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# Characterization of specificity of bacterial community structure within the burrow environment of the marine polychaete *Hediste (Nereis) diversicolor*

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**Abstract**

Bioturbation is known to stimulate microbial communities, especially in macrofaunal burrows where the abundance and activities of bacteria are increased. Until now, these microbial communities have been poorly characterized and an important ecological question remains: do burrow walls harbor similar or specific communities compared with anoxic and surface sediments? The bacterial community structure of coastal sediments inhabited by the polychaete worm *Hediste diversicolor* was investigated. Surface, burrow wall and anoxic sediments were collected at the Carteau beach (Gulf of Fos, Mediterranean Sea). Bacterial diversity was determined by analyzing small subunit ribosomal RNA (16S rRNA) sequences from three clone libraries (168, 179 and 129 sequences for the surface, burrow wall and anoxic sediments, respectively). Libraries revealed 306 different operational taxonomic units (OTUs) belonging to at least 15 bacterial phyla. Bioinformatic analyses and comparisons between the three clone libraries showed that the burrow walls harbored a specific bacterial community structure which differed from the surface and anoxic environments. More similarities were nevertheless found with the surface assemblage. Inside the burrow walls, the bacterial community was characterized by high biodiversity, which probably results from the biogeochemical heterogeneity of the burrow system.

*Keywords: Hediste diversicolor* burrow; Bacterial communities; Biodiversity; Bioturbation; Sediments

## 1. Introduction

It is well recognized that macrofaunal bioturbation reshapes the physical, chemical and biological properties of aquatic sediments, inducing, in most cases, higher rates of organic matter mineralization (e.g. Kristensen, 1985; Aller, 1994; Sun et al., 1999; Reise, 2002; Gilbert et al., 2003). Within sediments, a wide range of activities such as mucus-lined tube construction, periodic water flushing, maintenance of the structure, feeding activities (e.g. filter-feeding, grazing and gardening) and excretion of feces and liquid metabolites, modify solute and particle distribution (e.g. Kristensen, 1984; Aller and Yingst, 1985; Riisgård and Banta, 1998; Webb and Eyre, 2004; Meysman et al., 2005; Costa et al., 2006). Through these different behaviors, macrofauna can modify environmental variables. Indeed, the mucus layer that stabilizes the burrow wall provides labile organic matter and enables trapping of more or less fresh particles, enhancing the organic matter content in the burrow system (Defretin, 1971; Kristensen, 1985; Aller and Aller, 1986; Papaspyrou et al., 2005). The mucus layer may also act as a barrier to solute diffusion that establishes steep chemical gradients between anoxic sediment and burrow lumen (Aller and Yingst, 1978; Boudreau and Marinelli, 1994). The production of biogenic structures, enhancing the size of the sediment−water interface, and periodical water flushing, greatly increase solute exchanges between overlying water and anoxic sediments, e.g. oxygen renewal and toxic metabolite removal (Davey, 1994; Kristensen, 1984; Forster and Graf, 1992, 1995; Fenchel,

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1996; Aller, 2001; Pischedda et al., 2008; Bertics and Ziebis, 2010). The periodic character of ventilation and steep gradients also induce pH and redox oscillations in the structure, at frequencies depending on the macrofaunal organisms (Aller, 1988; Kristensen, 2000). One major consequence of macrofaunal bioturbation is thus establishment of a highly heterogeneous system associated with dynamic and constantly changing biogeochemical variables of large amplitude, on both small spatial (mm, cm) and short temporal (s, min) scales (Glud et al., 1998; Aller, 2001; Wenzhöfer et al., 2001; Polerecky et al., 2006; Glud, 2008; Pischedda et al., 2008).

Microorganisms are essential for ecosystem functioning as they are the primary recyclers of nutrients through mineralization of organic matter (Whitman et al., 1998). In most benthic environments, they are profoundly affected by macrofaunal bioturbation and induced sediment heterogeneity. Indeed, bioturbation has been shown to increase total microbial abundance and viable microbial biomass (e.g. Aller and Yingst, 1978; Alongi, 1985; Steward et al., 1996). Furthermore, it may also stimulate the activity of microbes (e.g. Kristensen et al., 1985, 1991; Phillips and Lovell, 1999) and lead to establishment of specific microbial communities associated with burrows of macroorganisms (e.g. Marinelli et al., 2002; Matsui et al., 2004). However, only a few studies have compared the bacterial community structure of the burrow wall with surface and/or surrounding sediments. They were based on phospholipid fatty acid (PLFA) (Dobbs and Guckert, 1988; Steward et al., 1996; Marinelli et al., 2002) or terminal restriction fragment length polymorphism (T-RFLP) (Laverock et al., 2010) analyses, nucleic acid analyses such as 5S rRNA (Lucas et al., 2003) and 16S rRNA gene fingerprinting or cloning (Matsui et al., 2004; Papaspyrou et al., 2005, 2006; Laverock et al., 2010). Those studies pointed out that the crustacean or annelid burrow walls presented specific microbial communities. As suggested by Papaspyrou et al. (2006), burrow walls should not be considered simply as an extension of the sediment−water interface, as they are usually characterized by unique physical and chemical properties and microbial communities (Kristensen, 1985; Aller and Aller, 1986; Fenchel, 1996; Papaspyrou et al., 2005). However, except in studies using T-RFLP or fingerprinting techniques, previous works only focused on particular microbial groups such as sulfate-reducing bacteria and ammonia- and nitrite-oxidizing bacteria (Matsui et al., 2004; Satoh et al., 2007). Thus, overall communities have not been thus far extensively characterized so as to compare these microenvironments.

The purpose of the present work was to gain knowledge forming a baseline for future research on the burrow wall bacterial community structure of the representative gallery-building species *Hediste diversicolor* (O.F. Müller, 1776). The main objective was to confirm the possible existence of a specific community associated with burrow walls. In situ sediments inhabited by communities of this polychaete worm were sampled in the Gulf of Fos in order to compare the bacterial structure of surface, burrow wall and anoxic sediments. For that, three clone libraries consisting of 476 rRNA sequences in total were constructed and analyzed using bioinformatic tools.

## 2. Material and methods

### 2.1. Study site and sampling

Sampling was performed in May 2008 at low tide (0−1 m water depth) in the Saint Antoine canal mouth of the Carteau cove (43°22′30″ N, 4°50′20″ E; Gulf of Fos, Mediterranean Sea). This area exhibits muddy-sand sediments and provides a habitat for a dense community of *H. diversicolor*. This polychaete builds a semi-permanent U- or Y-shaped mucus-lined burrow extending 6−12 cm into sediments. It actively renews burrow water by peristaltic or undulatory body movements with successions of active ventilation periods followed by rest periods (Kristensen, 1981; Davey, 1994). *H. diversicolor* is active for about 50% of the time with 4.8 min ventilation periods followed by 4.6 min of inactivity at 15 °C and at a salinity of 18‰ (Miron and Kristensen, 1993). Temporal patterns of bioirrigation vary according to several factors, including feeding activity (Kristensen, 2001), sulfide concentration inside burrows (Miron and Kristensen, 1993), temperature and salinity of seawater (Kristensen, 1983).

Because of heterogeneity of the sediment matrices (including burrow wall) and because only weak amounts of sediments from the burrow walls can be sampled (thickness of about 2 mm), sediment subsamples were pooled together. More specifically, from five sediment squares (30 × 30 cm; shovel sampling), fifty subsamples (∼3 ml) of sediment from surface (*S*), burrow wall (*BW*) and anoxic (*An*) compartments were randomly sampled. The surface sediments were sampled by gently scraping a 2 mm layer of sediments on top of the square with an ethanol-clean stainless steel microspatula. For sampling of the burrow wall (*BW*) and anoxic (*An*) sediments, sediment squares were gently broken and sampling of light brown oxidized burrow walls (2 mm layer) and dark brown reduced sediments was randomly performed between a 0 and 15 cm depth. After collection, subsamples of each compartment (*S*, *BW* and *An*) were pooled per habitat in order to obtain a global mean picture of each habitat community. Pooled samples were then immediately frozen on dry ice and stored at −80 °C until analyses.

### 2.2. Sediment analyses

The sediment grain size distribution was determined using a Malvern Mastersizer S long bed Ver. 2.18 FR, after 30 s of sonication. Sediment porosity was calculated from water loss after drying of sediment at 60 °C overnight. The organic matter content was measured as loss upon ignition (475 °C, 4 h; Schumacher, 2002).

### 2.3. DNA recovery and amplification

Total genomic DNA extraction was performed from 250 to 300 mg of homogenized sampled sediments using the

PowerSoil™ DNA isolation kit (MoBio Laboratories, Inc., USA) according to the manufacturer's recommendations. Samples were stored at −20 °C for less than 1 month before use.

Total genomic DNA of *S*, *BW* and *An* sediment samples were used as templates for the 16S rRNA gene amplification. A combination of reverse primer 907 RA (5′-CCGT CAATTCMTTTRAGTTT-3′, Thermo Scientific) and forward primer Eu5 Bac (5′-AGAGTTTGATNMTGGCTCAGA-3′, Thermo Hybaid) was used. The reaction mixture contained 10−15 ng of DNA quantified beforehand by spectrofluorimetry (BioPhotometer, Eppendorf), 1× PCR buffer (10× reaction buffer spiked with $Mg^{2+}$: 500 mM KCl, 100 mM Tris−HCl pH 8.3 at 25 °C, 15 mM $Mg^{2+}$), 10 μM each of the forward and reverse primers, 10 mM of dNTP (Eppendorf) and 2.5U of Taq DNA polymerase (Eppendorf). The initial denaturating step of 3 min at 94 °C was followed by 30 cycles of 1 min at 94 °C, 45 s at 55 °C, and 1 min at 72 °C with a final extension step of 5 min at 72 °C. Successful amplification and size of PCR products (900 bp) was checked by electrophoresis in 1.0% agarose in 1 × Tris−Borate−EDTA buffer. PCR was done in triplicate for each sample.

### 2.4. Cloning and sequencing

Bands of expected sizes were excised and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) in accordance with the manufacturer's instructions, and the triplicates were pooled. A quantity of 40−50 ng of DNA fragments (quantified beforehand by spectrofluorimetry, BioPhotometer, Eppendorf) were then cloned using pGEM-T-easy vector (Promega) and transformed using thermocompetent JM109 cells (Promega) according to the manufacturer's recommendations.

For each of the surface, burrow wall and anoxic sediment samples, 192 recombinant clones randomly selected were sent out for Sanger sequencing with a M13 forward primer (GATC Biotech, Konstanz, Germany, http://www.gatc-biotech.fr/fr/index.php).

### 2.5. Clone library characterization, analyses and comparison

Sequence treatment was performed with the pregap4 tool of the Staden Package Program (Staden, 1996; http://staden.sourceforge.net/) and sequence orientation was checked with the Orientation Checker tool (Ashelford et al., 2006; http://www.bioinformaticstoolkit.org/Squirrel/index.html). Sequences were then aligned with Clustal X (Thompson et al., 1997; ftp://ftp.ebi.ac.uk/pub/software/clustalw2) and checked for chimeric PCR artifacts using Mallard software (Ashelford et al., 2006; http://www.bioinformatics-toolkit.org/Mallard/index.html). Potential chimeric sequences were removed from the clone libraries. After these previous treatments, the number of sequences subjected to phylogenetic analyses was 168, 179 and 129 for the *S*, *BW* and *An* clone libraries, respectively. All sequences have been deposited in the Genbank database under accession numbers FJ753072 to FJ753240, FJ752762 to FJ752941 and FJ752942 to FJ753071 for the *S*, *BW* and *An* clone libraries, respectively.

Sequences were compared with those deposited in the GenBank database using BLASTN software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A phylogenetic tree was constructed for the burrow wall community with the Mega 4.1 Beta program (Tamura et al., 2007; http://www.megasoftware.net/index.html) using the neighbor-joining algorithm; 1000 bootstrap resamplings were performed to estimate the reproducibility of the tree.

Operational taxonomic unit (OTU) assignments for *S*, *BW* and *An* clone libraries were performed using the furthest neighbor clustering algorithm of the DOTUR program (Schloss and Handelsman, 2005; http://schloss.micro.umass.edu/software/dotur.html). This program involved preliminary production of a distance matrix which was done on the Greengenes website after alignment of sequences with the NAST tool (DeSantis et al., 2006; http://greengenes.lbl.gov/cgi-bin/nph-index.cgi). A 3% distance level between sequences was considered the cutoff to consider distinct OTUs. Based on these OTU assignments, the validity of sampled clones for species diversity in natural samples was evaluated by coverage value (*C*, in %) according to the following equation (Good, 1953): $C = (1 - n_1/N) \times 100$, where $n_1$ is the number of OTUs appearing only once in the library and *N* is the total number of OTUs. Rarefaction analysis and diversity indices for *S*, *BW* and *An* clone libraries were also performed with DOTUR based on OUT assignments (3% distance level). The observed richness in OTUs (*R*, rarefied for 129 sequences and measured from rarefaction curves), the Bootstrap richness estimator, Chao1 richness, ACE richness, the Jackknife estimator as well as Shannon−Weaver ($H'$) and Simpson ($1 - D$) indices of diversity were calculated for the three clone libraries. Moreover, OTU evenness was calculated with the Pielou indices $J' = H'/\ln(R)$ and Simpson evenness $D' = (1 - D)/(1 - (1/R))$. Mean pairwise divergence ($\pi$), i.e., the mean number of base pairs (bp) that differ between two randomly chosen sequences, was calculated with JM109 ARLEQUIN software (Excoffier et al., 2005; http://lgb.unige.ch/arlequin/) for each library (intra-library) and for the three pooled libraries together (inter-libraries). Software SONS version 1.0 (Schloss and Handelsman, 2006; http://schloss.micro.umass.edu/software/sons.html) was used to evaluate the percentage of shared OTUs between libraries.

To determine similarities between clone libraries, pairwise comparisons between non-dereplicated sequences (i.e. the possibility of finding a sequence several times was left in) of clone libraries were performed using ∫-LIBSHUFF, following the instructions of the authors (Schloss et al., 2004; http://www.plantpath.wisc.edu/joh/s-libshuff.html). *P*-values were corrected according to the Bonferroni correction (Sokal and Rohlf, 1995): $B = 1 - (1 - \alpha)^{1/N}$, where $\alpha$ is the significance level and *N*, the number of comparisons. In our case, $B = 0.00167$ with $\alpha = 0.01$ and $N = 6$. To determine differences between clone libraries, cluster analysis was performed

with the program Unifrac (Lozupone et al., 2006; http://bmf2.colorado.edu/unifrac/index.psp). Jackknife analysis was carried out to test the robustness of the cluster.

## 3. Results

### 3.1. Sediment characteristics

Sediment characteristics were quantified for the surface, burrow wall and anoxic sediment samples (Table 1). The three compartments did not exhibit similar grain size distribution. Indeed, the surface sediment was primarily composed of sands (57.8 ± 11.3%; $n = 4$) as described with the Wentworth scale, whereas burrow wall and anoxic sediments were mainly formed by silts (51.0 ± 4.6% and 53.8 ± 9.8% for *BW* and *An* respectively; $n = 4$). Surface and burrow wall sediments exhibited the same porosity values (0.53 ± 0.05 and 0.53 ± 0.03, respectively; $n = 4$ for each). The anoxic sediment presented lower porosity (0.45 ± 0.02; $n = 4$). The organic matter content ranged from 1.59 ± 0.39% ($n = 4$) for surface sediments to 2.71 ± 0.34% ($n = 4$) for the burrow wall with an intermediate value of 2.03 ± 0.67% ($n = 4$) for the anoxic compartment.

### 3.2. Differences between surface (S), burrow wall (BW) and anoxic bacterial communities (An)

A total of 476 clones were retrieved in the three clone libraries: 168, 179 and 129 clones for the surface, burrow wall and anoxic sediment samples, respectively. It is important to remember that because of PCR and cloning biases (possibly differential ligation efficiencies of the different amplicons) (Van Elsas and Boersma, 2011), the clone library approach used in this study cannot give a truly exact or exhaustive picture of the bacterial communities of the different environments studied. In order to evaluate the effectiveness with which libraries were sampled, rarefaction analyses were performed. For each library, the resulting curve did not reach its asymptotic phase, illustrating the incompletely described diversity of the studied microbial communities (Fig. 1). Nevertheless, Good's indices ($C$) calculation showed that libraries covered 46, 34 and 40% of bacterial community diversity for *S*, *BW* and *An* sediments, respectively ($OTU_{0.03}$ definition).

Table 1
Sediment grain size distribution, porosity and organic matter content (%) of surface (*S*), burrow wall (*BW*) and anoxic (*An*) sediments in the *H. diversicolor* environment ($n = 4$ for each).

| | *S* | | *BW* | | *An* | |
|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | Mean | SD |
| Grain size distribution | | | | | | |
| Clay (<4 μm) | 14.2 | 3.2 | 28.9 | 5.6 | 26.7 | 5.8 |
| Silts (4–63 μm) | 28.0 | 2.4 | 51.0 | 4.6 | 53.8 | 9.8 |
| Sand (63 μm–2 mm) | 57.8 | 11.3 | 20.1 | 6.5 | 19.5 | 4.9 |
| Porosity | 0.53 | 0.05 | 0.53 | 0.03 | 0.45 | 0.02 |
| Organic matter content | 1.59 | 0.39 | 2.71 | 0.34 | 2.03 | 0.67 |

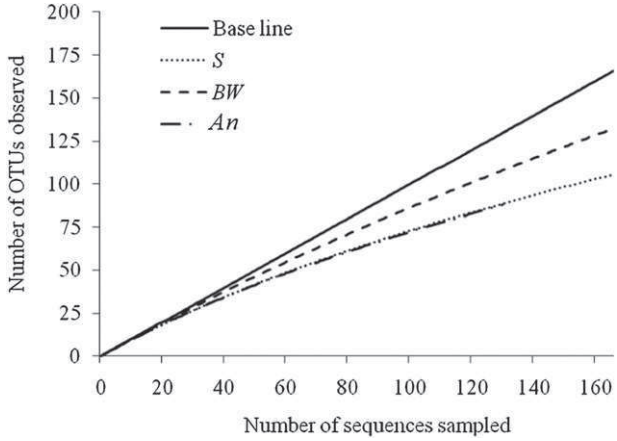


Fig. 1. Rarefaction curves of 16S rRNA libraries from surface (*S*), burrow wall (*BW*) and anoxic (*An*) sediment samples in *H. diversicolor* inhabited sediments.

The taxonomic affiliation of *S*, *BW* and *An* sequences was determined based on BLAST results (Fig. 2). Almost all sequences (i.e. 95%) were related to uncultured bacteria, which was not surprising considering that only few bacterial species inhabiting sediment can be cultured (Amann et al., 1995). Except for a few unclassified bacteria, sequences belonged to 10, 9, and 8 major bacterial phyla for surface, burrow wall and anoxic sediments, respectively. Clones could be classified into *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, *Epsilonproteobacteria* and an unclassified class in the phylum *Proteobacteria*; *Flavobacteria*, *Sphingobacteria* and an unclassified class in the phylum *Bacteroidetes*; *Acidobacteriales* and *Holophagae* in the phylum of *Acidobacteria*, as well as the phyla of *Chloroflexi*, *Cyanobacteria*, *Fibrobacteres*, *Firmicutes*, *Nitrospirae*, *Planctomycetes*, *Spirochete*s and *Verrucomicrobia*.

However, the structure of the bacterial communities presented important differences between the three investigated environments. The *Proteobacteria* were the most abundant phyla, accounting for more than 66% in each library (Fig. 2). Within this phylum, the community structure of surface sediments had a higher proportion of *Alphaproteobacteria* (21.4%) and *Gammaproteobacteria* (33.3%) compared with those of burrow wall (15.1% and 28.5%, respectively) and anoxic sediments (10.9% and 21.7%, respectively). *Deltaproteobacteria* mostly occurred in anoxic sediments (36%) and were less common in the burrow wall sediments (21%) and surface sediments (18%). *Betaproteobacteria* existed only in small proportions in surface (0.6%) and burrow wall (1.1%) sediments, as well as *Nitrospirae* (1.2 and 1.1% in *S* and *BW*, respectively). The oxygenic photosynthetic *Cyanobacteria* only occurred in surface sediments (4.2%) as did the *Fibrobacteres* but at lesser proportions (0.6%). In contrast, the *Spirochetes* were only found in clone libraries of burrow wall and anoxic sediments (1.1 and 2.3%, respectively). All samples harbored *Epsilonproteobacteria* (0.6–1.7%), *Chloroflexi* (0.6–2.3%), *Firmicutes* (0.6–0.8%) and *Verrucomicrobia* (1.6–2.4%) in small proportions, and *Acidobacteria* (3–6.1%), *Bacteroidetes* (3.1%–7.1%) and *Planctomycetes*

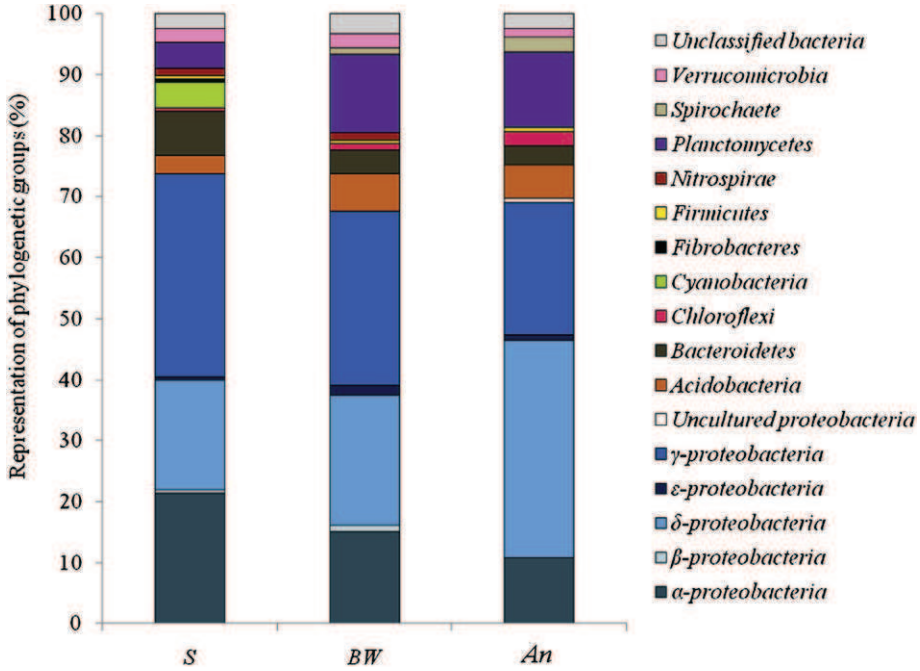


Fig. 2. Relative representation of phylogenetic groups in surface (*S*), burrow wall (*BW*) and anoxic (*An*) sediment samples in *H. diversicolor* inhabited sediments.

(4.2−12.8%) in greater proportions. They all also exhibited a small proportion of unclassified bacteria, 2.4%, 3.4%, and 2.3% for *S*, *BW* and *An* communities, respectively.

In the case of the *Deltaproteobacteria*, samples mainly contained the *Myxococcales* aerobic order (8.6%) as well as anaerobic orders implicated in the sulfur cycle like sulfur- and sulfate-reducing *Desulfuromonadales* (e.g. *Desulfuromonas* sp.) (25.8%), and *Desulfobacterales* (e.g. *Desulfosarcina variabilis*; Table 2) (53.8%). The majority of *Deltaproteobacteria* hosted in burrow wall sediments belonged essentially to the *Desulfobacterales* and unclassified *Deltaproteobacteria*. The surface and anoxic clones were members of the *Desulfobacterales* and *Desulfuromonadales*. Through the three clone libraries, more than the half of the *Alphaproteobacteria* belonged to *Rhodobacterales* (61.3%), with the remaining clones belonging to the orders of *Rhodospirillales* (14.7%), *Rhizobiales* (9.3%), *Kordiimonadales* (6.7%), *Parvularculales* (2.7%) or *Sphingomonadales* (4%). Among these *Alphaproteobacteria*, more than half were found in surface sediments and 25 and 22%, respectively, were clones from the burrow wall and anoxic sediments. The clones in the *Gammaproteobacteria* are shared between several orders such as the *Oceanospirillales* (20.5%), *Chromatiales* (19.7%), *Alteromonadales* (13.1%), *Pseudomonadales* (10.7%) and a non-negligible part of unclassified *Gammaproteobacteria* (36.0%). Among the *Gammaproteobacteria*, the clones belong mainly to surface and burrow wall sediments.

Table 2
Percentage of the most represented orders in *Alphaproteobacteria*, *Deltaproteobacteria* and *Gammaproteobacteria* in the overall data set (total) and in each of the library surface (*S*), burrow wall (*BW*) and anoxic (*An*) sediments ($S + BW + An = 100\%$ of total).

| | Total | *S* | *BW* | *An* |
|---|---|---|---|---|
| *Alphaproteobacteria* (75 seq.) | | | | |
| *Rhodobacterales* | 61.3 | 50 | 39.1 | 10.9 |
| *Rhodospirillales* | 14.7 | 27.3 | 18.2 | 54.6 |
| *Rhizobiales* | 9.3 | 57.1 | – | 42.9 |
| *Kordiimonadales* | 6.7 | – | 100 | – |
| *Parvularculales* | 2.7 | – | 100 | – |
| *Sphingomonadales* | 4 | 100 | – | – |
| *Deltaproteobacteria* (93 seq.) | | | | |
| *Desulfobacterales* | 53.8 | 22 | 54 | 24 |
| *Desulfuromonadales* | 25.8 | 54.2 | 12.5 | 33.3 |
| *Myxococcales* | 8.6 | 25 | 50 | 25 |
| Others | 11.8 | – | 100 | – |
| *Gammaproteobacteria* (122 seq.) | | | | |
| *Oceanospirillales* | 20.5 | 48 | 40 | 12 |
| *Chromatiales* | 19.7 | 50 | 37.5 | 12.5 |
| *Alteromonadales* | 13.1 | 68.7 | 25 | 6.3 |
| *Pseudomonadales* | 10.7 | 7.6 | 46.2 | 46.2 |
| Others | 36.0 | 31.8 | 43.2 | 25 |

The sequences of surface, burrow wall and anoxic clone libraries were assigned in 107, 141 and 88 OTUs, respectively (Table 3). Among the whole data set of sequences (336 OTUs), only 30 sequences were shared by at least two clone libraries. Global diversity indices pointed out that diversity was higher for the burrow wall bacterial community, with a Shannon indice value of 4.86 (varying between 4.74 and 4.97) compared with 4.47 (varying between 4.34 and 4.67) and 4.31 (varying between 4.16 and 4.42) in surface and anoxic sediments, respectively, and a Simpson index value ($1 - D$) of 0.997 for the burrow wall clone library (0.991 for both the surface and anoxic libraries). Moreover, rarefaction analyses and richness indices clearly showed that the burrow wall bacterial community exhibits higher species richness (Fig. 1 and Table 3) compared to surface and anoxic communities which appeared to have similar biodiversity (despite the fact

Table 3
Statistics of clone libraries for the surface ($S$), burrow wall ($BW$) and anoxic ($An$) sediments (97% similarities).

| Clone libraries | Surface | | | Burrow wall | | | Anoxic | | |
|---|---|---|---|---|---|---|---|---|---|
| | Value/Mean | Lower limit | Upper limit | Value/Mean | Lower limit | Upper limit | Value/Mean | Lower limit | Upper limit |
| Number of clones | 129 | – | – | 179 | – | – | 169 | – | – |
| Number of OTUs | 107 | – | – | 141 | – | – | 88 | – | – |
| Species richness | | | | | | | | | |
| R | 89 | – | – | 108 | – | – | 88 | – | – |
| Boot | 137.2 | – | – | 185.7 | – | – | 113.9 | – | – |
| ACE | 289.3 | 210.5 | 430.3 | 478.8 | 340.0 | 714.4 | 256.1 | 176.0 | 409.1 |
| Chao1 | 235.6 | 175.7 | 347.9 | 550.7 | 367.2 | 882.9 | 236.6 | 161.9 | 386.8 |
| Jack | 245.3 | 198.7 | 291.8 | 1171.6 | 669.3 | 1673.8 | 259.9 | 195.3 | 324.4 |
| Diversity indices | | | | | | | | | |
| Shannon | 4.47 | 4.34 | 4.61 | 4.86 | 4.74 | 4.97 | 4.31 | 4.16 | 4.46 |
| Simpson ($1 - D$) | 0.991 | – | – | 0.997 | – | – | 0.991 | – | – |
| Species evenness | | | | | | | | | |
| Pielou index | 0.957 | 0.928 | 0.986 | 0.981 | 0.958 | 1.004 | 0.962 | 0.929 | 0.995 |
| Simpson evenness | 1.000 | – | – | 1.004 | – | – | 1.002 | – | – |
| Mean divergence ($\pi$, bp) | | | | | | | | | |
| Intra-libraries | 345.2 | – | – | 342.2 | – | – | 358.3 | – | – |
| Inter-libraries | $S$–$BW$: 348.2 | – | – | $S$–$An$: 357.4 | – | – | $BW$–$An$: 354.8 | – | – |

that they presented different bacterial structures). This was not surprising and most likely linked to the high microorganism diversity revealed by culture-independent methods and by the microscale heterogeneity of biogeochemical/environmental parameters of marine sediments (Amann et al., 1995; Aller et al., 1998; Hughes et al., 2001). At 97% nucleotide identity, Chao1 and ACE indices were two times higher within the burrow wall than in surface or anoxic sediments. These results are consistent with previously reported taxon richness from various sediments (Kemp and Aller, 2004). Species evenness, estimated via both the Pielou and Simpson evenness indices (Table 3), was high for all compartments. The burrow wall showed higher taxon evenness than surface and anoxic sediment at 97% nucleotide identity.

All three statistical pairwise clone library comparisons with the $\int$-LIBSHUFF method showed significant differences, with all corrected $P$-values = 0.00167 for a confidence interval of 0.01 ($P$-values = 0.0000 without any correction). In particular, comparisons revealed that sequences of burrow walls were composed of significantly different OTUs compared with surface and anoxic sediments. This was in agreement with the fact that clone libraries seemed to exhibit higher levels of specific sequences, with the burrow wall community presenting a slightly larger proportion (77.4%) compared to surface (69.4%) and anoxic (73.9%) sediment communities (Table 4). These significant differences are observed despite the fact that the different subsamples from each environment were pooled together before analysis. If there were only non-specific heterogeneous assemblages of bacterial species in the sediment matrices, then this pooling step would probably have led to no observable differences between the different environments. This is obviously not the case, which reinforces the hypothesis of a specific bacterial community associated with burrow wall sediments.

The three bacterial communities shared only a modest proportion of OTUs (6.7%). The burrow wall community shared more OTUs with the surface (15.1%; Table 4) compared with the anoxic community (7.5%); likewise, the surface community shared more OTUs with the burrow wall (19.8%) compared with the anoxic community (10.8%; Table 4). Moreover, mean pairwise divergence between sequences from different libraries (Table 3) was greater for the surface–anoxic and burrow wall–anoxic associations (357.4 and 354.8 bp, respectively) and slightly lower for the surface–burrow wall association (348.2 bp). Both these observations suggested that the bacterial community of burrow wall sediments resembled the community of the surface more than that of anoxic sediments. This was also statistically supported by cluster analysis associated with Jackknife analysis shown in Fig. 3.

## 4. Discussion

Concerning the increased abundance and activity observed for *H. diversicolor* burrow wall bacterial assemblage, the

Table 4
Fraction of shared OTUs (%) between the clone libraries of the surface ($S$), burrow wall ($BW$) and anoxic ($An$) sediments, and fraction of unique sequences in samples.

| | $S$ | $BW$ | $An$ | Specificity |
|---|---|---|---|---|
| $S$ | – | 19.8 | 10.8 | 69.4 |
| $BW$ | 15.1 | – | 7.5 | 77.4 |
| $An$ | 13.6 | 12.5 | – | 73.9 |

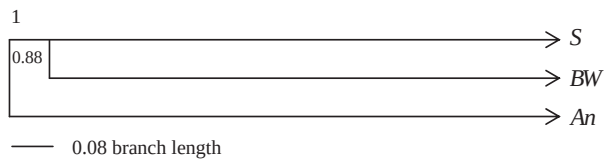


Fig. 3. Jackknife environment cluster analysis of surface ($S$), burrow wall ($BW$) and anoxic ($An$) clone libraries. Values are Jackknife fractions.

differences in the structure of bacterial communities may also be attributed to the burrow system and worm behavior and ecology (e.g. Goňi-Urriza et al., 1999; Marinelli et al., 2002; Lucas et al., 2003; Papaspyrou et al., 2006). As described in Table 5, the microbial community in the burrow wall has to deal with and to adapt to sediment characteristics (e.g. sediment grain size distribution, porosity and organic matter content; Table 2) and physicochemical conditions that are very different from those of the surface and surrounding anoxic sediments. Within the first millimeters, the organic-rich surface sediments generally represent a well-aerated dynamic place in part due to water current. Surface sediments also exhibit environmental variables that may vary on a diel pattern. Conversely, anoxic sediments are depleted in oxygen and water movements are limited by sediment compaction, resulting in a more stable environment compared with the surface. Finally, as previously described, the burrow environment is characterized by environmental variables which widely oscillate with the frequency of the worm's ventilation activity, and a higher quantity of labile organic material.

Considering the similarities between communities, the few studies that compared bacterial assemblages of surface, burrow wall and anoxic sediments found contrasting results. Based on 16S rRNA gene DGGE fingerprinting, Papaspyrou et al. (2006) found more similarities between the communities of burrow wall and anoxic sediments whereas, based on PLFA and T-RFLP analyses, respectively, Steward et al. (1996) and Laverock et al. (2010) found more similarities between the burrow wall and the surface communities. These results do not point to a general rule. The age of the burrow can explain this in part, as structural characteristics are evolving with time (e.g. construction, maintenance). Once abandoned, the old burrow structure becomes a non-bioirrigated anoxic system which can be progressively recolonized by surrounding anaerobic microbes (Diaz and Cutter, 2001). Hence, succession with time is an important question to address in order to explain the diversity of the bacterial community structure of burrows. For instance, using a burrow mimic system and PLFA analysis, Marinelli et al. (2002) demonstrated that burrows with a longer residence time were characterized by higher microbial biomass and a distinct anaerobe signature, compared to burrows having shorter residence times. Finally, differences between bacterial communities may also depend on sedimentary matrix characteristics. In a recent study, Bertics and Ziebis (2009) showed that when geochemical parameters were alike, microbial communities associated with burrows of two crustaceans, the ghost shrimp *Neotrypaea californiensis* and the fiddler crab *Uca crenulata*, showed significant similarity to sediment surface communities. However, as previously mentioned, in the case of *H. diversicolor*, the burrow wall matrix is markedly different from that of the surface sediment (Tables 1 and 5).

Rarefaction curves clearly showed that the bacterial community of burrow walls exhibited substantially higher diversity. Due to construction and ventilation of burrows in otherwise anoxic sediments, thus redefining the biogeochemical conditions, *H. diversicolor* introduces a high level of heterogeneity into the sediments that seems to maintain a higher level of microbial diversity. Indeed, this biogeochemical heterogeneity most likely implies: (1) the providing of numerous microbial ecological niches which succeed in space and time and probably favor establishment of new and better adapted core taxa; and (2) proliferation of rare taxa from the sediment diversity reservoir (Pedrós-Alió, 2006). Moreover, burrow walls may be enriched with microbes brought into the burrow structure from overlying water through periodical burrow flushing or with microbes migrating from the nearby surrounding environment (oxic and anoxic) or transported by particles reworked during construction and maintenance of the biogenic structure (Reichardt et al., 1991). Furthermore, the community may also be enriched by microbes associated with the worms or other organisms attracted by this particular environment. Microbes may originate simply from the worm body or from the fecal pellets produced (ingested microbes,

Table 5
Comparison of surface (*S*), burrow wall (*BW*) and anoxic (*An*) ecosystem characteristics.

| Properties | *S* | *BW* | *An* | References |
|---|---|---|---|---|
| Porosity | Higher | Variable | Lower | c |
| Water dynamic | Water current (advection) and diffusive boundary layer | Bio-irrigation | Molecular diffusion in muddy sediment | f,i |
| Variability of environmental conditions | Dynamics on large scale (hours, days) | Dynamics on small scale (min) | More stable | d,e,g |
| Organic matter content | Labile (phytoplankton, detritus) | Labile (mucus, phytoplankton, detritus) | Refractory | a |
| Oxygen | Aerobic, non-limiting | Oscillating | Anaerobic | d,e,h |
| Solutes | Non-limiting | Oscillating | ± Limiting | d |
| pH | Stable, neutral | Oscillating (±2 units) | Stable, more acid | b |

[a] e.g. Aller and Aller, 1986; Papaspyrou et al., 2005.
[b] e.g. Kristensen, 2000; Zhu et al., 2006.
[c] e.g. Papaspyrou et al., 2006; Meysman et al., 2007.
[d] e.g. Aller, 1994; Aller et al., 1998; Kristensen, 2000.
[e] e.g. Glud, 2008.
[f] e.g. Forster and Graf, 1995; Kristensen, 2001.
[g] e.g. Glud et al., 1999.
[h] e.g. Revsbech and Jorgensen, 1986; Aller, 1988.
[i] e.g. Berner, 1980; Aller, 1982; Jorgensen and Revsbech, 1985; Glud et al., 2007.

endogenous intestinal tract microflora) through excretion. Indeed, differential digestion of bacteria passing through the digestive tract of *H. diversicolor*, as well as enteric bacteria release during excretion have been demonstrated (e.g. Wilde and Plante, 2002; Lucas et al., 2003; Grossi et al., 2006). These selected bacteria may enrich the burrow wall community. Finally, macrofaunal bioturbation activities as well as inferred sediment heterogeneity induce an important microbial mixing of bacterial populations suitable to the expansion of phylogenetic and metabolic diversity of microbes.

In conclusion, based on the comparison and bioinformatic analysis of clone libraries, the present work contributes to the understanding of the microbiology of bioturbated sediments. Despite the probable existence of variability among the communities of the different burrow walls (e.g. age of the burrow), as shown in this study, the biogeochemical constraints of this particular environment lead to selection of a specific community associated with burrow walls, with significantly higher diversity compared with the nearby surface and anoxic sediments. These results provide further evidence of the determinant role of macrobenthic invertebrates in microbial diversity in marine sediments. Clearly, further studies are now needed to improve our knowledge of the fine-scale phylogenetic architecture and functioning of the various bacterial communities associated with the complex ecosystem formed by burrow walls of marine polychaetes.

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## Appendix. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.resmic.2011.07.008.

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