuary (Mantel statistic based on Pearson
347 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
355 microbial communities (Reed & Martiny, 2012; Zinger *et al.*, 2011) and environmental gradients are
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
363 river water salinity in muddy fine-grained sediments, and it probably remains close to the long term
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

Microbial Diversity of the Humber Estuary

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

Microbial Diversity of the Humber Estuary

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 *Flavobacteriaceae*. 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 chemolithotrophs 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
412 food-borne and waterborne pathogens) and faecal pollution (Levican *et al.*, 2014). The dominance of
413 *Campylobacterales* in the subsurface sediments from S3 and the low bacterial diversity measured could
414 be due to the sampling of a specialist niche in S-reducing geochemical conditions. However other
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.

Microbial Diversity of the Humber Estuary

417 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^a) was relatively uniform along the
421 Humber estuary, which appears to be 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 to 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^a and D_2^a (Figure 5b
432 and 5c) revealed a decreasing trend of microbial diversity in terms of common and dominant OTUs
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 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

Microbial Diversity of the Humber Estuary

441 variation. The surface sample (S3s) showed D_1^a and D_2^a 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^a and D_2^a 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
446 environmental pressure had produced a specialist niche that favoured just a few bacterial species at this
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 (Bühning *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
462 (Bertics & Ziebis, 2009; Lavergne *et al.*, 2017; Liu *et al.*, 2014; Musat *et al.*, 2006; O'Sullivan *et al.*,
463 2013).

Microbial Diversity of the Humber Estuary

464 Overall, salinity, ammonium in porewater and reduced iron in solids were the set of
465 environmental variables that best explained the variability of our dataset. Although the significance of
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
476 also be enhancing the uniformity of the microbial populations in the freshwater end of the Humber.
477 Apart from the resuspension, other external parameters (temperature, wind, tidal cycle, light exposure,
478 organic matter, benthic fauna and microphytobenthic 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).

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

Microbial Diversity of the Humber Estuary

488 the varying salinity conditions (there will be less of a direct link between the geochemistry and the
489 bacterial community in frequently disturbed estuarine sediments (O'Sullivan *et al.*, 2013)). The second
490 compositional unit may develop in the more strongly reducing and less frequently disturbed subsurface
491 sediments of the mid and outer estuary mudflats which is in agreement with the multivariate analysis
492 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^α and D_2^α) 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 mathematical 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**

Microbial Diversity of the Humber Estuary

510 *The authors declare that the research was conducted in the absence of any commercial or*
511 *financial relationships that could be construed as a potential conflict of interest.*

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