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RESEARCH ARTICLE

Influence of oyster culture on biogeochemistry and bacterial community structure at the sediment–water interface

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Abstract

Bacterial community structure and some biogeochemical parameters were studied in the sediment of two Pacific oyster farming sites, Aber Benoît (AB) and Rivière d'Auray (RA) in Brittany (France), to examine the ecological impact of oysters and to evaluate the emission of sulfide and ammonia from sediment. At AB, the organic matter accumulated in the sediment beneath the oyster tables was rapidly mineralized, with strong fluxes of ammonia and sulfide that reached 1014 and 215 μmol m^{-2} h^{-1}, respectively, in June 2007. At RA, the fluxes were about half as strong on average and better distributed through the year. The ammonia and sulfide concentrations in the overlying water never reached levels that would be toxic to oysters in either site, nor did hypoxia occur. Total culturable bacteria (TCB) varied greatly according to the temperature: from 1.6×10^4 to 9.4×10^7 cell g^{-1} sediment. Inversely, the bacterial community structure remained surprising stable through the seasons, marginally influenced by the presence of oysters and by temperature. Bacterial communities appeared to be characteristic of the sites, with only one common phylotype, *Vibrio aestuarianus*, a potential oyster pathogen. These data refine the hypothesis of seawater toxicity to oysters because of ammonia and sulfide fluxes and show that the measured environmental factors had only a weak influence on bacterial community structure.

Introduction

Since the 1990s, culture of the oyster *Crassostrea gigas* in France has been subject to mortality episodes, which generally occur in June as soon as the temperature reaches 19 °C. Over the period since they began, these mortality episodes have increased dramatically. Since 2008, they have been seen to start at temperatures around 16 °C, because of the appearance of a new type of OsHv1 herpes virus that is much more virulent than the previously known strain (Segarra *et al.*, 2010). Before this development, a multidisciplinary research project known as Morest (Samain & McCombie, 2008) was conducted to study the original mortality syndrome. The conclusions of Morest underlined the involvement of multiple factors, including the genetic and physiological status of the oysters, occurrence of pathogens, and environmental factors including temperature, pollutants, food availability, and sediment proximity. For this last factor, it was demonstrated that mortality rate increased with the closeness to the sediment at which the oysters were reared. Indeed, mortality of oysters reared directly on or a few centimeters above the sediment was significantly higher than that of oysters reared on oyster racks 50–70 cm above it. The beginning of the mortality episodes appeared to be concomitant with the release of hydrogen sulfide and ammonia from the sediment in June, as a result of mineralization of organic matter (OM) especially that generated by oyster farming itself. Before the mortality, a growth deficit was generally recorded for oysters grown directly on bottom, suggesting that chronic toxicity was caused by a factor from the sediment (Soletchnik *et al.*, 2005; Samain & McCombie, 2008). As early as the 1950s, a study showed how the intensive oyster culture could modify the surface sediment through oyster biodeposition and how, in turn, this modified sediment could disturb

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oyster growth and survival (Susumu & Takeo, 1955). At Katsurashina, an oyster farming area in Japan, the sediment was found to contain high levels of sulfide and ammonia, which are known to be toxic to macroorganisms (Van Sprang *et al.*, 2000). In the Etang de Thau (south of France), where there is semi-intensive culture of oysters attached to ropes, the benthic fluxes of ammonia and oxygen demand at the water–sediment interface were, respectively, 1–5 and 1.8–3 times higher underneath the suspended oysters than outside the culture area (Mazouni *et al.*, 1996). It is also well known that bacteria are the main actors in the degradation of OM in the aquatic environment (Kemp, 1990), leading to the production of reduced compounds including methane, the reduced forms of Mn and Fe, ammonia, and hydrogen sulfide.

Bacterial abundance tends to increase noticeably in sediment under intensive fish farming (Rajendran & Nagatomo, 1999; Caruso *et al.*, 2003). This increase is accompanied by a shift in the bacterial community composition and activities (Vezzulli *et al.*, 2002). For oysters, two studies, one carried out in the USA (Richardson *et al.*, 2008) and the other in Japan (Asami *et al.*, 2005), have also reported a substantial change in microbial communities and an increase in sulfide production underneath cultured oysters.

This study was undertaken to verify whether the presence of oysters could significantly modify the biotic and abiotic characteristics of sediment by measuring some biogeochemical parameters. Ammonia, sulfide, nitrate, and phosphate were measured in both overlying and pore waters to evaluate diffusive fluxes, and high-resolution measurements of oxygen profiles were performed to determine the oxygen penetration depth. In addition, organic matter (OM) was measured in the solid phase. Bacterial community structure and composition were studied to determine which biogeochemical parameters influenced it and to identify the potential functional groups and any oyster pathogens present.

Materials and methods

Site descriptions

Sampling was performed in two estuarine areas of France where oyster farming is highly developed: (1) the downstream part of Aber Benoît (4°36′W and 48°36′N); and (2) an oyster culture site (Fort-Espagnol) near the mouth of Rivière d'Auray (2°58′W and 47°36′N).

Aber Benoît (Fig. 1, Table 1) is an estuary 31 km in length, with a catchment area of 140 km^2, situated in the northwest of Finistère (Brittany). The average water flow in spring is 0.418 $m^3\ s^{-1}$, but this site is subjected to strong seawater currents (up to 3.5 $m\ s^{-1}$) because of the high tidal amplitude, and salinity ranges from 2.4% to 3.4%. Anthropic activities, which mainly center on animal husbandry, lead to moderate discharges of OM, nitrate-rich fertilizer, and sometimes pesticides into Aber Benoît. However, the dilution owing to high tide (5 m of tidal amplitude) and currents prevent serious pollution. The oyster-growing area (250 ha) is located both within and between Aber Benoît and Aber Wrac'h. Before 2008, no summer mortality occurred and the temperatures never reached 19 °C.

The estuarine area of Auray (Fig. 1, Table 1) is 56.4 km in length and represents the western part of the Gulf of Morbihan in south Brittany. It is influenced by anthropic activities, mainly agriculture, on a catchment area of 800 km^2 around the two principal rivers, the Loch and the Sal. These rivers flow into Rivière d'Auray, with an average flow of 2.99 $m^3\ s^{-1}$ for the Loch and about half this for the Sal. The tidal flux allows a renewal of 50% of the Gulf's water every 10 days (20 tides) and produces a current of up to 4 $m\ s^{-1}$. In the downstream part of Rivière d'Auray, which is used for oyster farming, salinity can vary from 2.7% to 3.5%. Oyster mortality has occurred almost every year since the phenomenon began, notably because the temperatures exceed 19 °C in summer.

The sediment of Aber Benoît is sandy-mud with a deep gray color, while Rivière d'Auray sediment is black muddy-sand. Sediments at both sites have high mean organic carbon (OC) contents (Fig. 3).

Sample collection

Sediment samples were collected on a monthly basis from June 2007 to September 2007. In 2008, samples were taken in February, March, May, and June. At each site, six bags containing either 100 or 250 15-month-old oysters were attached to oyster tables, 50 cm above the sediment, in April 2007. The mean size of the oysters was 6.2 ± 0.9 cm, for a mean weight of 13.8 ± 1.9 g. Sediment samples were subsequently collected just beneath the experimental oyster bags ('Oyster' stations) and at 30 m away from them ('Reference' stations). Samples for this study were all collected under similar hydrodynamic conditions (moderate spring tide, ebb tide). For biogeochemical parameter analysis, three replicate sediment samples were collected from 20 cm depth with 15 cm of overlying water, using PVC tubes of 40 cm length and 9 cm diameter, as described in Mudroch & Azcue (1995). Any disturbance of the sediment–water interface was carefully avoided.

For bacterial counts and bacterial community structure analysis, samples were taken to 5 cm depth at three randomly selected points spaced about 1 m apart, using Falcon tubes (50 mL volume, 3 cm diameter) severed at

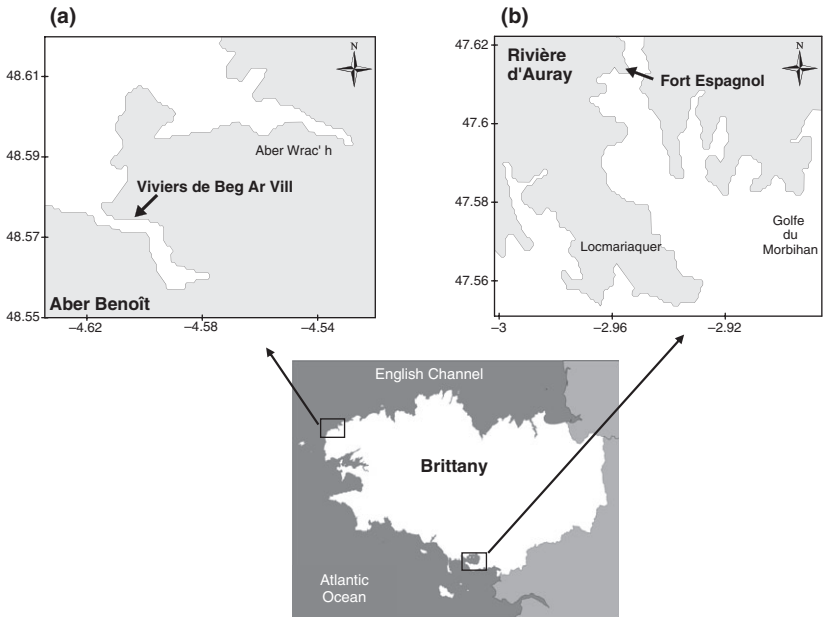

Fig. 1. Map showing (a) Aber Benoît and (b) Rivière d'Auray in the gulf of Morbihan. Locations of the sampling sites are indicated by arrows.

Table 1. Main characteristics of Aber Benoît and Rivière d'Auray

	Aber Benoît	Rivière d'Auray
Location	North-west Brittany 4°36′W and 48°36′N	In the gulf of Morbihan South Brittany 2°58′W and 47°36′N
Catchment area	140 km^2	800 km^2
Average spring water flow	0.418 $m^3\ s^{-1}$	2.99 $m^3\ s^{-1}$ for the Loch river about 1.5 $m^3\ s^{-1}$ for the Sal river
Anthropic influences	+	++
Oyster culture Annual production/area	1500 t/38 ha	4500 t/1635 ha
Summer mortality of oyster before 2008	No	Yes
Depth at zero tide	14 m	20 m
Mean tidal range	6 m	5 m
Seawater current	Up to 3.5 m s^{-1}	Up to 4 m s^{-1}
Sediment	Sandy-mud	Muddy-sand
Salinity (%)	2.4–3.4	2.7–3.5
Temperature range (this study)	11.1–18.1 °C	10.8–21.8 °C

6 cm. In 2008, four replicate sediment samples were collected. The quantity of sediment collected per sample to be used for bacterial analyses was the same as that used in most other microbiological studies (Caruso *et al.*, 2003; Richardson *et al.*, 2008). The relative similarity of samples from a single station in terms of counts and bacterial community structure demonstrated *a posteriori* that they were representative of the stations.

For bacterial counting, 5 g of each sediment sample was suspended in 10 mL of sterile filtered seawater and homogenized by vortexing for 5 min. The supernatants were then serial 10-fold diluted in sterile seawater and plated on marine agar (Difco) for total culturable bacteria counts (TBC), and on TCBS for vibrios. As in previous studies examining the impact of fish farming or temperature on bacteria in the sediment (Gonzalez-Acosta *et al.*, 2006; Castine *et al.*, 2009), culturable bacteria counting was chosen in preference to direct counting of bacteria stained with orange acridine or DAPI because this method is more sensitive to changes in physicochemical parameters such as OM and temperature. *Vibrionacaea*, notably those that can grow on TCBS, are important for marine farming because this family includes most pathogens of farmed marine animals, especially those of *C. gigas* (Gay *et al.*, 2004; Garnier *et al.*, 2007). The remainder of each of the sediment samples was stored at − 80 °C until DNA extraction.

Sediment treatments

All overlying and pore water treatments were performed at the sampling sites after the collection of sediment cores. An aliquot of overlying water was collected immediately for further nutrient and sulfide analyses. High-resolution

vertical profiling of dissolved O_2 was then carried out both on the overlying water and on the sediment core, using miniaturized Clark-type oxygen sensors (Unisense OX500; http://www.unisense.com) coupled with a picoammeter (Unisense PA 2000) and a micromanipulator (Unisense MM33) according to Duchemin *et al.* (2005). These microsensors (tip diameter: 500 μm) are well suited to performing high-resolution measurements of O_2 profiles and determining O_2 penetration depth without disturbing the sediment (Revsbech *et al.*, 1980). Subsequently, within < 30 min, one core from each station was sliced into eight horizontal layers down to a total depth of 8 cm (4 slices of 0.5 cm from the top down to 2 cm depth, 2 slices of 1 cm down to 4 cm, and 2 slices of 2 cm down to 8 cm). In July, each core was sliced down to 8 cm whereas on the other dates, only one core was sliced down to 8 cm, the two other cores only being sliced down to 1 cm (slices: 0–0.5 and 0.5–1 cm). For every level, a sub-sample was centrifuged at 2075 ***g*** and 4 °C for 20 min in a Whatman VectaSpin 20TM centrifuge tube filter (0.45 μm) in an inert atmosphere (N_2) to collect pore waters, which were then stored at − 20 °C. Finally, a sub-sample of wet sediment was frozen for subsequent organic C analysis.

The pore water was acidified to pH *c.* 2 and an aliquot frozen for later analysis of phosphate, nitrate, and ammonia. Another aliquot was diluted 50-fold and preserved by adding zinc chloride to precipitate the sulfide as zinc sulfide.

Biogeochemical parameter analyses and flux determination

After thawing, an aliquot of the pore water was diluted 10-fold before nutrient analysis. Ammonia (NH_4^+), nitrate (NO_3^-), and phosphate (PO_4^{3-}) were analyzed by segmented flow analysis (Aminot *et al.*, 2009). Hydrogen sulfide (H_2S, HS^-, S^{2-}) was measured using the colorimetric methylene blue method according to Fonselius *et al.*(1999).

OC was measured using a vario EL-III CN elemental analyzer after decarbonation. Diffusive fluxes were calculated using the Fick's first law adapted for sediments (Berner & Honjo, 1981):

$$\mathrm{Fd} = -\Phi \times D_s\left(\frac{\mathrm{d}C}{\mathrm{d}z}\right), \quad (1)$$

where Fd is the rate of efflux (μmol m^{-2} day^{-1}), Φ is the sediment porosity (dimensionless) of the upper sediment sample, D_s is the bulk diffusion coefficient (m^{-2} day^{-1}), and d*C*/d*z* is the concentration gradient at the sediment–water interface (μmol m^{-4}). For ammonia, nitrate, and phosphate, d*C*/d*z* was calculated from linear regression on the concentration values of water at the bottom and in the sediment just below the interface. In the case of HS^-, the concentration gradient was calculated for the depth interval with the greatest concentration gradient change, as was the corresponding porosity (Sahling *et al.*, 2002). Therefore, the sulfide flux represented the maximum of the ascendant flux observed locally in the sediment, rather than the flux across the sediment–water interface. Consequently, ammonia and sulfide fluxes could not be directly compared. D_s was corrected for tortuosity, that is, $D_s = \frac{D_0}{\theta^2}$, where θ is the tortuosity (dimensionless) and D_0 is the diffusion coefficient in water for PO_4^{3-}, NH_4^+ or HS^- (m^2 day^{-1}). The diffusion coefficient in water (D_0) is corrected for the *in situ* bottom water temperatures (Li & Gregory, 1974), and the value of θ is assumed to be equal to $\sqrt{1 - 2 \times \ln \phi}$ (Boudreau, 1996).

DNA extraction

DNA was extracted from 5 g of sediment according to the SDS-based lysis method of Zhou *et al.* (1996) and was suspended in 500 μL of sterile deionized water. Finally, the DNA was purified with the Wizard® DNA Clean-Up System (Promega, Madison, WI), according to the manufacturer's instructions. After quantitation by optical density, the DNA was diluted to 10 ng μL^{-1} for PCR amplification.

PCR amplification and DGGE analysis

As in most microbial analyses on intertidal sediment, bacteria were studied to gain an overall view of the microbial community. Archaea were not considered because these microorganisms are more specialized in specific functions such as methanogenesis and ammonium oxidation, parameters that were not taken into account in this study. The primers chosen are universal for the amplification of any bacterium, without selection of a particular group. The primers 341f-GC (5′-GC-clamp-CCTTACGGGAGGCAGCA-3′) and 518r (5′-ATTACCGCGGCTGCTGG-3′) (Muyzer *et al.*, 1993) were used to amplify a 230-bp fragment of the V3 region of the bacterial 16S rRNA gene. A 40-bp GC-clamp (CGCCCGCCGCGCGCGGCGGGCGGGGGCACGGGGGG) was attached to the 5′ end of the forward primer (Muyzer *et al.*, 1993). The reaction mixture contained 20 ng DNA, 1 μM of each primer, 2 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate, and 1 U AmpliTaq DNA polymerase (Invitrogen, life technologies) with 1 × its buffer in milliQ water, to give a final volume of 25 μL.

A PCR was carried out in a thermocycler (My CyclerTM thermal cycler; Biorad) using the touchdown program described in Murray *et al.* (1996), with slight modifications: initial denaturation at 94 °C for 3 min, followed by 10 cycles with denaturation at 94 °C for 30 s and touchdown annealing from 65 to 56 °C for 30 s. The annealing temperature was decreased 1 °C for each cycle and was then set at 55 °C for 20 cycles, with primer extension at 72 °C for 30 s. The final extension was run at 72 °C for 30 min to achieve fragment elongation according to Syvyk *et al.* (2008), prior to cooling at 4 °C.

PCR products were loaded onto 8% (w/v) polyacrylamide gels (acrylamide/bis-acrylamide 37.5 : 1; Bio-Rad laboratories, France) cast in 1 × TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0). These gels were made with a denaturing gradient ranging from 40% to 80% (100% denaturing mixture contained 7 M urea and 40% deionized formamide). DGGE was carried out with the D-code DGGE system (Bio-Rad laboratories). Electrophoresis was conducted in 1 × TAE buffer at a constant voltage of 80 V and a temperature of 60 °C for 18 h. After electrophoresis, gels were stained with SYBR Gold diluted at 1/10 000 for 30 min and rinsed with 0.5 × TAE for 15 min. The gels were read using a fluorimager (typhoon 9400; Amersham) with fluorescence at 540 nm, followed by image analysis.

Sequencing and phylogenetic analysis of DGGE bands

Interesting DGGE bands (17 in Aber Benoît and 18 in Rivière d'Auray) were chosen according to their intensity and presence/absence criteria and excised from the gels. These were reamplified using the usual primers (341f with GC-clamp and 518r). The bands were excised from a second gel and reamplified with 341f without the GC-clamp and 518r. The amplified products were then cloned into PCR II plasmids using a TA cloning kit and transformed into the *Escherichia coli* DH5α (Fisher Scientific SA, France) according to the manufacturer's instructions. Four positive clones per band were randomly chosen for sequencing. The sequencing was performed using the SP6 promoter primer and a 16-capillary ABI3130 XL sequencer. The sequences were then analyzed using Bioedit software and compared to the GenBank database using its online software and the Basic Local Alignment Search Tool algorithm (BLAST) (Altschul *et al.*, 1997). The sequences were deposited in Genbank, where they were assigned the numbers FN555177–FN555194 and FN555208–FN555229.

DGGE and statistical analysis

DGGE banding profiles from all sediment samples were analyzed using GEL COMPARII software (Applied Maths, Kortrijk, Belgium) to obtain a matrix consisting of the relative intensity and position of each DNA band. Although GelCompar normalization was performed using external ladders, gel effect was significant. To avoid misinterpretation owing to superimposition of gel and treatment effects, analyses were only performed on individual gels or between gels without significant gel effects. For each site, the 2007 profiles were analyzed separately from those of 2008, because the samples taken in these 2 years had been loaded onto separate gels. The DGGE data matrix was used to generate a triangular similarity matrix based on the Bray–Curtis coefficient, using PRIMER software (PRIMER-E, Plymouth, UK). Multidimensional scaling (MDS) (Kruskal & Wish, 1978), representing the similarity ranks of community structure between samples, was then derived from the matrices. The resulting MDS map shows every band pattern as a point, so relative changes among different DGGE patterns can be visualized and interpreted as the distances between the points. The more similar the DGGE banding patterns, the smaller the interpoint distance. The analysis of similarity (ANOSIM) routine was used to examine the statistical significance (significance data reported as *P*-values) of differences between the DGGE profiles from Oyster station samples and Reference station samples, and differences according to temporal variation.

The Spearman correlation coefficient and associated *P* significance level (obtained by a permutation test using 5000 permutations) were computed to quantitate the correlation between the rank similarity matrices obtained for environmental variables (using Euclidean distance) on the one hand and genetic structure (using Bray–Curtis dissimilarity index) on the other (Clarke & Ainsworth, 1993).

Results

Biogeochemical parameters of overlying water and sediment

The overlying water temperature in Aber Benoît varied between 14.7 and 18.1 °C over the spring, summer, and autumn (Table 2). In Rivière d'Auray, over the same seasons, it ranged from 14.7 to 21.8 °C (Table 3). In February and March 2008, it varied between 9 and 11 °C in both sites.

NH_4^+ and PO_4^{3-} concentrations in overlying waters at Aber Benoît were always lower than 3.5 and 1.5 μM, respectively, at both Oyster and Reference stations. NO_3^- concentrations varied from 29 μM (Oyster in July) to 66 μM (Reference station in June). In Rivière d'Auray, NH_4^+ and PO_4^{3-} concentrations never exceeded 6 and 1 μM, respectively, whereas NO_3^- concentrations varied

Table 2. Some biogeochemical characteristics of Aber Benoît sediment. Values of NO_3^-, PO_4^{3-} are means ± (SD) of calculated fluxes based on three replicates

		NO_3^- (μmol m^{-2} h^{-1})		PO_4^{3-}(μmol m^{-2} h^{-1})	
Sampling months	Temperature in °C	Reference station	Oyster station	Reference station	Oyster station
June 2007	16.8	− 39 (2)	− 30 (2)	0 (0)	34 (6)
July 2007	18.1	− 35 (0)	− 22 (4)	0.1 (0)	0.7 (0.1)
August 2007	16.6	− 28 (9)	− 25 (7)	1 (0)	2 (1)
September 2007	15.1	− 36 (2)	− 27 (2)	1.3 (0.3)	0.6 (0.1)
March 2008	11.1	− 60 (12)	− 52 (1)	1 (0)	1 (0)
May 2008	14.7	− 31 (4)	− 30 (8)	1 (1)	3 (2)
June 2008	15.5	− 43 (8)	− 36 (8)	2 (0)	45 (40)

Table 3. Some biogeochemical characteristics of the Rivière d'Auray sediment. Values of NO_3^-, PO_4^{3-} are means ± (SD) of calculated fluxes based on three replicates

		NO_3^- (μmol m^{-2} h^{-1})		PO_4^{3-} (μmol m^{-2} h^{-1})	
Sampling months	Temperature in °C	Reference station	Oyster station	Reference station	Oyster station
June 2007	20.6	− 2 (0)	− 1 (0)	2 (1)	9 (2)
July 2007	21.8	− 21 (36)	− 1 (1)	3.7 (1.6)	2.6 (0.1)
August 2007	19.6	2 (2)	0 (0)	2 (2)	12 (2)
September 2007	14.7	2 (2)	0 (0)	1.6 (0.6)	19.2 (20.4)
March 2008	10.8	− 74 (8)	− 77 (13)	1 (1)	1.8 (0.2)
May 2008	18.2	− 33 (4)	− 45 (7)	6 (4)	17 (15)
June 2008	18.3	− 5 (8)	− 3 (5)	2 (1)	10 (2)

from about 1 μM (July) to 70 μM (March) both at Oyster and Reference stations. Sulfide was never detected in the overlying waters at either site. Abnormally low dissolved oxygen concentrations (i.e. hypoxia) were never measured at either the Oyster or Reference stations; oxygen concentrations varied from 165 to 300 μM.

At both sites, according to the oxygen profiles, sediments were always anoxic below 2–3 mm depth and no significant differences between Oyster and Reference stations could be observed; Fig. 2 shows the example of May 2008. The other biogeochemical parameters varied according to site, season, and station (Tables 2 and 3, Figs 3–5).

In Aber Benoît, a minor enrichment in OC generally occurred beneath the oyster tables, making the level 1.2 times higher on average (949 μmol g^{-1}, 1.14% dry sediment) than at the Reference station, with a peak in May 2008 (2072 μmol g^{-1}, 2.49% dry sediment). During June 2007 and June 2008, peaks of NH_4^+ fluxes (six times higher than the other months) were recorded at the Oyster station. Ascendant sulfide fluxes (Fig. 5) significantly increased at the Oyster station in May, July, and August 2008 (up to 215 μmol m^{-2} h^{-1}), while they were always close to zero at the Reference station. Phosphate fluxes were weak and almost all values of nitrate flux were negative, indicating that strong redox occurred in the sediment. Positive correlations were found between temperature and nitrate flux ($R = 0.713$, $P < 0.05$) and between phosphate and ammonia fluxes ($R = 0.808$, $P < 0.05$).

In Rivière d'Auray, OC ranged from 959 to 2300 μmol g^{-1} (1.15–2.76% dry sediment), with an average of 2.07% dry sediment at Reference and 1.71% at the Oyster station. The OC values at Reference remained high in winter but were more variable at the Oyster station. The ammonia flux (Fig. 4) was half as much in Reference as Oyster. In contrast, the ascendant sulfide flux, concentrated in two peaks in September 2007 and May 2008, was five times higher in Reference than in Oyster.

Phosphate flux reached 2–19.2 μmol g^{-1} for Oyster, while the nitrate flux was negative, reflecting the anoxia of the sediment. OC and ammonia flux were only positively correlated in Rivière d'Auray underneath the oyster tables ($R = 0.939$ $P < 0.05$), and phosphate and ammonia fluxes showed the same pattern ($R = 0.639$, $P < 0.05$).

Bacterial counts

Total culturable bacteria (marine agar) and vibrio (TCBS) counts varied according to the season. Their numbers, expressed in log10 (Fig. 6a and b), were positively correlated with the temperature (R ranged from 0.641 to 0.877, $P < 0.05$), but not with OC or nutrient fluxes. Total culturable bacteria (TCB) reached only

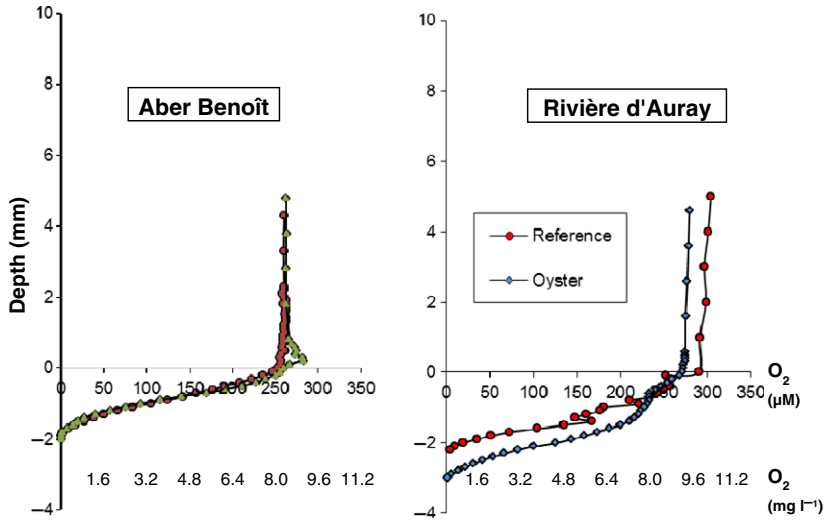

Fig. 2. Oxygen microprofiles as a function of depth (mm) at oyster and reference sites in Aber Benoît and Rivière d'Auray (May 2008). The *X* axis, represents the sediment water interface. O_2 is expressed both in mg L^{-1} and µM.

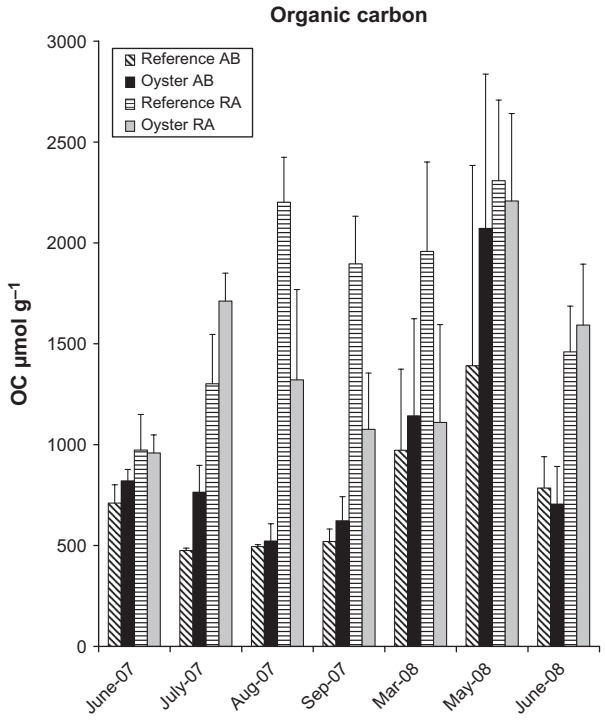

Fig. 3. OC values in Aber Benoît and Rivière d'Auray. Values are means (± SD) based on three replicates.

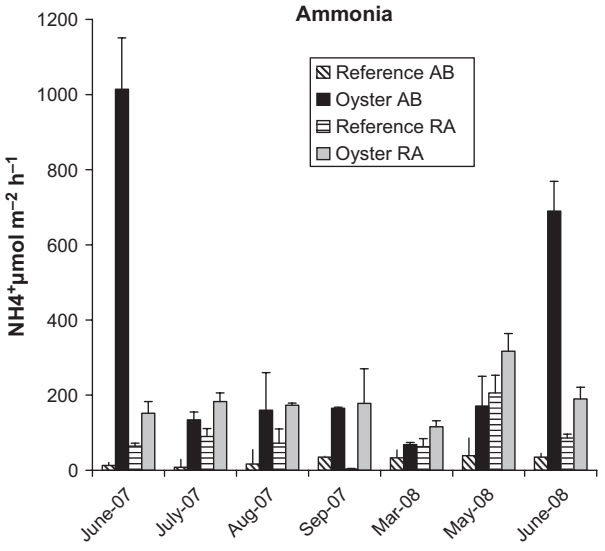

Fig. 4. Histogram of NH_4^+ values in Aber Benoît and Rivière d'Auray. Values are means (± SD) of calculated fluxes based on three replicates.

3.10^6 CFU g^{-1} in Aber Benoît but reached up to 10^8 CFU g^{-1} in Rivière d'Auray. However, the vibrios/THB ratio was seven times higher on average in Aber Benoit than in Rivière d'Auray. The TCB and vibrio concentrations in Oyster and Reference stations for both sites were of the same order, but significantly different in June, July, and August 2007.

DGGE profiles and phylogenetic analysis

Amplification and DGGE analysis of the 16S rRNA gene fragments from Rivière d'Auray and Aber Benoît showed different banding patterns (Fig. 7), but appeared to be

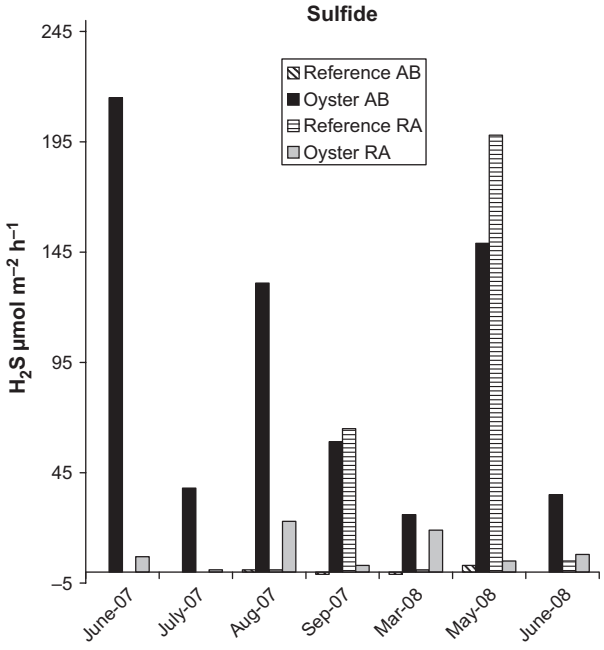

Fig. 5. Histogram of sulfide values in Aber Benoît and Rivière d'Auray. Values are the calculated fluxes from one sediment sample.

little different between replicates, stations, or sampling dates within a single site.

In Aber Benoît, the total number of bands detected was 24.1 (± 2.9) for Oyster, with the lowest number in February and the highest in September. For Aber Benoît Reference, the total number of detected bands was 23.5 (± 2.6), with the lowest number in June 2008 and the highest in September 2007.

In Rivière d'Auray, the number of bands ranged from 15 to 24 for Oyster and from 20 to 28 for Reference. The lowest number of bands occurred in September at both sampling stations, and the highest numbers were observed in 2008 during February and March for Oyster, and February and June for Reference.

In Aber Benoît, no band was found to be station specific, and each band was detected at least once. The same pattern occurred in Rivière d'Auray except in February 2008, where three bands (27, 30, 34) were specific to Oyster and two (40, 42) to Reference.

At each site, four bands (no. 1, 4, 9, and 15 in Aber Benoît and 20, 21, 25, and 33 in Rivière d'Auray) were frequently present at both stations throughout the survey, although these varied widely in strength. The DNA was

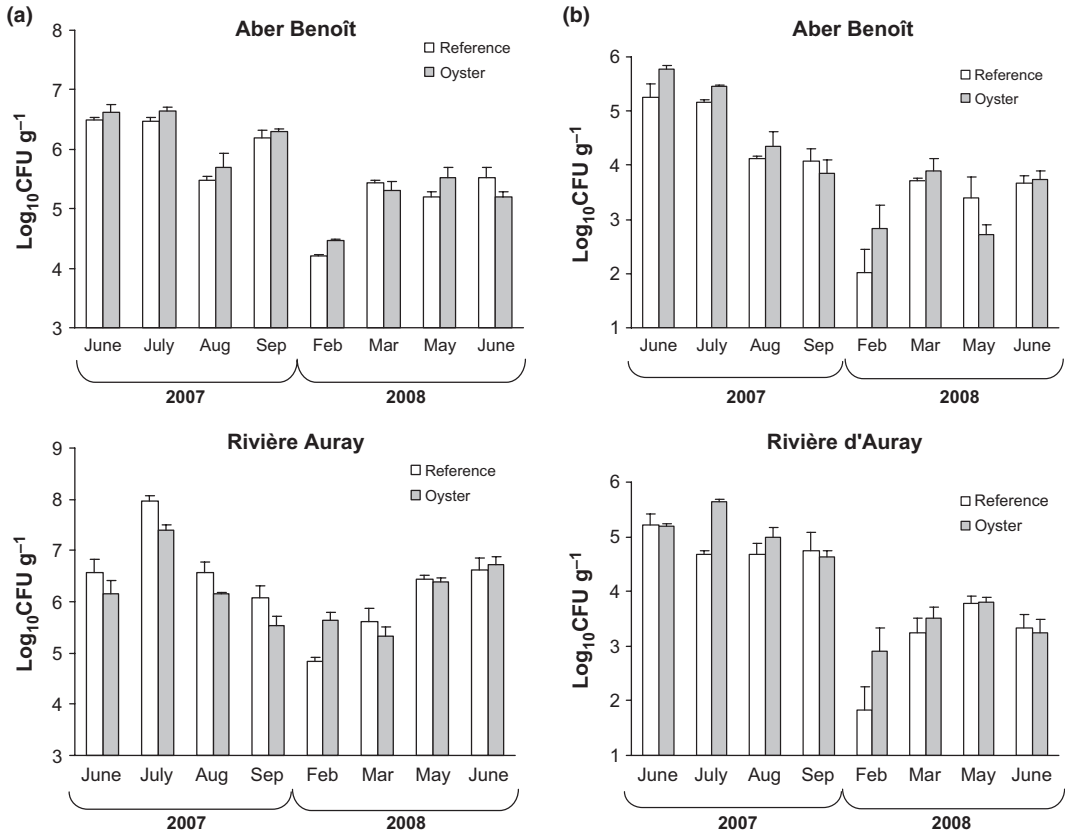

Fig. 6. Culturable bacterial counts in Aber Benoît and Rivière d'Auray: (a) Total culturable bacteria (Marine Agar) and (b) vibrios (TCBS), according to dates.

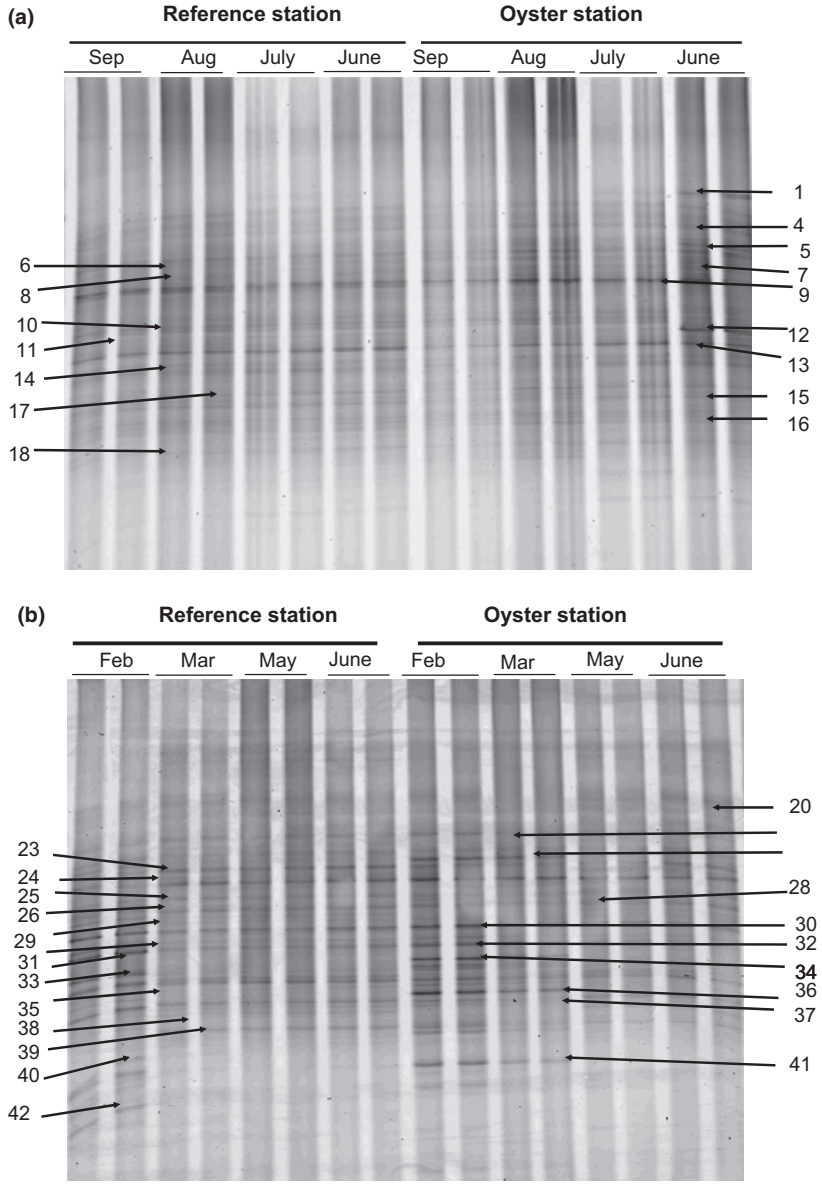

Fig. 7. DGGE profiles of sediments sampled (a) in Aber Benoît in 2007 and (b) in Rivière d'Auray in 2008. The sequenced bands are numbered.

successfully sequenced from 18 bands for Rivière d'Auray and 17 for Aber Benoît. Most bands corresponded to a unique sequence (Tables 4 and 5), except some that contained several different sequences. These sequences were affiliated with seven major phylogenetic groups including *Acidobacter*, *Bacilli*, *Bacteroidetes*, *Verrucomicrobia*, α-, δ-, γ-*Proteobacteria*, and one unclassified environmental strain. The members of γ-*Proteobacteria* were the most numerous, with many uncultured bacteria and some vibrios, such as *Vibrio aestuarianus*, *Vibrio anguillarum*, or *Vibrio ordalii*. Two sequences corresponded to the functional group of sulfate-reducing bacteria (bands 1 and 28).

Bacterial community structure

First, replicates from the same sampling station were compared to check for similarity. In AB, the MDS analyses of the 2007 profiles (Fig. 8) showed that the replicates of both stations for the same date were clustered together or were in close relation to one another. This implied that there was a variation between successive months but that oyster tables only had a significant influence in June, as assessed by the ANOSIM analyses.

In June and July 2007 in Rivière d'Auray, the three replicates from Oyster and three from Reference grouped separately (Fig. 9), but these became mixed in

Table 4. Sequence analysis of bands excised from DGGE gels of Aber Benoît sediment. Letters correspond to clones of the same band

Band no.	Phylum or class (family)	Accession number	Most related species in Genbank	Homology (%)
1	*Deltaproteobacteria* (*Desulfobacteriaceae*)	FN555177	Sulphate-reducing bacterium JHA1	96
4a	*Gammaproteobacteria*	FN555178	Uncultured bacterium	96
5	*Acidobacteria*	FN555179	Uncultured bacterium clone C08P3MbH	94
6	*Gammaproteobacteria*	FN555180	Uncultured bacterium clone AV19F59b	98
7	*Flavobacteria*	FN555181	*Winogradskyella* sp. K7-7	98
8	*Flavobacteria*	FN555182	Uncultured bacterium clone Mn3b-B11	98
9	*Gammaproteobacteria*	FN555183	Uncultured bacterium clone AN05aug-063	98
10	*Gammaproteobacteria*	FN555184	Uncultured bacterium clone NY06dec-099	97
11-a	*Verrucomicrobia*	FN555185	Uncultured *verrucomicrobium* bacterium clone LD1-PB2	95
11-bc	*Deltaproteobacteria* (*Desulfobacteraceae*)	FN555186	Uncultured *Desulfobacterium* sp clone S57	98
12-acd	*Gammaproteobacteria* (*Vibrionaceae*)	FN555188	*Vibrio aestuarianus* 01/064	99
12-b	*Gammaproteobacteria*	FN555187	Uncultured *gammaproteobacterium* clone B05_RAMPDAY15	97
13	*Alphaproteobacteria*; (*Rhodobacteraceae*)	FN555189	*Roseovarius aestuarii*	97
14	*Gammaproteobacteria*	FN555190	Uncultured bacterium Ld1-1	98
15	*Deltaproteobacteria*	FN555191	Uncultured *deltaproteobacterium* clone YS-UMF5_122	98
16	*Alphaproteobacteria*	FN555192	Uncultured *alphaproteobacterium* clone CL33-G06	98
17	*Alphaproteobacteria* (*Rhodospirillaceae*)	FN555193	Uncultured bacterium clone C15cm.A08	97
18	*Verrucomicrobia*	FN555194	Uncultured bacterium clone L2-B01	98

Table 5. Sequence analysis of bands excised from DGGE gels of Rivière d'Auray sediment. Letters correspond to clones

Band no.	Phylum or class (family)	Accession number	Most related species in Genbank	Homology (%)
22	*Gammaproteobacteria* (*Vibrionaceae*)	FN555208	*Vibrio anguillarum*	98
			Vibrio ordalii	98
23	*Gammaproteobacteria*	FN555209	Uncultured bacterium isolate JH12_C45	98
24	*Gammaproteobacteria*	FN555210	Uncultured bacterium clone NY04sep-017	97
27	*Gammaproteobacteria*	FN555211	Uncultured bacterium clone OS02-TRNA-74 16S	98
28	*Deltaproteobacteria*	FN555212	*Desulfobacterium* sp AN05aug-096	97
29-ab	*Gammaproteobacteria* (*Vibrionaceae*)	FN555213	*Vibrio aestuarianus* strain 01/031	99
29-c	*Alphaproteobacteria*	FN555214	Uncultured *Rhodobacteraceae* bacterium clone Q8-C10	98
30	*Gammaproteobacteria*	FN555215	Uncultured *gammaproteobacterium* clone SIMO-4102	96
31	*Flavobacteria*	FN555216	Unculture *Bacteroidetes* bacterium clone CL18-D02	92
33	*Gammaproteobacteria*	FN555217	*Microbulbifer thermotolerans*	97
34	*Bacilli* (*Bacillaceae*)	FN555218	*Bacillus* sp. GN-M06-10	97
35	*Deltaproteobacteria*	FN555219	Uncultured *Deltaproteobacterium* clone YS-UMF5_80	98
36-a	*Bacilli* (*Paenibacillaceae*)	FN555220	*Brevibacillus laterosporus* strain 42S9	96
36-b	*Bacilli* (*Bacillaceae*)	FN555221	*Pontibacillus sp. R527*	96
37	*Gammaproteobacteria*	FN555222	*Microbulbifer thermotolerans*	96
38	*Gammaproteobacteria*	FN555223	*Marinobacter taiwanensis* strain GI-1701	98
39	*Bacilli* (*Bacillaceae*)	FN555224	Uncultured bacterium clone MFC-10	98
40	*Bacilli* (*Bacillaceae*)	FN555225	Bacterium enrichment culture clone heteroA8_4W	96
41-ab	*Acidobacteria*	FN555226	Uncultured *Acidobacteriaceae* bacterium clone NdGal159	97
41-c	*Unclassified bacterium*	FN555227	Uncultured bacterium clone G2-01	93
41-d	*Gammaproteobacteria*	FN555228	*Marinobacter sp.*	98
42	*Bacilli* (*Bacillaceae*)	FN555229	Bacterium enrichment culture clone 080213-ABM-Febex	96

the following months. There were, however, some extreme points at Reference in July and August 2007. The ANOSIM analysis revealed variation between the consecutive months of June and July for both stations, August and September, for Oyster and also revealed a significant effect of oyster bags on bacterial community structure for all sampling dates except July.

In 2008, all replicates from Aber Benoît were dispersed and only influenced by date between February and March at Reference, and by oyster bag in February.

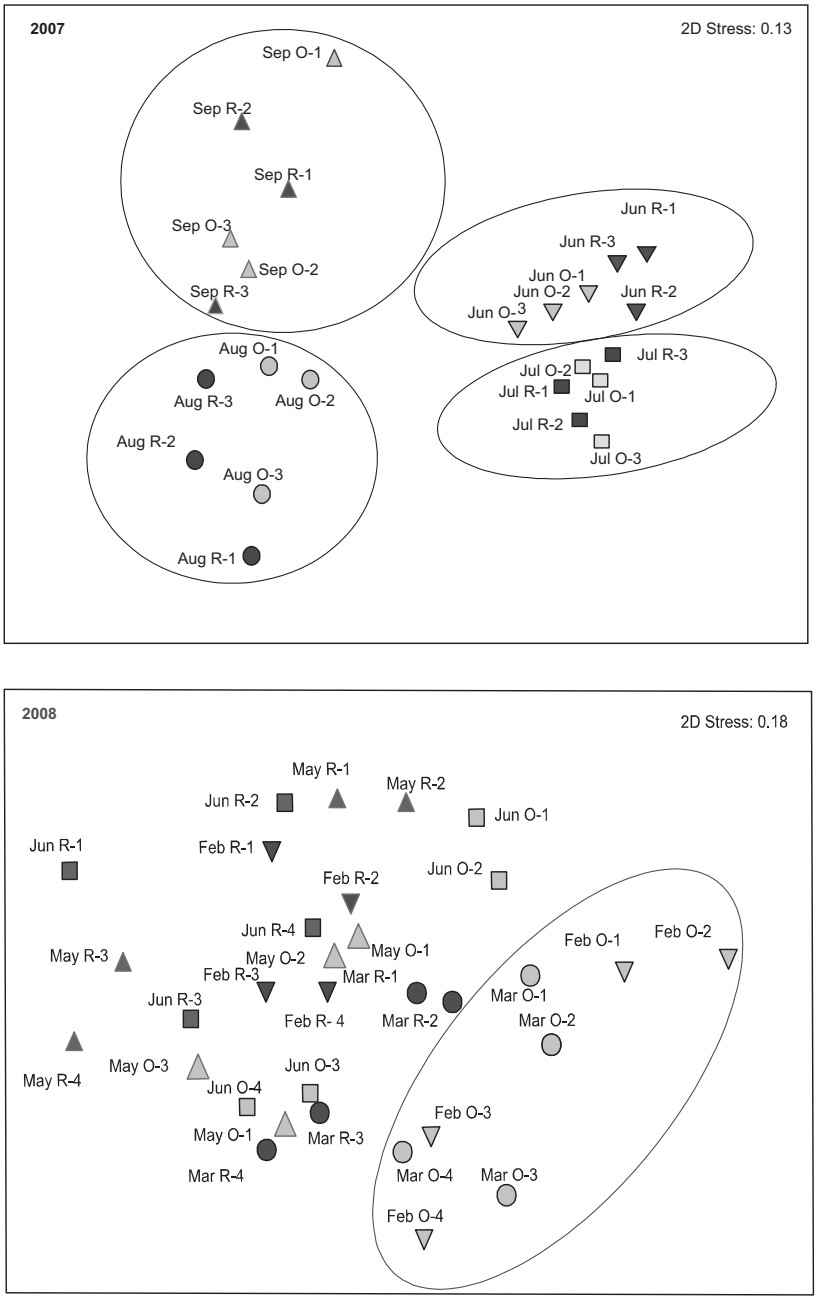

Fig. 8. MDS maps derived from DGGE band profiles of all 24 samples taken in 2007 and all 32 samples taken in 2008, showing spatial and temporal variations in bacterial diversity in the sediment of Aber Benoît. The ANOSIM were performed to test for significant differences between dates and between sites with oysters (O) or without (R). Influence of oyster tables in June 2007 (R = 0.956, 10%) and February 2008 (R = 0.656, 2.9%). Variation between successive months between June and July 2007 (Reference: R = 0.926, 10%; Oyster: R = 1, 10%) and between February and March 2008 (Oyster: R = 0.771, 2.9%).

Most of the profiles from Rivière d'Auray were grouped together, except those from Reference in February and March and those from Oyster in March, which formed separate clusters. This grouping indicates an effect of date and oyster bag on the bacterial community structure at the beginning of the year only, confirmed by ANOSIM analyses, which was not observed later on.

Using Spearman correlation rank analysis, temperature showed a significant relation with the bacterial profiles of Aber Benoît in June–July 2007, in August–September

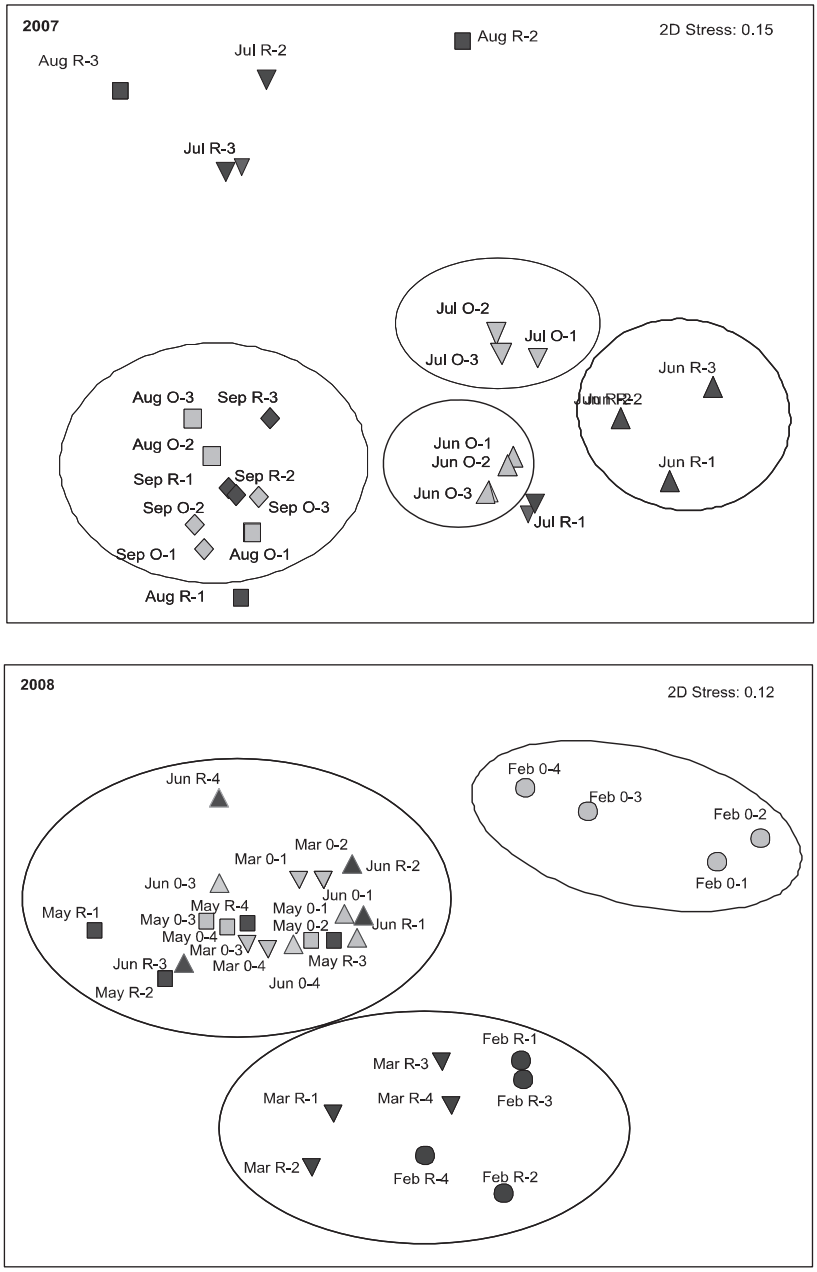

Fig. 9. MDS maps derived from DGGE band profiles of all 24 samples taken in 2007 and all 32 samples taken in 2008, showing spatial and temporal variations of bacterial diversity in the sediment of Rivière d'Auray. The ANOSIM were performed to test for significant differences between dates and between sites with oysters (O) or without (R). Influence of oyster tables in June 2007 (R = 0.815, 10%), August 2007 (R = 0.593, 10%), September 2007 (R = 1, 10%), February 2008 (R = 1, 10%) and March 2008 (R = 1, 0.1%). Variation between successive months between June and July (Oyster: R = 1, 10%; Reference: R = 0.519, 10%) and between August and September (Oyster: R = 0.667, 2.9%).

2007, and in 2008. In Rivière d'Auray, however, temperature was only significantly related to the profiles in June–July (Table 6). The nitrate fluxes were also significantly related to the bacterial community structure in June–July in Aber Benoît, and in August–September in Rivière d'Auray. Phosphate flux also had a significant correlation with community structure in August–September.

Discussion

Some previous studies have demonstrated that oysters on tables induce a long-term accumulation of OM in the sediment beneath them (Mazouni *et al.*, 1996), because of feces and pseudofeces deposits they produced. In contrast, other studies have shown no measurable impact of oyster

Table 6. Statistical significance of bacterial structure changes as a function of different parameters. Spearman correlation was used to test for the correlation between bacterial structures and environmental parameters. Values in brackets are Rho and *P*

Parameter	Aber Benoît	Rivière d'Auray
Temperature	June–July 2007 (0.325, 0.018) August–September 2007 (0.413, 0.03) All months in 2008 (0.139, 0.06)	June–July 2007 (0.398, 0.01)
Nitrate flux	June–July (0.198, 0.083)	June–July (0.412, 0.032) August–September (0.307, 0.066)
Phosphate flux		August–September (0.213, 0.046).

cultures on benthic sediment biogeochemistry, because of a highly dynamic environment and low oyster production intensity (Crawford *et al.*, 2003; Mallet *et al.*, 2006). In the present case, the impact of oysters on OM was only seasonal. In Aber Benoît, OM accumulated beneath the oyster tables from March to May, but decreased quickly in the May–June period. For Rivière d'Auray, two peaks of OC were measured underneath the oyster tables in May and in July, but they decreased progressively in the following months. The sediment of the Rivière d'Auray Reference station contained 1.2 times more OC and, therefore, appeared atypical; this was probably due to the accumulation and degradation of stranded algae (macrophytes) regularly observed there.

In Aber Benoît, fluxes of ammonia at the sediment–water interface and maxima of the ascendant sulfide fluxes in the sediment resulting from OM mineralization were much higher on average at the Oyster station than at the Reference station, 13- and 653-fold, respectively. This suggests an efficient and rapid degradation of OM underneath oyster tables. For Rivière d'Auray, the fluxes of the Oyster sediment were better distributed through the year, with a lower mean level (1.8 times less for ammonia and 10 times less for sulfide) than at Aber Benoît. The difference between Oyster and Reference stations at Rivière d'Auray was also less marked for the ammonia flux (only 2.2 times higher in average in Oyster) than for the sulfide flux (four times lower). These fluxes did not induce a high ammonia concentration in the overlying seawater, which never reached a level toxic to oysters. Neither sulfide nor anoxia was detected, despite low oxygen penetration depths into the sediment. This means that the high ascendant sulfide flux did not result in a high efflux from the sediment and highlights the differing behavior of ammonia and sulfide fluxes. Most of the ascendant sulfide flux would have been intercepted in the superficial sediment through precipitation with Fe^{2+} (from FeS) or oxidation by aerobic chemotrophic sulfide oxidizers or phototrophic sulfide oxidizers. Unfortunately, DGGE band sequencing did not reveal clear specialist chemotrophic or phototrophic sulfide oxidizers, although some Rhodobacter strains possess capacities to oxidize sulfide. Therefore, the effects of the changes in sediment biogeochemistry on oyster health, previously reported in the Morest project (Samain & McCombie, 2008), could not have been caused by ammonia and sulfide fluxes such as these, unless considerable quantities of these nutrients are suddenly released by a sediment resuspension event.

The intensity of mineralization reflected by these fluxes may depend on the nature of newly deposited OM, which is probably mainly feces and pseudofeces from oysters (Asami *et al.*, 2005), although no analysis was made in the present study to confirm this point. OM appeared more easily degradable in Aber Benoît than in Rivière d'Auray, even though the mean temperature was 2 °C lower. Otherwise, the transport of feces and pseudofeces from the Oyster station to the Reference station appeared limited in Aber Benoît, because biomineralization remained weak at Reference despite the small distance between the stations. The current generated by high tides may disperse the feces and pseudofeces from oysters across large area and thus dilute them.

Abundance of TCB and vibrios was not greatly influenced by OM or correlated with the ammonia and sulfide fluxes, which may reflect the activity of these microorganisms. At the Oyster station of Aber Benoît in May, when the peak of OC appeared, TCB reached only 3.4×10^5 cell g^{-1}. The OC decreased to 705 μmol g^{-1} in the following month, while TCB continued to increase up to 1.4×10^6 cell g^{-1}. Thus, the relationship between OM and culturable bacteria seems more complex than that described by Vezzulli *et al.* (2002), Vezzulli & Fabiano (2006), and Richardson *et al.* (2008). In these previous studies, abundance of TCB in the sediment increased with the same magnitude as OC content. It is possible that the most active bacteria were not culturable and/or that their activity was not directly related to their number. The relationship between abundance and bacterial activity in sediment has not yet been investigated in any depth. Overall, the main factor explaining the fluctuation of bacterial concentrations was temperature.

There was, however, a temperature difference of just 1.3 °C less in June 2008 than in June 2007 in AB, which cannot completely explain why the bacterial concentrations were lower in June 2008. In addition, these lower bacterial concentrations coincided with a metabolic slowdown of bacterial activity, revealed by the weaker fluxes of ammonia and sulfide. A large dispersion of bacterial

profiles also occurred in 2008, as shown by the MDS analyses, while in 2007 they were grouped by sampling date. The nature of newly deposited OM and its distribution within stations could induce a decrease in growth of culturable bacteria compared with June 2007, and a greater diversity of bacterial populations.

At Rivière d'Auray, the bacterial concentrations (TCB) reached similar levels in June 2008 as in June 2007, although the temperature was 2.3 °C less in 2008. The bacterial profiles from the two stations became similar from May onwards, whereas the replicates clustered separately the year before. This difference coincided with a higher amount of OM in the sediment, which was apparently degraded slowly. It could be the nature and distribution of newly deposited OM that stimulates the bacterial growth and homogeneity of profiles between stations in this case. Nevertheless, factors modifying the bacterial community structure can remain unknown (Boer *et al.*, 2009).

The fact that vibrios were more abundant in AB (1% TCB in average) than in RA (0.16%) may be due to a better degradability of OM at AB, because these bacteria are particularly organotrophic. Among these vibrios, *V. aestuarianus*, which is pathogenic to *C. gigas*, was frequently detected; however, it is difficult to estimate the true risk it posed to the oysters in terms of weakening effects or as a direct cause of mortality. To examine these issues, it would be necessary to prove that the *V. aestuarianus* strains that infected the oysters came from the sediment. It has been shown that some strains collected in a previous study (Azandegbe *et al.*, 2010) and later identified (results not shown) were slightly different from the virulent strains isolated in oysters.

The bacterial community structure of Aber Benoît sediment is significantly affected by the temperature. The predominant influence of this factor on bacterial communities agrees with previous studies (Gonzalez-Acosta *et al.*, 2006; Tabuchi *et al.*, 2010). Its impact on the bacterial community structure was less noticeable in Rivière d'Auray, where there was only a significant effect in June and July 2007. Inversely, the effect of oysters was more often significant in Rivière d'Auray than in Aber Benoît, with five of seven dates showing a significant interstation difference rather than only two. The correlations with the other biogeochemical parameters, including ammonia, phosphate, and nitrate, were very scattered.

The majority of the DGGE bands were affiliated to the γ-*Proteobacteria*. Several studies have already shown the importance of this bacterial class in marine sediments. δ-*Proteobacteria*, which is generally in second position, was less represented in the present study (Gray & Herwig, 1996; Urakawa *et al.*, 1999; Bowman & McCuaig, 2003; Bowman *et al.*, 2003; Bissett *et al.*, 2006).

Interestingly, the second bacterial class in Rivière d'Auray was the *Bacilli*. This group is generally scarce in aquatic environments except in polluted areas, notably those contaminated by hydrocarbons (Cavallo *et al.*, 1999; Chikere *et al.*, 2009). In addition, one strain of *Bacillus* found (band no. 34) is closely related to a *Bacillus* sp. of terrestrial origin and may, therefore, be an indicator of river input. Two strains that are members of the *Desulfobacterium* genus, found at the Oyster station of Aber Benoît (band no. 1) and Rivière d'Auray (band no. 28), were only ones that could be attributed to a specific metabolism.

Phylogenetic groups were similar between the two sites, except for *Bacilli* (RA) and *Verrucomicrobia* (AB). In contrast, the phylotypes within each group were different, except for *V. aestuarianus*. Finally, the sediment of each site harbored its own bacterial community regardless of the influence of oyster culture. In a recent study, van der Zaan *et al.* (2010) reported a degree of stability in terms of abundance and functional groups in sediment bacterial flora exposed to pollutants. Consequently, as suggested by Bissett *et al.* (2006), the sediment may have the ability to maintain diverse communities despite the disruption caused by changes in OM input.

In conclusion, the oyster tables in Aber Benoît were probably the source of the greater quantity of OM measured underneath them. This OM was more rapidly degraded than in Rivière d'Auray, despite the temperature being 2 °C lower on average. The substantial ammonia and sulfide fluxes, which may result from this degradation, were insufficient to induce seawater toxicity, except maybe in the event of sediment resuspension. These flux variations did not coincide with any change in bacterial community structure that were little influenced by the temperature and the input of OM. The identified bacteria belonged to bacterial groups that are common in sediment. Among the vibrios, *V. aestuarianus* could represent a threat for oysters as it is pathogenic to them.

One reason why the bacterial community structures were found to be so static is perhaps because the methods were not sensitive enough to reveal metabolically active bacteria.

In the future study, the metabolically active bacteria would probably be easier to detect whether bacterial profiles could be determined from direct RNA extraction including rRNA (Nimnoi *et al.*, 2011), rather from template DNA. In addition, future work would need to focus on particular functional groups using specific primers and real-time PCR to monitor their abundances (van der Zaan *et al.*, 2010).

To confirm that the ammonia and sulfide do not reach the threshold of toxicity, they could be monitored with specific electrodes, as used by Borum *et al.* (2005) and

Berner (1963). The physiological status of oysters could be regularly examined by measuring the expression of some genes or enzymes involved in stress responses, as already performed in some previous studies (Le Moullac *et al.*, 2007; Kawabe & Yokoyama, 2012).

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