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

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ABSTRACT: Intertidal mudflats are fluctuating environments that support highly diverse microbial communities. The highly variable physico-chemical conditions complicate the understanding of the environmental controls on diversity patterns in estuarine systems. We investigated the bacterial diversity in the surface and subsurface sediments along the salinity gradient of the Humber estuary (UK) using amplicon sequencing of the 16S rRNA gene and correlated its variations with environmental variables. The sediment depths sampled were selected based on the local resuspension patterns. In general, bacterial communities showed similar composition at the different sites and depths, with *Proteobacteria* being the most abundant phylum. The richness of operational 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 non-metric multi-dimensional scaling (NMDS) analysis only in the mid- and outer estuary samples, where redox transitions with depth were more abrupt. Salinity, porewater ammonium concentration and acid-extractable Fe(II) in solids were the subset of environmental factors that best correlated with community dissimilarities. Analysis of regional diversity indicated that the dataset may include 2 potentially distinct communities: (1) a near-surface community that is the product of regular mixing and transport and is subjected to a wide range of salinity conditions, and (2) a bacterial community indigenous to the more reducing subsurface sediments of the mudflats of the mid- and outer estuary.

KEY WORDS: Microbial diversity · Hill numbers · Intertidal sediments · Salinity gradient · 16S rRNA · Illumina MiSeq sequencing

INTRODUCTION

Estuaries are transitional environments where substantial physico-chemical 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 physico-chemical characteristics (e.g. pH, temperature, salinity, particle size, turbidity, sulfate concentration, organic matter, light exposure and river flow seasonal fluctuations), which can affect the stability and composition of microbial communities along the estuarine continuum (Crump et al. 1999, O'Sullivan et al. 2013, Liu et al. 2014, Wei et al. 2016). Although it is widely accepted that microbial communities are sensitive to salinity variations (e.g. Lozupone & Knight 2007), no consensus on other physico-chemical factors controlling microbial abundance in estuarine systems has yet emerged

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(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, Zinger et al. 2011, Reed & Martiny 2013), the relationship between microbial composition and ecosystem functioning remains unclear (Bertics & Ziebis 2009, Reed & Martiny 2013). Quantifying the microbial community variations along estuarine gradients will improve our understanding of their role in these ecosystems and their response to environmental change (Reed & Martiny 2013, Bier et al. 2015).

Salinity is a major abiotic factor controlling the patterns of benthic and pelagic diversity in estuaries (Crump et al. 1999, 2004, Attrill 2002, Lozupone & Knight 2007, Elliott & Whitfield 2011, Herlemann et al. 2011, Telesh et al. 2011, Campbell & Kirchman 2013, L. Zhang et al. 2014, Lallias et al. 2015). The variation of macrozoobenthos in estuaries has been traditionally explained using the conceptual model known as Remane's concept (Remane 1934) (Fig. 1), which was developed for the non-tidal Baltic Sea and models 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 salinity ('critical salinity zone' sensu Khlebovich 1968), which can be explained by the decline in the number of non-tolerant species (marine and freshwater specialists) in the transitional waters (Elliott & Whitfield 2011). However, despite several modifications (Schubert et al. 2011, Telesh et al. 2011, Whitfield et al. 2012) and critiques (Barnes 1989, Bulger et al. 1993, Attrill 2002, Attrill & Rundle 2002), Remane's model has significant limitations as a description of diversity in estuarine systems. Telesh et al. (2011) conducted a meta-analysis of large datasets from previous studies in the Baltic Sea and found that protists showed a diversity maximum in the 'critical salinity zone' (Fig. 1). Subsequently, Telesh et al. (2013) proposed that salinity stress may create niches in 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 (Fig. 1), possibly due to the mixing of freshwater and marine communities.

In tidal estuaries, the impacts of large salinity variations on pelagic microbial community composition, activity and diversity have been well investigated (Feng et al. 2009, Campbell & Kirchman 2013, Liu et al. 2014, Wei et al. 2016). However, the corresponding impacts on benthic communities have received much less attention (Klier et al. 2018). Benthic microbial communities will experience different environmental stresses to pelagic organisms and may be expected to exhibit higher bacterial biomass, higher richness and different diversity patterns (Zinger et al. 2011). For example, vertical stratification of sediment geochemistry influences the composition and function of benthic microbial communities (Musat et al. 2006, Canfield & Thamdrup 2009, O'Sullivan et al. 2013, Liu et al. 2014, Lavergne et al. 2017). Nevertheless, sediments in tidal estuaries are frequently dis-

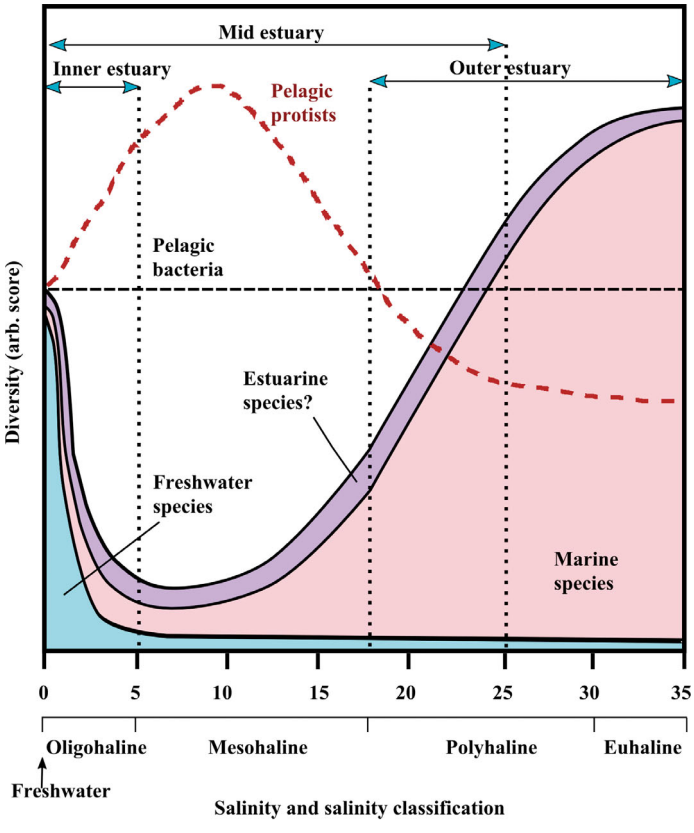

Fig. 1. Diversity variation patterns along a salinity gradient. Coloured areas represent Remane's (1934) conceptual model for the variation in macrobenthic biodiversity (after Whitfield et al. 2012, Skarlato & Telesh 2017). Variations in the diversity of pelagic protists (Telesh et al. 2011) and planktonic bacteria (Herlemann et al. 2011) are shown as dashed lines (red and black, respectively). The dotted lines indicate boundaries for the salinity zonation defined for the Humber estuary (see 'Materials and methods')

turbed 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, Hewson et al. 2007, Feng et al. 2009). Consequently, sediment dynamics may also be an important environmental factor shaping estuarine microbial diversity.

Recently, high-throughput sequencing techniques have become widely available (Buttigieg & Ramette 2014, Liu et al. 2014, Bier et al. 2015). These techniques offer an opportunity to investigate microbial communities in more depth. However, challenges remain as the very large datasets produced reveal the extremely diverse nature of microbiota, which is difficult to evaluate rigorously with the traditional mathematical and statistical approaches to biodiversity estimation (Buttigieg & Ramette 2014, Oulas et al. 2015, Kang et al. 2016). 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 in order to control the variability associated with rare taxa, sampling issues and other biases associated with experimental procedures (Chao et al. 2014, Kang et al. 2016).

Our general aims were to (1) describe the bacterial communities in estuarine sediments at centimetre-scale resolution, (2) identify microbial diversity trends along the salinity gradient and (3) investigate how the environmental variables control such trends. As a first approach, we studied in detail 4 stations along a salinity gradient in the Humber estuary (UK) which, until now, had been sampled only once at low tide during summer. We have extensively studied the Humber Estuary in the past, observing that only the top few mm of the sediments are resuspended during regular tidal cycles, while the entire top 10 cm of sediment were only resuspended during a powerful storm (Mortimer et al. 1999a,b). The current sampling strategy was based on these earlier observations. Hence, samples were collected at 2 depths: surface sediments that are frequency mobilised during the tidal cycle and subsurface sediments that are only resuspended by seasonal storms, which occur once or twice a year in the Humber (House et al. 1997, Mortimer et al. 1999b). Sequencing data from amplicon sequences of the V4 hyper-variable region of the 16S rRNA gene were processed, and the benthic bacterial community composition was correlated with geochemical data using multivariate statistics to infer the environmental drivers controlling microbial diversity patterns and test whether sediment depth has an impact on microbial diversity.

MATERIALS AND METHODS

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 (Fig. 2). Its catchment area is 24 240 km^2

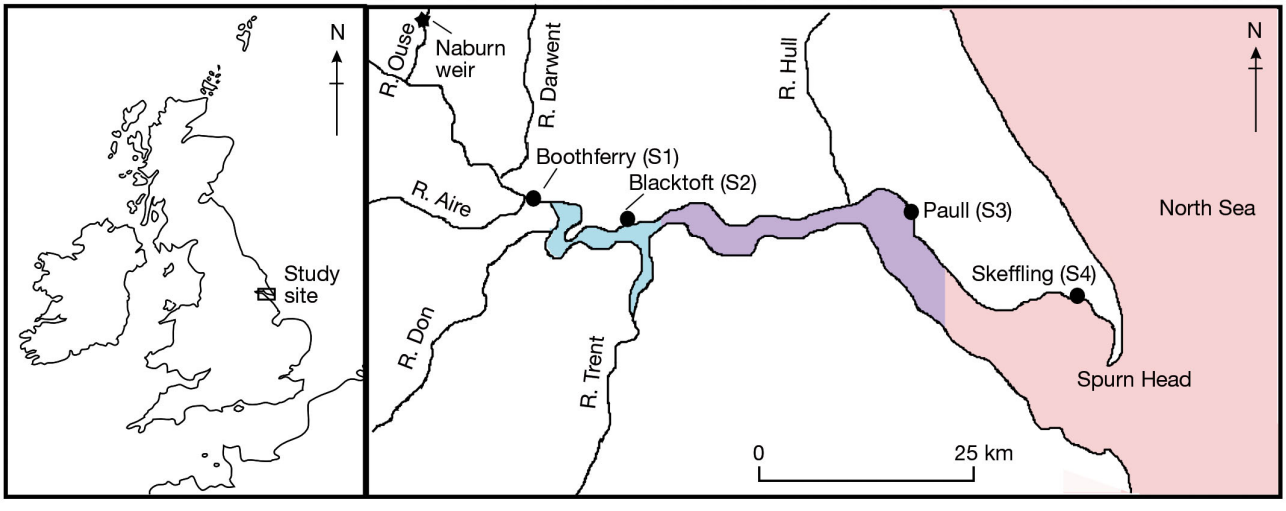

Fig. 2. Humber Estuary (UK) with the sampling sites (S1: Boothferry, S2: Blacktoft, S3: Paull and S4: Skeffling) and the salinity variation zones (blue: ≤5, inner estuary; purple: 0–25, mid-estuary; pink: 18–35, outer estuary)

(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 is situated at the inner estuary, although it moves seasonally with the river flow (Uncles et al. 1999). Water column salinity records from 14 locations on the Humber over a period of ~25 yr 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 (see Supplement 1 at www.int-res.com/articles/suppl/a081p277_supp.pdf). 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 (from freshwater to oligohaline water) (blue area in Fig. 2; see also annotation in Fig. 1). Secondly, the mid-estuary extends from 60 to 100 km downstream of Naburn weir. In this zone, the water column salinity ranges between 0 and ~25 (purple area in Fig. 2; see also annotation in Fig. 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 to seawater salinity (pink area in Fig. 2; see also annotation in Fig. 1), which includes polyhaline to euhaline waters.

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 15 July 2014. The 4 sites were at Boothferry (S1), Blacktoft (S2), Paull (S3) and Skeffling (S4), and they were selected to span the salinity range. A sample of the surface (s) (0–1 cm) and subsurface (d) (5–10 cm) sediment was recovered from each location in 1 l acid-washed containers and 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.

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 with a bucket and transferred into 2 l acid-washed polythene containers. Porewater was recovered from sediment subsamples by centrifugation (30 min, 6000 × *g*) in the laboratory. All water and porewater samples were filtered (0.2 µm Minisart®) and stored at 4 or −20°C, as appropriate, for further analysis. Nutrient concentrations were determined by ion chromatography (sulfate and chloride) on a Dionex CD20, and colorimetrically (nitrate, nitrite and 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 for 0.5 N HCl-extractable iron followed by ferrozine assay (Lovley & Phillips 1987, Viollier et al. 2000). Acid volatile sulfide (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 (60°C) 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 Sulfur and Carbon Analyser. All physico-chemical analyses of sediment and water samples were carried out in triplicate.

DNA extraction, amplicon sequencing and sequence analyses

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

DNA samples (1 ng $µl^{-1}$ in 20 µl aqueous solution) were sent for sequencing at the Centre for Genomic Research (CGR), University of Liverpool, where Illumina adapters and barcodes were attached to DNA fragments in a 2-step PCR amplification that targets the hyper-variable V4 region of the 16S rRNA gene.

The protocol was based on Caporaso et al. (2011) and uses the forward target-specific primer 5′-GTG CCA GCM GCC GCG GTA A-3′ (F515, Turner et al. 1999) and the reverse target-specific primer 5′-GGA CTA CHV GGG TWT CTA AT-3′ (R806, Caporaso et al. 2011). Pooled amplicons were paired-end sequenced on the Illumina MiSeq platform (2× 250 bp) generating ~12 M paired-end reads. The raw fastq files were trimmed for the presence of Illumina adapter sequences using Cutadapt version 1.2.1 (Martin 2011) by the CGR. The option -O 3 was used, so the 3′ end of any reads which match the adapter sequence for 3 bp or more were trimmed. The reads were further trimmed using Sickle version 1.200 with a minimum window quality score of 20. Reads shorter than 10 bp were removed after trimming. If only one of a read pair passed this filter, it was included in the R0 file. The trimmed reads were processed using the UPARSE pipeline (Edgar 2013) within the USEARCH software package (version 8.1.1861) (Edgar 2010) installed on a Linux OS platform. First, overlapping paired-end reads were assembled using the *fastq_mergepairs* command. The reads from each sample were then quality-filtered using the *fastq_filter* command (expected error cutoff was set at 1.0 and length truncation was not applied), re-labelled and de-replicated before they were randomly subsampled (500 000 paired-end reads with an average length of 296 bp) to produce a manageable sample size for combined analysis (~4 M reads). After further de-replication of the combined pool of reads, clustering and chimera filtering was performed simultaneously within the pipeline by using the *cluster_otus* command (with the *minsize 2* option to specify a minimum abundance of 2 and discard singletons). The sequence identity threshold was fixed at 97% to define operational taxonomic units (OTUs). The *utax* command was applied for taxonomic assignment using the RDP 16S rRNA training database (RDP15) and a confidence value of 0.7 to give a reasonable trade-off between sensitivity and error rate in the taxonomy prediction. The entire dataset (~6 M paired-end reads) was then allocated to the OTUs using the *usearch_global* command, and the results were reported in an OTU-table. OTUs which were not classified to the bacterial phylum level with a confidence >0.7, or were classified as *Archaea*, were not included in the diversity and statistical analyses. Sequence reads are available at the National Center for Biotechnology Information (NCBI) under the Sequence Read Archive (SRA) accession number SPR105158.

Statistical analyses

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

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

where S is the total number of species (OTUs in this study), and p_i is the proportion of individuals belonging to the i^{th} species in the dataset. The degree of weighting is controlled by the index q (increasing q places progressively more weight on the high-abundance species in a population and discounts rare species) (Hill 1973, Jost 2006, 2007, Chao et al. 2014, Kang et al. 2016). All Hill numbers are in units of 'species' (OTUs). Three Hill numbers were used to evaluate the alpha-diversity (D_q^{α}) of each individual sample: D_0^{α} (species 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 (see Table S5 in Supplement 3). The assemblage or 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 ($^{\bullet}D_1^{\alpha}$), using the Whittaker multiplicative law ($^{\bullet}D_1^{\alpha} \times D_1^{\beta} = D_1^{\gamma}$) (Whittaker 1972). $^{\bullet}D_1^{\alpha}$ compensates for unequal sample sizes, so is not the arithmetic average of the alpha diversities of the individual samples (see Supplement 3).

All statistical analyses were performed in RStudio (R version 3.4.2) (RStudio Team 2015) using the 'vegan' package (Oksanen et al. 2013). The microbial community data were input as a matrix of the relative abundance of each OTU in each of the 8 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 2-dimensional space. Non-parametric multivariate analysis of variance (PERMANOVA) (Anderson 2001) was used to assess the similarity in the microbial abundance among groups of samples (samples were grouped by depth, zone of the estuary and/or

sampling location, 999 permutations). 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 performed to study the significance of the BIOENV results. The environmental data used for the BIOENV analysis included: salinity; ammonium, nitrate, sulfate, iron and manganese porewater concentrations; TOC content; pyrite and total iron in solids; percentage of acid extractable Fe(II) in solids; iron associated with pyrite; and particle size.

RESULTS

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 at S1) to high salinity water at the sea end of the estuary (26.1 at S4). Porewater salinity was slightly lower than the water column salinity at 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 $S4_s$, nitrate concentrations in the porewater were lower than

Table 1. Physico-chemical properties of the water column, sediment porewater and sediment at the study sites (S1–S4; see Fig. 2). Suffixes 's' and 'd' refer to surface and subsurface sediments, respectively. Particle grain size is expressed as the upper bound diameter of 50% of cumulative percentage of particles by volume (D_{50}). AVS: acid volatile sulfide; TOC: total organic carbon; TS: total sulfur. Where shown, ± values are SD. DL: detection limit; nd: not detected

	Water column			
	S1	S2	S3	S4
Salinity	0.4	3.5	21.6	26.1
pH	7.87	7.52	7.90	8.02
Eh (mV)	+151 ± 24	+109 ± 23	+75 ± 8	+75 ± 4
Temperature (°C)	20.0	19.7	19.2	19.5
Conductivity (mS cm^{-1})	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							
	$S1_s$	$S1_d$	$S2_s$	$S2_d$	$S3_s$	$S3_d$	$S4_s$	$S4_d$
Porewater salinity	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							
	$S1_s$	$S1_d$	$S2_s$	$S2_d$	$S3_s$	$S3_d$	$S4_s$	$S4_d$
Acid-extractable Fe (µmol g^{-1})	106 ± 1	116 ± 10	106 ± 6	105 ± 4	123 ± 3	206 ± 8	93 ± 9	191 ± 28
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
% Fe-Pyrite	0.08	0.10	0.09	0.10	0.10	0.12	0.12	0.18
% Fe-AVS	nd	nd	nd	nd	<DL	0.01	<DL	0.09
%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
% Water content	42	39	41	28	65	44	64	40

those in the water column, whereas ammonium concentrations were higher, especially in the sites where more reducing sediments were found. Sulfate 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 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.

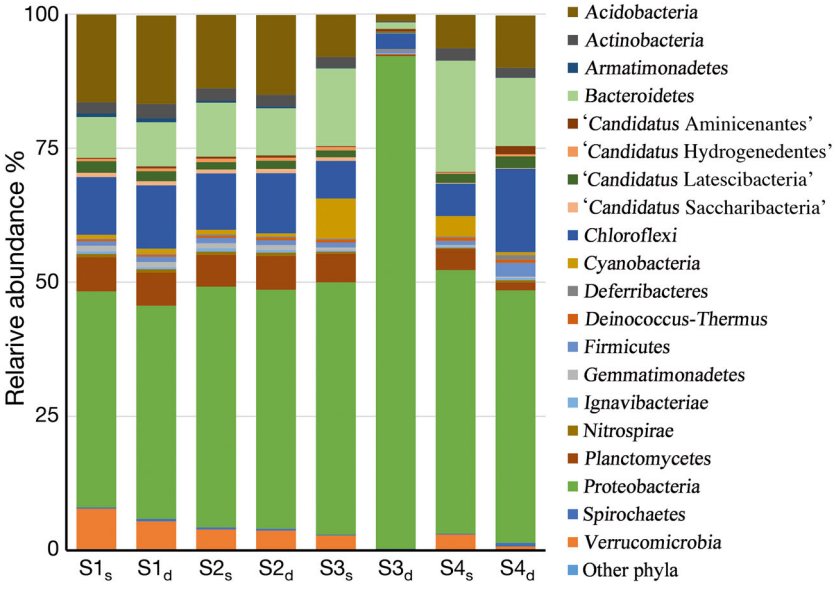

Fig. 3. Taxonomical composition of the bacterial community at the phylum level. Phyla with relative abundance below 0.1% are grouped as 'Other phyla'. Sampling sites (see Fig. 2) were S1: Boothferry, S2: Blacktoft, S3: Paull and S4: Skeffling; 's' and 'd' refer to surface and subsurface sediments, respectively

Bacterial community composition and diversity along the salinity gradient

The Illumina MiSeq run yielded >500000 paired-end reads per sample after quality control (Table S6 in Supplement 3). This dataset was randomly sampled to give exactly 500000 reads per sample. The combined pool of 4 million reads was used to identify the characteristic OTUs in the regional dataset. A total of 3596003 reads in the combined pool passed the chimera check, and these were clustered into OTUs (>97% sequence identity), and assigned to taxonomic groups. The entire dataset of 6179119 reads was then allocated to these OTUs. The OTUs classified as *Archaea* (4% of non-chimeric reads) and OTUs which were not classified to the bacterial phylum level with a confidence >0.7 (14% of non-chimeric reads) were excluded from further analyses. This resulted in 5064424 reads that were allocated to 7656 OTUs.

Twenty phyla individually represented more than 0.1% on average of the total reads (Fig. 3), 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 samples had a similar composition, with the exception of the sample of subsurface sediment from Paull ($S3_d$). In this sample, *Proteobacteria* were dominant, accounting for 92% of the OTUs present versus the 45% (on average) that *Proteobacteria* represented at the other sites. Further information about the classification of each bacterial community to the class and order level can be found in Supplement 2 (Tables S1–S4).

A 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 $S3_d$ 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; Table S2). *Betaproteobacteria* was the next most numerous class in the inner estuary samples, with 9% of total reads, but had <3% of total reads in the outer estuary. On the other hand, it was notable that the abundance of *Deltaproteobacteria* was similar in all inner estuary samples and in the outer estuary surface samples (~7% of total reads), but represented ~17% of $S4_d$. 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 $S4_d$.

Acidobacteria was the second most abundant bacterial phylum, representing ~15% of the total reads

in the inner estuary, but only ~8% of reads in the outer estuary samples. Within the *Acidobacteria*, subdivision 6 (class *Acidobacteriia*) was most numerous in the inner estuary (~6% of total reads) but was only 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. *Flavobacteriaceae* 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 2 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 Fig. 4a. The average richness at the different sites and sediment depths was ~5000 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 (Fig. 4b,c). These differences in OTU relative abundance between the inner and the outer zones of the estuary were significant (PERMANOVA analysis indicated $p < 0.05$, F.Model was >3.3 if grouping by zone, and 2.2 if grouping by sampling location; see details in Supplement 8). 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} were 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

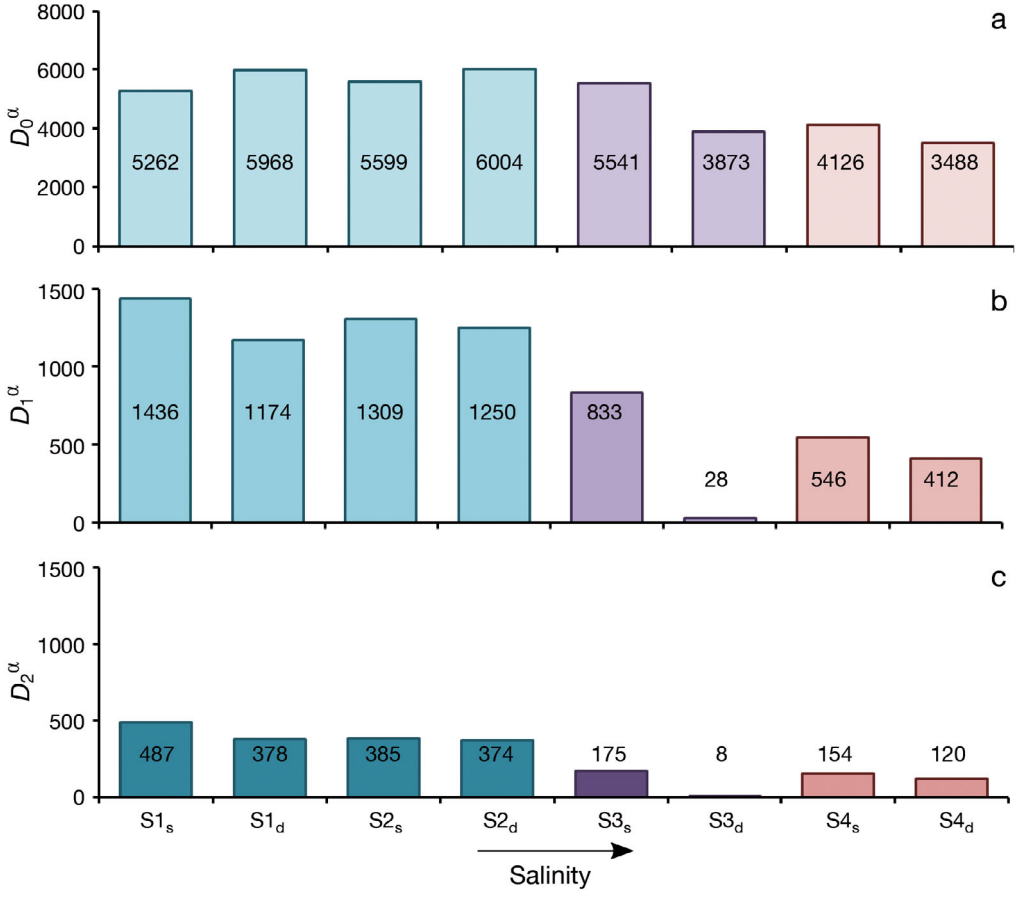

Fig. 4. Alpha-diversity (D_q^{α}) at each location measured with Hill numbers (D_q) of different order (q = 0, 1 and 2) which represent: (a) operational taxonomic unit (OTU) richness (D_0^{α}), (b) common OTUs (D_1^{α}) and (c) dominant OTUs (D_2^{α}). The colours of the bars follow the colour code for the inner (blue), mid- (purple) and outer (pink) estuary defined by salinity variation range (colour darkens as q increases from D_0^{α} to D_2^{α}). Sampling sites (see Fig. 2) were S1: Boothferry, S2: Blacktoft, S3: Paull and S4: Skeffling; 's' and 'd' refer to surface and subsurface sediments, respectively. For more details, see the 'Materials and methods'

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 OTUs; the regional diversity (D_1^{γ}) was 934 OTUs, which, following Whittaker's multiplicative law, ($D_1^{\beta} = D_1^{\gamma}/{}^{*}D_1^{\alpha}$), gave a beta component (D_1^{β}) of 2.

NMDS analysis indicated that the variation of species frequencies in the samples is well represented in 2 dimensions (Fig. 5, stress value < 0.05). The NMDS ordination showed the split between the inner estuary samples, which were ordinated in a relatively close group, and the outer estuary samples that were progressively more distant from the inner estuary group. The mid- and outer estuary samples were also separated by depth, but there were too few samples to determine whether this difference was significant ($p > 0.05$, F.Model = 0.84, see Supplement 8).

The BIOENV analysis showed that salinity, ammonium concentration in porewater and acid-extractable Fe(II) in solids were the subset of environmental variables that best correlated (0.94) with the community composition of the different sites along the Humber estuary (Mantel statistic based on Pearson correlation, $R = 0.72$, $p < 0.05$) (see Supplement 7).

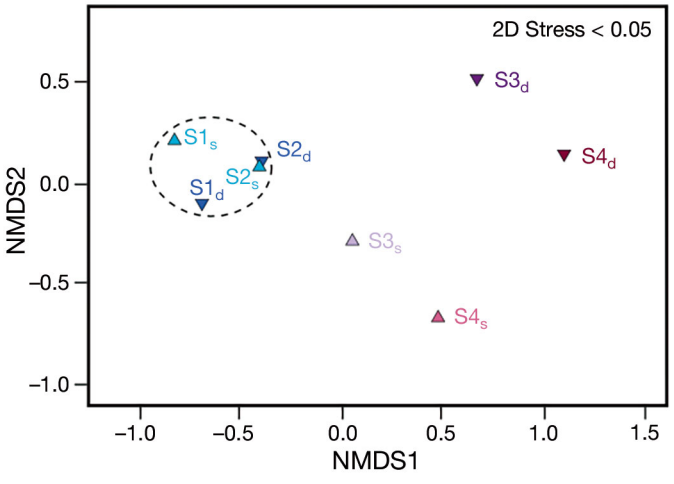

Fig. 5. Non-metric multidimensional scaling (NMDS) ordination for dissimilarities in the bacterial community distribution among samples based on Bray-Curtis distances. Samples are colour-coded according to the salinity variation zones (blue: inner, purple: mid-, pink: outer estuary). Lighter (darker) colours represent surface (subsurface) sediment samples. The dashed ellipse has been added to indicate the inner estuary samples. Sampling sites (see Fig. 2) were S1: Boothferry, S2: Blacktoft, S3: Paull and S4: Skeffling; 's' and 'd' refer to surface and subsurface sediments, respectively

DISCUSSION

Environmental variability along the Humber estuary

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

The large-scale spatial gradients in salinity and nutrient concentrations observed in this study are reflective of natural environmental gradients expected within estuarine systems (Crump et al. 2004, Liu et al. 2014, Jeffries et al. 2016). Overall, the mid-estuary river waters experience the widest salinity variation in the Humber. However, porewater salinity is expected to change more slowly than estuarine water salinity in muddy, fine-grained sediments due to their lower permeability (Harrison & Phizacklea 1987, Musat et al. 2006), and, therefore, it probably varies less and remains close to the long-term average salinity of the overlying waters. Concentrations of nitrate decreased in the water column towards the outer estuary, while sulfate increased with increasing proportions of seawater mixed in the water column. The main difference between the inner and the mid-/outer estuarine sediments was the more reducing nature of the latter. The sediments recovered from the mudflats of the mid- and outer estuary showed some iron enrichment compared to the sites from the inner estuary. Iron and ammonium concentrations in the porewater also increased toward the marine end of the system, as did the proportion of acid-extractable Fe(II) 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 have reported that the subsurface sediments of the outer estuary Humber mudflats can be sulfidic (Mortimer et al. 1999a, Andrews et al. 2000). 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 (Harrison & Phizacklea 1987, Blanchard et al. 2000, Bühring et al. 2005, Musat et al. 2006).

Bacterial community composition along the salinity gradient

Taxonomically, all samples except for $S3_d$ had a similar composition. *Proteobacteria* was the most represented phylum in all bacterial communities, followed by *Acidobacteria*, *Bacteroidetes* and *Chloroflexi*. This distribution of phyla was consistent with other studies in coastal and estuarine sediments (Wang et al. 2012, Halliday et al. 2014, Liu et al. 2014, Jeffries et al. 2016, Wei et al. 2016, Pavloudi et al. 2017). 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, Romanenko et al. 2004, Lin & Shieh 2006, Spring et al. 2009), so this increased abundance may be related to increasing salinity (Pavloudi et al. 2017). Furthermore, the increase in abundance of reads from the order *Desulfobacterales* in sample $S4_d$ could be a response to the salinity and redox conditions in the outer estuary subsurface sediments, as this order contains strictly anaerobic sulfate-reducing bacteria that are most frequently found in tidal mudflats and marine habitats (Mußmann et al. 2005, Wilms et al. 2006, Gittel et al. 2008, Kuever 2014, Pavloudi et al. 2017). There was also an increase in the abundance of *Bacteroidetes* along the estuary, and particularly of species in the family *Flavobacteriaceae*. The marine genera of *Flavobacteriaceae* are a major component of the oceanic microbial biomass in the pelagic zone (Kirchman 2002, McBride 2014) and have also been found in tidal sediments (Jung et al. 2005, Choi & Cho 2006, Wilms et al. 2006). *Acidobacteria* are ubiquitous and abundant in nature, and especially in soils (Barns et al. 1999, Rappé & Giovannoni 2003). A decrease in the abundance of *Acidobacteria* along the estuary (as a result of a decrease in the abundance of subdivision 6) was observed, which may be related to soil inputs at the inner estuary. Members of subdivision 6 (Class *Acidobacteriia*) are 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 $S3_d$ 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 (Labrenz et al. 2005, Campbell et al. 2006, Grote et al. 2008, Bruckner et al. 2013, Jeffries et al. 2016), and is occasionally abundant (Wang et al. 2012). *Epsilonproteobacteria* have been suggested to be among 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 $S3_d$) are microaerophilic chemolithotrophs that can couple the oxidation of sulfur compounds or hydrogen to the reduction of manganese, oxygen or nitrate (Thamdrup et al. 2000, Labrenz et al. 2005, Campbell et al. 2006, Grote et al. 2008, Bruckner et al. 2013). 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 low bacterial diversity measured in sample $S3_d$ was unexpected and could be due to the sampling of a specialist niche (the dominance of *Campylobacterales* may be related to the reducing geochemical conditions at this location) (Teske et al. 1996, Llobet-Brossa et al. 1998, Thamdrup et al. 2000). 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.

Trends and environmental drivers of microbial diversity

Ever since the publication of Remane's model, there has been substantial interest in the role of salinity stress in shaping estuarine biodiversity (Attrill 2002, Whitfield et al. 2012). In this study, we found that the OTU richness of benthic bacteria (as meas-

ured by D_0^{α}) was relatively uniform along the Humber estuary, which appears to agree with previous reports of uniform bacterial richness along a salinity gradient (Hewson et al. 2007, Herlemann et al. 2011, W. Zhang et al. 2014). In contrast, Pavloudi et al. (2017) found that the total number of OTUs showed a negative relationship with increasing salinity. However, due to the hyperdiverse nature of microorganisms in many ecosystems, richness can give a distorted view of microbial diversity because it gives equal weight to common and rare taxa (i.e. richness takes no account of OTU relative abundance). Also, it is seldom possible to evaluate richness accurately, as it is extremely difficult to adequately sample rare taxa even with high-throughput sequencing technologies (Kang et al. 2016). Therefore Hill numbers of higher order (q = 1 or 2) are considered to be a more suitable mathematical approach to microbial diversity that give consistent measures of the prominence of common or dominant species in a community since they are not sensitive to sequencing depth (Kang et al. 2016).

The analysis of the microbial diversity in the Humber mudflats using D_1^{α} and D_2^{α} revealed a decreasing trend of microbial diversity in terms of common and dominant OTUs with increasing salinity. The common and dominant OTUs in the mid- and outer estuary samples were only about 40 and 35% of the average number of common and dominant OTUs, respectively, in the inner estuary. This indicated a change towards a community structure with a smaller number of more abundant OTUs along the estuarine salinity gradient. Other studies also reported a similar decreasing trend in pelagic and benthic bacterial diversity along the salinity gradient (Campbell & Kirchman 2013, Liu et al. 2014, L. Zhang et al. 2014, Wang et al. 2015), which may be in part be explained by the influence of the riverine inputs on the inner estuary communities (Crump et al. 1999, Rappé et al. 2000, L. Zhang et al. 2014, Monard et al. 2016). Generally, Site 3 fitted this trend, despite being in the area of highest salinity variation. The surface sample ($S3_s$) 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 ($S3_d$) 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. 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, all of the inner estuary samples were clustered together in this analysis, suggesting that the bacterial populations of the inner estuary mudflats were not significantly different between depths. The colour pattern in the heat map (see Fig. S3 in Supplement 6) 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 (Crump et al. 1999, Bühring et al. 2005, Musat et al. 2006, Feng et al. 2009, Lavergne et al. 2017). 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- and 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 (Musat et al. 2006, Bertics & Ziebis 2009, O'Sullivan et al. 2013, Liu et al. 2014, Lavergne et al. 2017).

Overall, salinity, ammonium concentration in porewater and acid-extractable Fe(II) 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 covary with salinity along the gradient. For example, Liu et al. (2014) found that sulfate concentration might be hidden by salinity as a driver for the distinct distribution of methanogens and sulfate-reducing bacteria between fresh- and seawater sediments. Stronger redox stratification would be expected in the less frequently disturbed subsurface sediments, which in the more sulfidic mid- and outer Humber mudflats may provide the geochemical conditions for more specialist communities to develop (Hewson & Fuhrman 2004, Bertics & Ziebis 2009). We suggest that the weaker redox stratification in the inner Humber estuary is likely the reason for the similarity of the microbial populations between depths, al-

though 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 as mentioned before. Apart from the resuspension, other external parameters (temperature, wind, tidal cycle, light exposure, organic matter, benthic fauna and microphytobenthic activity) probably 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, Orvain et al. 2014, Lavergne et al. 2017).

The regional microbial diversity of the Humber estuary (D_1^{γ} = 934 OTUs) indicated that many of the OTUs that were common in individual samples were common within the regional dataset. Further, the beta diversity, calculated for common species (D_1^{β} ~ 2) can be interpreted as there being 2 distinct assemblages dispersed amongst the various local communities of the region. We suggest that the first of these compositional units may be a community that is subjected to resuspension into the water column and laterally transported along the estuary. Hence, this community may be 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.

Conclusions

This study has provided initial insight into the microbial diversity of the Humber estuary. Although no biological replicates were used, 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, which challenges the Remane concept. 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 represents a larger proportion of the population. The discovery of this trend along the salinity gradient illustrated the importance of using a rigorous and consistent mathematical 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 physico-chemical conditions (sediment resuspension and mixing) may also have an impact on the bacterial community composition. Future investigations with a wider sampling strategy and more biological replicates would be needed to confirm these findings, as well as to explore more deeply the effects of these and other biotic and abiotic variables on microbial diversity through different seasons.

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