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

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Abstract

Sediments from intertidal mudflats are fluctuating environments that support very diverse microbial communities. The highly variable physicochemical conditions complicate the understanding of the environmental controls on diversity patterns in estuarine systems. This study investigated bacterial diversity and community composition in surface (0-1 cm) and subsurface (5-10 cm) sediments 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 selected based on the local remobilisation frequency patterns. In general, bacterial communities showed similar composition at the different sites and depths, with Proteobacteria being the most abundant phylum. Richness of operationally defined taxonomic units (OTUs) was uniform along the

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

1 Introduction

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

Salinity is known to be a major abiotic factor controlling the patterns of benthic and pelagic 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, 2007; Telesh *et al.*, 2011; Zhang *et al.*, 2014a). The variation of macrozoobenthos in estuaries has been traditionally explained using the conceptual model known as Remane's concept (Remane, 1934) (Figure 1), which was developed for the non-tidal Baltic Sea, and it models the species richness along a salinity gradient. It concludes that there is a relationship between species diversity and salinity. Species diversity reaches a minimum (*Artenminimum*) in the region of 5-8 psu salinity ('the critical salinity zone', Khlebovich, 1968) because the number of brackish specialists does not compensate for the decline of the marine and freshwater species richness (Elliott & Whitfield, 2011). However, despite several modifications (Schubert *et al.*, 2011; Telesh *et al.*, 2011; Whitfield *et al.*, 2012) and critiques (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 a meta-analysis of large data sets from previous studies in the Baltic Sea and found that protists showed a diversity maximum in the 'critical salinity zone' (Figure 1). Subsequently, Telesh *et al.* (2013) 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. 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 trend with salinity (Figure 1) possibly due to the mixing of freshwater and marine communities.

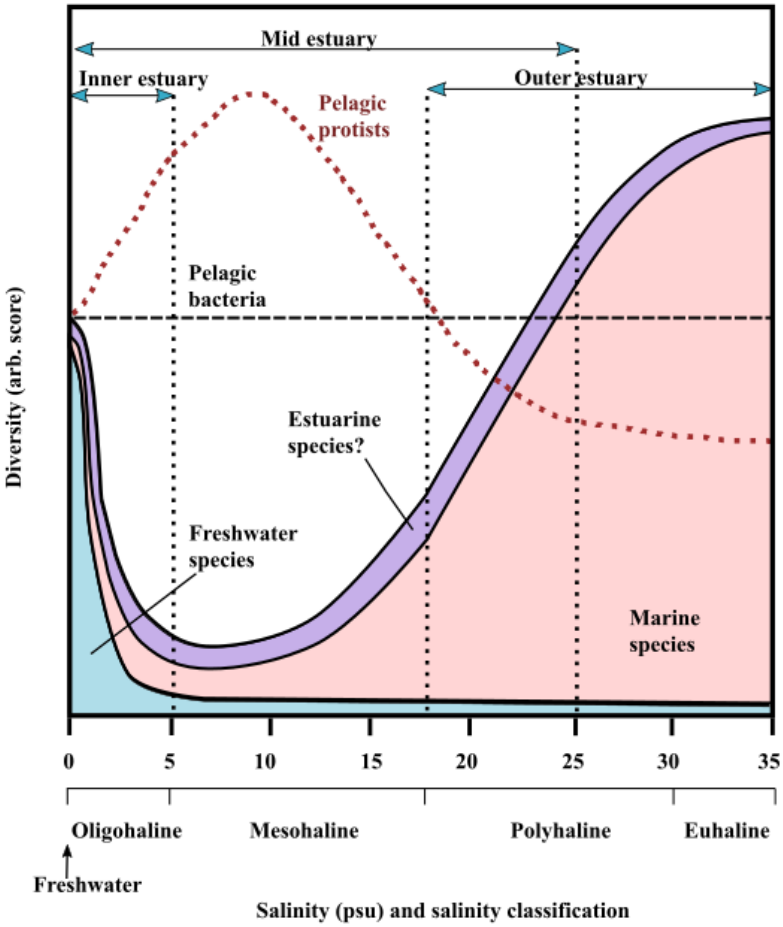

Figure 1: Diversity variation patterns along a salinity gradient. Coloured areas represent the Remane's conceptual model for the variation in macrobenthic biodiversity (after Whitfield *et al.* 2012). Variations in the diversity of pelagic protists (Telesh *et al.*, 2011) and planktonic bacteria (Herlemann *et al.*, 2011) 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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Although it is widely accepted that microbial communities are sensitive to environmental change (Lozupone & Knight, 2007), no consensus on the factors controlling microbial abundance in estuarine systems has yet emerged (Elliott & Whitfield, 2011; Telesh *et al.*, 2013). In tidal estuaries, the large salinity variations are expected to impact on bacterial community composition, activity and diversity (Campbell & Kirchman, 2013; Feng *et al.*, 2009; Liu *et al.*, 2014; Wei *et al.*, 2016). Benthic microbial communities will experience different environmental stresses to pelagic organisms, and may 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 & Thamdrup, 2009; Lavergne *et al.*, 2017; Liu *et al.*, 2014; Musat *et al.*, 2006; O'Sullivan *et al.*, 2013). However, sediments in tidal estuaries are frequently disturbed and thus may not exhibit clear links between geochemical zones and the bacterial communities present, particularly since geochemical profiles tend to re-establish more quickly than diversity profiles within the sediments (O'Sullivan *et al.*, 2013). Moreover, sediment resuspension facilitates the interaction and mixing of microbial 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 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

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

The aims of this study were: 1) to describe the bacterial communities in estuarine sediments at centimetre scale resolution, 2) to identify microbial diversity trends along the salinity gradient, and 3) to investigate how the environmental variables control such trends. This work has focused on intertidal sediments of the Humber estuary (UK) which were sampled during the same tidal cycle at low tide in summer conditions. The authors have extensively sampled the Humber Estuary in the past, observing that tidal resuspension moved just the few top mm of sediment, and during this intensive sampling, the entire top 10 cm of sediment were only removed during a powerful storm (Mortimer *et al.*, 1998; Mortimer *et al.*, 1999). The sampling strategy was based in this observed remobilisation patterns, and thus samples were collected at two depths; surface sediments that are frequency mobilised on the tidal cycle; and subsurface sediments that are only mobilised during medium/moderate resuspension events 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 benthic community composition was correlated with geochemical data using multivariate statistics to identify the environmental drivers controlling microbial diversity patterns and test whether sediment depth has an impact on microbial diversity.

2 Material and Methods

2.1 Field sites and sample collection

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

heritage (Figure 2). Its catchment area is 24,240 km^2 (20% of the area of England), it has 150 km^2 of mudflats, and the region of freshwater-saltwater mixing stretches from Naburn Weir on the Ouse, and Cromwell weir on the Trent, to the mouth of the estuary at Spurn Head. The Humber represents the main UK freshwater input to the North Sea. Generally the estuarine turbidity maximum (ETM) is situated at the inner estuary although it moves seasonally with the river flow (Uncles *et al.*, 1998a). Water column salinity records from 14 locations on the Humber over a period of ~25 years have been 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. Firstly, the inner estuary extends from 0 to 60 km below Naburn weir (the tidal limit of the Ouse system) where the water column salinity is always ≤5 psu (from freshwater to oligohaline water) (blue area in Figure 2 and 3, see also annotation in Figure 1). Secondly, the mid estuary extends from 60 to 100 km downstream of Naburn weir, and in this zone the water column salinity ranges between 0 to ~25 psu (purple area in Figure 2 and 3, see annotation in Figure 1), which includes oligohaline, mesohaline and polyhaline waters. Finally, the outer estuary extends from 100 km below Naburn weir to open coastal waters. Here the water column salinity typically varies from ~18 psu to seawater (35 psu) (pink area in Figure 2 and 3, see annotation in Figure 1), which includes polyhaline to euhaline waters.

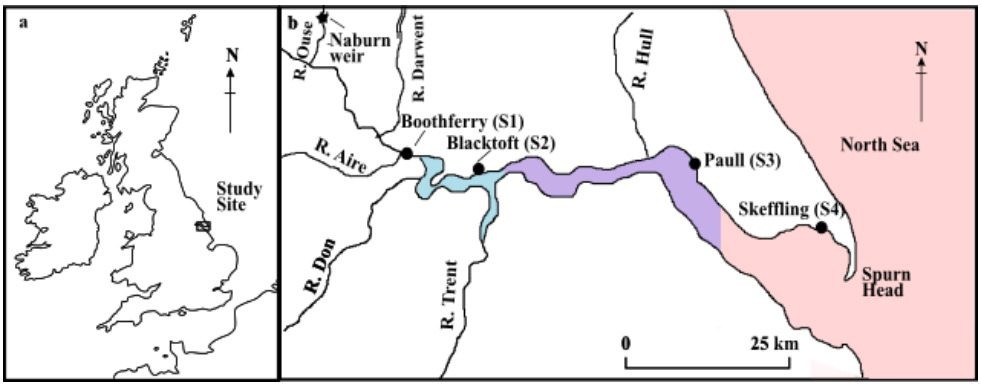

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Figure 2: Map of the Humber Estuary (UK) with the sampling locations (Boothferry (S1), Blacktoft (S2), Paull (S3), and Skeffling (S4)) and the salinity variation zones (blue for ≤5psu; purple for 0-25 psu; and pink for 18-35 psu).

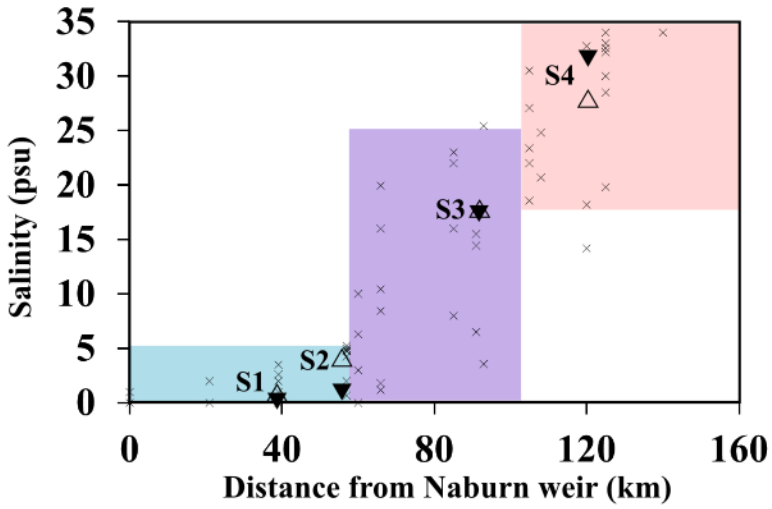

Figure 3 : Salinity zonation based on salinity records of different sites along the Humber estuary (x) (ABP Research 2000; Barnes & Owens, 1998; Burke *et al.*, 2005; Freestone, 1987; Fujii & Raffaelli, 2008; Garcia-Alonso *et al.*, 2011; Millward *et al.*, 2002; Mitchell, 1998; Mortimer *et al.*, 1999; NRA, 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 18-35 psu salinity range (pink area).The triangle markers indicate the porewater salinity measurements of this study (S1-S4) (empty and coloured markers for surface and subsurface porewater salinity 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.

2.2 Physical and chemical analysis of water and sediments

Water pH, conductivity and temperature were determined *in situ* using a Myron Ultrameter PsiII handheld multimeter. Water samples from each site were collected in 2L polythene containers. Porewater was recovered from sediment subsamples by centrifugation (30 min, 6000 *g*) in the laboratory. All water and porewater samples were filtered (0.2µm Minisart ®) and stored at 4 or −20°C, as appropriate, for further analysis. Nutrient concentrations were determined by ion chromatography (nitrate, nitrite, sulphate, and chloride) on a Dionex CD20, and colorimetrically (ammonium) on a continuous segmented flow analyser (SEAL AutoAnalyser 3 HR). Dissolved Mn and Fe were determined after acidification with 1% AnalaR HNO_3 (VWR) using ion coupled plasma-mass spectroscopy (Thermo Scientific ™ ICP-MS). Wet sediments were analysed for: particle size by laser diffraction on a Malvern Mastersizer 2000E and 0.5 N HCl extractable iron (Lovley & Phillips, 1987; Viollier, 2000). Acid volatile sulphide (AVS) (Canfield *et al.*, 1986) and pyrite (Fossing & Jørgensen, 1989) were extracted from freeze-dried sediments and quantified by weight. Finally, subsamples of ground and oven- dried sediments (60ºC) were acid washed with HCl 10% (v/v) prior to the total organic carbon (TOC) analysis by combustion with non-dispersive infrared detection on a LECO SC-144DR Sulphur and Carbon Analyser. All these physicochemical analysis of sediments and water 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
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'-GTGCCAGCMGCCGCGGTAA-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

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

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

indices, such as Shannon entropy or Gini-Simpson concentrations, can be converted to D_1^{α} and D_2^{α} by simple algebraic transformations (Supplementary Information, Table S6). The regional OTU diversity (gamma-diversity, D_1^{γ}) was calculated using the combined dataset. The beta-diversity, D_1^{β}, which reflects the proportion of regional diversity contained in a single average community, was calculated from the gamma diversity and the statistically weighed alpha-diversity , using Whittaker multiplicative law ($*D_1^{\alpha}$ x $D_1^{\beta} = D_1^{\gamma}$) (Whittaker, 1972). $*D_1^{\alpha}$ compensates for unequal sample sizes, so is not the arithmetic average of the alpha diversities of the individual samples (see Supplementary Information).

All the statistical analyses were performed with RStudio software (v 0.99.486) (RStudioTeam, 2015) using the package'vegan' (Oksanen *et al.*, 2013). The microbial community data were input as a matrix of the relative abundance of each OTU in each of the eight samples. Non-metric Multi-Dimensional Scaling (NMDS) analysis (distances based on Bray Curtis dissimilarity index) was used to graphically represent the similarity between bacterial assemblages in a two-dimensional space. Non-parametric multivariate analysis of variance (PERMANOVA) (Anderson, 2001) was used to assess the similarity in the microbial abundance among samples. BIOENV ('biota-environment') analysis (Clarke & Ainsworth, 1993) was also performed to further investigate the relationship between the microbial populations and the environmental variables using Spearman's rank correlation coefficient and Bray Curtis dissimilarities. This test finds the combination of environmental variables that best explain the patterns in the biological data. The Mantel test was also performed to study the significance of the BIOENV results. The environmental data used the BIOENV analysis included: salinity; ammonium, nitrate, sulphate, iron and manganese porewater concentrations; TOC content; pyrite and total iron in solids; particle size; percentage of acid extractable iron (II) in solids; and iron associated with pyrite.

3 Results

3.1 Environmental characterisation of the samples

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 freshwater end (0.4 psu at S1) to high salinity water at the sea end of the estuary (26.1 psu at S4). Porewater salinity was slightly lower than the water column salinity in all sites with the exception of S4. Nitrate concentration in the water column decreased along the estuary, while ammonium concentration increased slightly. With the exception of S4s, nitrate concentrations in the porewater were lower than those in the water column, whereas ammonium concentrations were higher, especially in the sites where more reducing sediments were found. Sulphate concentrations increased with salinity from 1 to 22 mM in the water column, and from 2 to 40 mM in the porewater (there was no trend with sediment depth). The total amount of iron in solids did not vary with sediment depth but increased along the estuary. The proportion of the acid extractable iron that was Fe(II) was constant in the surface sediment, however in the subsurface sediments it increased along the estuary. Sediments of the mid and outer estuary mudflats were also finer and contained slightly more TOC than sediments from the 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			
	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
NO_3^- (µM)	266	250	248	24
NO_2^- (µM)	1.6	1.6	0.4	0.7
NH_4^+ (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^- (µM)	0.2	0.4	0.1	0.3	0.9	<DL	1.0	<DL
NH_4^+ (µM)	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
Mn^{2+} (aq) (µM)	3.4	82.3	5.1	49	60	0	15	62
Sediment								
	S1s	***S1d***	***S2s***	***S2d***	***S3s***	***S3d***	***S4s***	***S4d***
(%) Acid extractable $Fe^{2+}_{(s)}$	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) (D_{50})	57	51	52	49	14	17	14	17

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 (see Supplementary Information; Table S7). This dataset was randomly sampled to give exactly 500,000 reads per sample. The combined pool of 4 million reads was used to identify the characteristic 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 groups. Then, the entire dataset of 6,179,119 reads were allocated to these OTUs. The OTUs classified as Archaea (4% of non-chimeric reads), and the OTUs which were not classified to the Bacteria phylum level with a confidence >0.7 (14% of non-chimeric reads) were excluded from further analysis. This resulted in 5,064,424 reads that were allocated to 7,656 OTUs that were classified to the Bacteria 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.

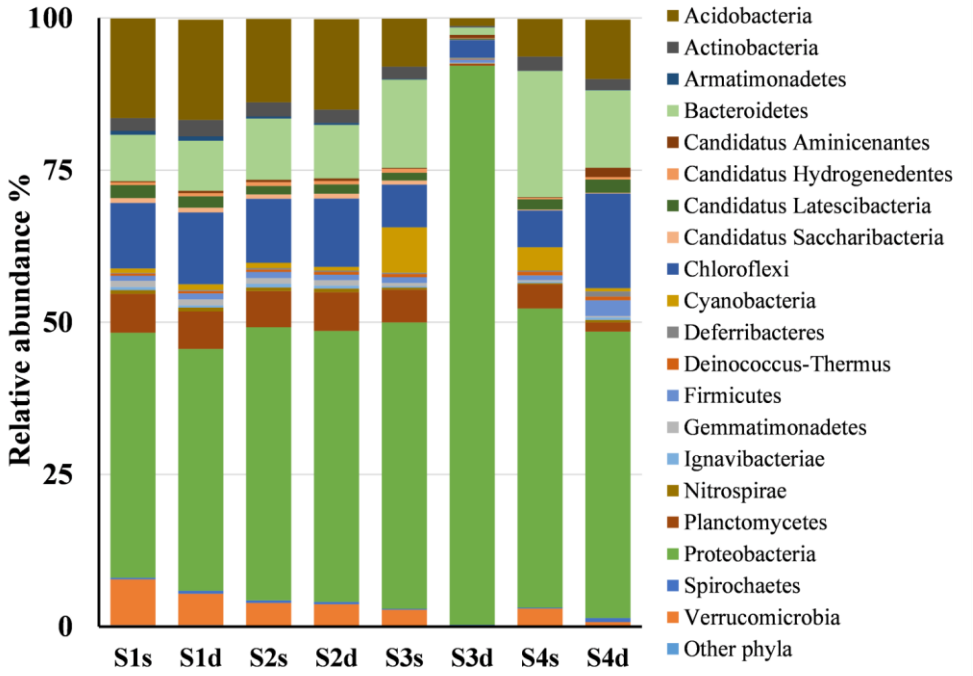

Figure 4: Taxonomical composition of the microbial community at Bacteria phylum level. Phyla with relative abundance below 0.1% are grouped as "Other phyla".

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

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

The OTU richness, D_0^{α}, in each sample is shown in Figure 5a. The average richness at the different sites and sediment depths was ~5,000 OTUs; although sites towards the outer estuary showed slightly lower D_0^{α}. Diversity measures that indicate the number of common OTUs (D_1^{α}) and dominant 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 of the estuary were significant (PERMANOVA analysis indicated $p < 0.05$). Between the innermost 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_1^{α} and D_2^{α} have been used to estimate the percentage of reads within the common and dominant OTUs. Common OTUs accounted for >80% of total sequence reads in all samples, and dominant OTUs accounted for 54-73% of total sequence reads in all samples. Therefore, the decrease observed in the 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 ($^{*}D_1^{\alpha}$) was 438; the regional diversity (D_1^{γ}) was 934; which following Whittaker's multiplicative law, ($D_1^{\beta} = D_1^{\gamma}/{}^{*}D_1^{\alpha}$), gave a beta component (D_1^{β}) of 2.

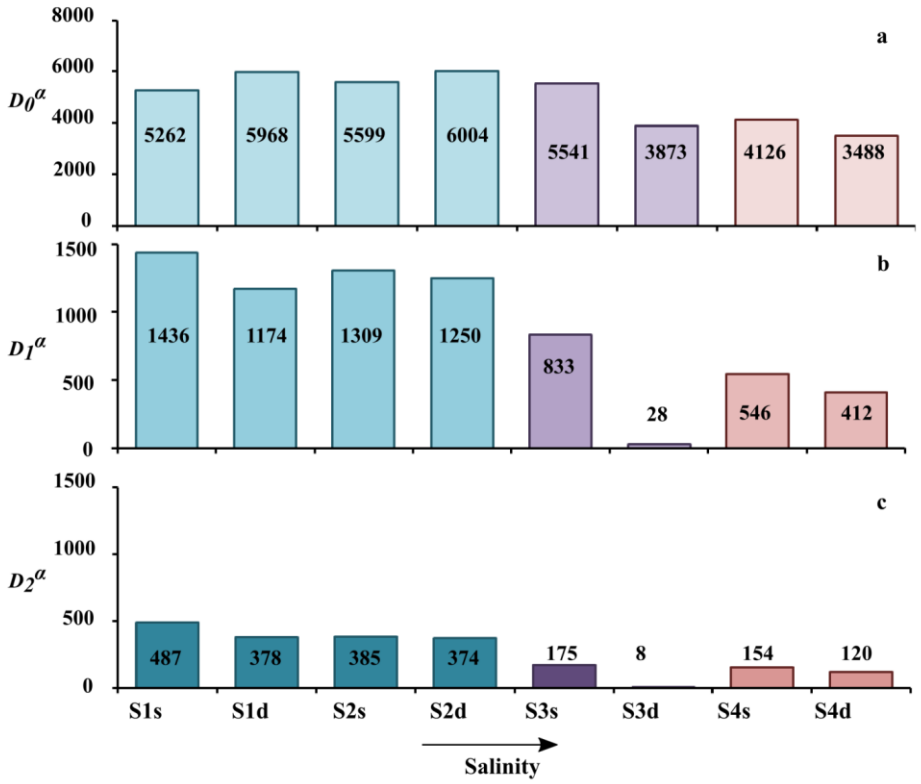

Figure : Alpha-diversity D_q^α values for each location (Hill numbers of order 0, 1, and 2): (a) D_0^α or OTUs richness; (b) D_1^α; and (c) D_2^α.The colour of the bars follows the colour code for the inner (blue), mid (purple) and outer (pink) estuary defined by salinity variation range~~, and colour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

NMDS analysis indicates that the variation of species frequencies in the samples is well represented in two-dimensions (Figure 6, stress value < 0.05). The NMDS ordination showed the split 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 ($p > 0.05$).

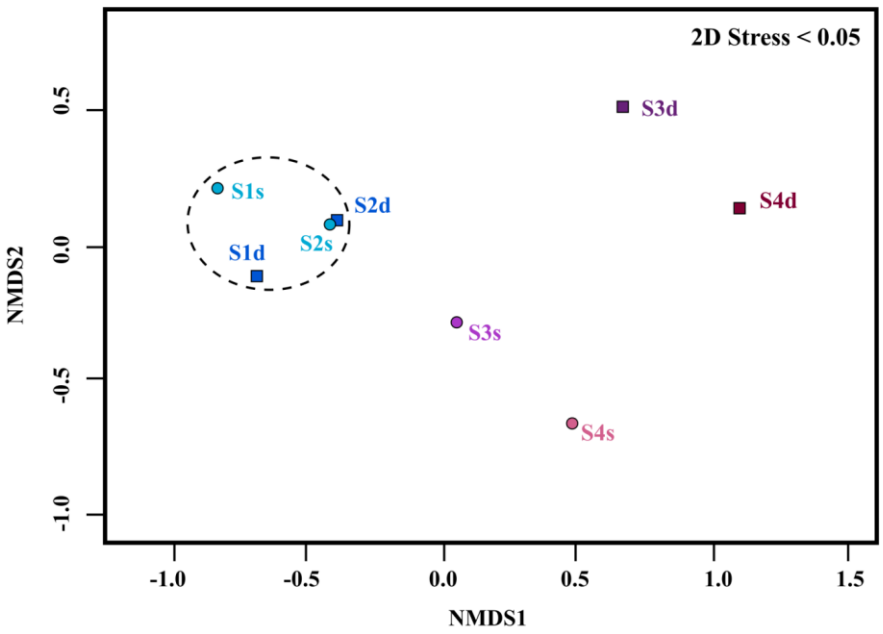

Figure 6: NNMDS ordination for dissimilarities in the bacterial community distribution among samples based on Bray-Curtis distances. Samples are colour-coded according to the salinity variation zones (inner (blue), mid (purple) and outer (pink) estuary). Surface sediment samples (circle markers) are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

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

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

the inner estuary. Iron and ammonium concentrations in the porewater increased also toward the marine end of the system, as well as the proportion of reduced iron from solids found in subsurface sediments. Field observations of the sediment colour at the mid and outer estuary sites (reddish-brown at the surface but dark grey-black in the subsurface) evidenced an abrupt redoxcline at these sites. Although H_2S concentrations were not measured and AVS concentrations were relatively low, others reported that the subsurface sediments of the outer estuary Humber mudflats can be sulfidic (Andrews *et al.*, 2000; Mortimer *et al.*, 1998). Such an abrupt redox change with depth was probably not developed at the inner estuary sites, where the subsurface sediments appeared to be poised between nitrate and iron reducing conditions. Sediment was finer in the samples from the mid and outer estuary, which may have further implications in the temperature gradients, organic matter turnover, and the erodibility of the sediments (Blanchard *et al.*, 2000; Bühring *et al.*, 2005; Harrison & Phizacklea, 1987; Musat *et al.*, 2006).

4.1 Bacterial community composition along the estuarine gradient

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

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

The taxonomic composition of sample S3d differed markedly from the other samples. Here the bacterial community was dominated by *Epsilonproteobacteria*. This taxonomic group has been found in other estuarine and coastal sediments and pelagic redoxclines (Bruckner *et al.*, 2013; Campbell *et al.*, 2006; Grote *et al.*, 2008; Jeffries *et al.*, 2016; Labrenz *et al.*, 2005), and is occasionally abundant (Wang *et al.*, 2012). *Epsilonproteobacteria* has been suggested to be one of the dominant microorganisms involved in the coupling of C, N and S cycles (Campbell *et al.*, 2006). Many *Epsilonproteobacteria* within the order of *Campylobacterales* (the most important in sample S3d) are microaerophilic chemolitotrophs that can couple the oxidation of sulphur compounds or hydrogen to the reduction of oxygen or nitrate (Bruckner *et al.*, 2013; Campbell *et al.*, 2006; Grote *et al.*, 2008; 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 *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 causes of these anomalous results (i.e. sampling or sequencing technology biases, or the proximity of shellfish to the sample) cannot be discarded.

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^{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} (Figure5b
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 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

variation. The surface sample (S3s) showed D_1^{α} and D_2^{α} measurements that were intermediate between the inner and outer estuary, which was not surprising given the regular resuspension and mixing processes of surface sediments by tidal forces. However, as mentioned above, the subsurface sample (S3d) showed lower D_1^{α} and D_2^{α} values than any other sample analysed. This could be associated with 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 location. Microbial DNA was extracted from <0.5 g of sediment, and thus very local geochemical effects could affect the bacterial community within individual samples.

NMDS ordination showed differences in the bacterial community associated with progression toward the outer estuary. Also, the NMDS analysis clustered all the inner estuary samples together, suggesting that the bacterial populations of the inner estuary mudflats were not significantly different between depths. The colour pattern in the heat map (see Supplementary Information) also showed these samples as being similar in their composition. The effects of the mixing at the ETM and the presence of more coarse sediments could enhance the homogenisation of surface and subsurface bacterial communities (Bühring *et al.*, 2005; Crump *et al.*, 1999; Feng *et al.*, 2009; Lavergne *et al.*, 2017; Musat *et al.*, 2006). The NMDS analysis also separated the subsurface mid and outer estuary samples from their surface counterparts, but insufficient samples were used to determine whether this trend was significant. Nevertheless, field observations and geochemical measurements indicated that subsurface mid/outer estuarine sediments were more reducing than the inner estuarine sediments. Other studies in similar environmental conditions suggested that such vertical stratification in the microbial 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.*, 2013).

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 salinity determining microbial compositions has been well documented; the importance of other environmental variables may be hidden as they co-vary with salinity along the gradient. For example, Liu *et al.* (2014) found that sulphate concentration might be hidden by salinity as a driver for the distinct distribution of methanogens and sulphate-reducing bacteria between fresh and seawater sediments. Stronger redox stratification would be expected in the less-frequently disturbed subsurface sediments, which in the more sulphidic mid and outer Humber mudflats, may provide the geochemical conditions for more specialist communities to develop (Bertics & Ziebis, 2009; Hewson & Fuhrman, 2004). We hypothesise that the weaker redox stratification in the inner Humber estuary is likely the reason of the similarity of the microbial populations between depths, although the coarser (i.e. more 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. Apart from the resuspension, other external parameters (temperature, wind, tidal cycle, light exposure, organic matter, benthic fauna and microphytobentic activity) will strongly influence the distribution of bacterial communities, especially in the surface sediment layer. These could cause important seasonal differences in microbial metabolism in different zones, as observed by different authors (Hubas *et al.*, 2007; Lavergne *et al.*, 2017; Orvain *et al.*, 2014).

The regional microbial diversity of the Humber estuary (D_1^{γ} = 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 ($D_1^{\beta} \sim 2$) 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..

4.3 Conclusions

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

Conflict of Interest

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

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