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# Benzo(a)pyrene inhibits the role of the bioturbator *Tubifex tubifex* in river sediment biogeochemistry

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## HIGHLIGHTS

- ▶ Influences of a PAH on microbial activities and worm bioturbation in river sediments
- ▶ Low direct impact of benzo(a)pyrene on micro-organisms
- ▶ Benzo(a)pyrene inhibited the influence of worms on microbial processes.
- ▶ Ecosystem services can be affected by sublethal concentrations of PAHs.

## ABSTRACT

The interactions between invertebrates and micro-organisms living in streambed sediments often play key roles in the regulation of nutrient and organic matter fluxes in aquatic ecosystems. However, benthic sediments also constitute a privileged compartment for the accumulation of persistent organic pollutants such as PAHs or PCBs that may affect the diversity, abundance and activity of benthic organisms. The objective of this study was to quantify the impact of sediment contamination with the PAH benzo(a)pyrene on the interaction between micro-organisms and the tubificid worm, *Tubifex tubifex*, which has been recognized as a major bioturbator in freshwater sediments. Sedimentary microcosms (slow filtration columns) contaminated or not with benzo(a)pyrene (3 tested concentrations: 0, 1 and 5 mg kg<sup>-1</sup>) at the sediment surface were incubated under laboratory conditions in the presence (100 individuals) or absence of *T. tubifex*. Although the surface sediment contaminations with 1 mg kg<sup>-1</sup> and 5 mg kg<sup>-1</sup> of benzo(a)pyrene did not affect tubificid worm survival, these contaminations significantly influenced the role played by *T. tubifex* in biogeochemical processes. Indeed, tubificid worms stimulated aerobic respiration, denitrification, dehydrogenase and hydrolytic activities of micro-organisms in uncontaminated sediments whereas such effects were inhibited in sediments polluted with benzo(a)pyrene. This inhibition was due to contaminant-induced changes in bioturbation (and especially bio-irrigation) activities of worms and their resulting effects on microbial processes. This study reveals the importance of sublethal concentrations of a contaminant on ecological processes in river sediments through affecting bioturbator-microbe interactions. Since they affect microbial processes involved in water purification processes, such impacts of sublethal concentrations of pollutants should be more often considered in ecosystem health assessment.

### Keywords:

Polluted sediments  
Benzo(a)pyrene  
Bioturbation  
Microbial activities  
Biogeochemical processes  
Slow infiltration columns

## 1. Introduction

Water-sediment interfaces are dynamic zones that regulate the fluxes of organic matter, nutrients, and contaminants in marine and freshwater ecosystems (Palmer et al., 1997; Covich et al., 2004). Ecological processes occurring at these interfaces are driven by interactions between the abiotic characteristics of the sedimentary habitat

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(e.g., organic matter quantity, sediment permeability) and the activities of resident organisms (Godbold et al., 2011; Mermillod-Blondin, 2011). Biogeochemical processes are principally mediated by micro-organisms but trophic and ecosystem engineering activities of macro-organisms often play a very significant role on these microbial processes (e.g. Aller, 1994; Traunspurger et al., 1997). For instance, bioturbation by U-shaped tube burrower and gallery-diffuser invertebrates may increase microbial respiration at the water–sediment interface of marine and lake systems by up to 250% (Svensson and Leonardson, 1996; Kristensen, 2000; Karlson et al., 2005). Although bioturbation activities can significantly enhance the degradation of organic matter and nutrient cycling in benthic systems, the ability of bioturbators to significantly impact these processes is largely mediated by environmental factors such as temperature (Przeslawski et al., 2009), organic matter quality and quantity (Nogaro et al., 2009; Michaud et al., 2010), hydrodynamics (Biles et al., 2003; Mermillod-Blondin, 2011; Navel et al., 2012) or the presence of pollutants (Lagauzère et al., 2009a).

Previous studies focusing on the interactions between contaminants and bioturbation processes aimed at quantifying not only the role of bioturbation on the fate of pollutants (Gilbert et al., 1996; Kure and Forbes, 1997; Grossi et al., 2002; Banta and Andersen, 2003; Granberg et al., 2008; Timmermann et al., 2011) but also the impacts of sublethal concentrations of pollutants on bioturbation activity (Gilbert et al., 1994; Madsen et al., 1997; Landrum et al., 2004; Ciutat et al., 2005; Lagauzère et al., 2009b). Some of these works demonstrated a negative influence of contaminants on sediment reworking and burrowing of benthic invertebrates. For example, Mulsow et al. (2002) showed that the burial rate induced by the polychaete *Heteromastus filiformis* was reduced by more than 50% in sediment contaminated by dichlorodiphenyltrichloroethane (DDT, concentration: 10 mg kg<sup>-1</sup> of sediment) compared to uncontaminated sediment. Landrum et al. (2004) also measured a significant decrease in sediment reworking activity of the oligochaete *Lumbriculus variegatus* due to sediment contamination with polychlorinated biphenyl (PCB). However, the impacts of pollutants on bioturbation activities were only studied in benthic habitats of marine and lake ecosystems. Although benthic sediments of streams and rivers are recognized as major sinks for organic and metallic pollutants (e.g., Reynoldson, 1987), the impacts of contaminants on bioturbation and associated ecological processes in such ecosystems (e.g. organic matter processing, denitrification, Mermillod-Blondin et al., 2002; Marshall and Hall, 2004) remain understudied.

Thus, the present study aimed at quantifying the impact of a streambed contamination not only on bioturbation activity but also on micro-organisms (bacterial abundance, community structure and microbial activities) and biogeochemical processes (aerobic respiration, denitrification) occurring in freshwater sediments. We employed a factorial experimental approach in which the presence of the freshwater tubificid worm *Tubifex tubifex* and sediment contamination were manipulated to address how these features may interact to determine interface functioning. The influences of *T. tubifex* on sediment reworking and microbial processes were studied across a gradient of sediment contamination. Specifically, three concentrations of benzo(a)pyrene (0, 1, and 5 mg kg<sup>-1</sup> of dry sediment) were used to assess the influence of surface sediment contamination by polycyclic aromatic hydrocarbons (PAHs). In our experiment, the tested concentrations of benzo(a)pyrene did not exceed 5 mg per kg in order to simulate realistic environmental conditions. We thus did not expect lethal effects of this contaminant on the survival of *T. tubifex* which is commonly encountered in PAH-contaminated sediments (Lafont and Vivier, 2006; Datry et al., 2003b; Nogaro et al., 2007). We rather predicted a negative relationship between the benzo(a)pyrene concentrations in sediments and the bioturbation activities of worms. Consequently, the stimulatory influence of *T. tubifex* on biogeochemical processes (e.g. aerobic respiration, denitrification) and micro-organisms (bacterial abundances, dehydrogenase and hydrolytic activities, community structure) would be significantly

reduced in sediment contaminated with benzo(a)pyrene compared to uncontaminated sediment.

## 2. Material & methods

### 2.1. Experimental design

To address how sediment contamination by benzo(a)pyrene modulates the effects of a bioturbator at the water–sediment interface of rivers, a factorial design was employed to manipulate the occurrence of *T. tubifex* (0 or 100 individuals) and the contamination of surface sediments with benzo(a)pyrene (3 tested concentrations) in microcosms. In addition to its high toxicity in the environment, benzo(a)pyrene has been selected because of its (with other PAHs) widespread occurrence in aquatic habitats and of its high concentration in urbanized areas where freshwater systems act as collectors of storm waters (Pitt et al., 1999; Datry et al., 2003a; Grapentine et al., 2004; Nogaro et al., 2007). For example, Datry et al. (2003a) reported benzo(a)pyrene concentrations of 5.6 mg kg<sup>-1</sup> of dry sediment collected in a stormwater infiltration basin. The worm *T. tubifex* was selected because it exhibits large populations in most freshwater habitats (Brinkhurst and Kennedy, 1965), it is strongly tolerant to pollutants (Lafont and Vivier, 2006) and it is recognized as a key bioturbator in freshwater sediments (McCall and Fisher, 1980). Indeed, *T. tubifex* is a common deposit feeder that produces fecal pellets at the water–sediment interface, creates biogenic structures and affects O<sub>2</sub> and nutrient concentrations in sediments (e.g. Nogaro and Mermillod-Blondin, 2009; Lagauzère et al., 2009b).

Surface sediments were contaminated by spiking fresh sand with benzo(a)pyrene (>99% dissolved in acetone, HPLC-grade, Sigma-Aldrich Co., France). Unpolluted fresh sand (benzo(a)pyrene concentrations < 5 µg kg<sup>-1</sup>, sediment grain size distribution: 8%, 23%, 43% and 26% of particles ranging between 10–150 µm, 150–300 µm, 300–500 µm and 500–800 µm, respectively) was collected in a braided channel of the Rhône river and elutriated to eliminate coarse particulate organic matter. Eighty grams of fresh sand were spiked with 0.14 mg or 0.70 mg of benzo(a)pyrene dissolved in acetone and vigorously mixed with a stainless-steel spoon during 5 min (Doick et al., 2003). For the uncontaminated treatment, fresh sand was only spiked with acetone. The same volume of acetone (1 mL) has been introduced into the sand used for the 3 benzo(a)pyrene treatments to prevent the co-variation of acetone and benzo(a)pyrene concentrations on results. After spiking, acetone was allowed to evaporate during 10 h under an extractor hood at room temperature (around 22 °C) and spiked sand was mixed with 80 g of fresh sand to minimize the influence of spiking on indigenous microbial populations (Brinch et al., 2002). Final benzo(a)pyrene concentrations obtained with this procedure were 0.003 ± 0.005, 1.08 ± 0.09 and 5.08 ± 0.23 mg kg<sup>-1</sup> of dry sand (measurements performed following the methodology described in Section 2.4.2.) for nominal concentrations of 0, 1 and 5 mg kg<sup>-1</sup> of dry sand, respectively. Sand spiking with benzo(a)pyrene and/or acetone did not influence the C, N or P contents of surface sediments at the start of the experiment. Analyses indicated that the amounts of total organic C, total N and P per dry mass of sand (means ± SD, n = 9, 3 replicates per benzo(a)pyrene treatment) were 900 ± 30 mg kg<sup>-1</sup>, 77 ± 6 mg kg<sup>-1</sup> and 3.8 ± 1.5 mg kg<sup>-1</sup>, respectively.

Experiments were carried out in slow filtration columns (height = 25 cm and inside diameter = 10 cm, Mermillod-Blondin et al., 2005) at constant temperature (15 ± 0.5 °C) under a 12:12 h light:dark cycle. Each Plexiglas® column (n = 18) was filled by a 13 cm-thick layer of fresh sand (same sand as those spiked with benzo(a)pyrene). This sandy matrix was topped by an additive 1 cm-thick layer of sand contaminated or not with benzo(a)pyrene (0, 1 and 5 mg kg<sup>-1</sup> of dry sand, see above). Six columns were set-up for each benzo(a)pyrene treatment. Our experimental design simulated a surface contamination of the sediment, a phenomenon classically observed in systems

impacted by urban storm waters (Winiarski et al., 2006). After sediment filling, all columns were provided by the top with chemically-controlled water ( $96 \text{ mg L}^{-1} \text{ NaHCO}_3$ ,  $39.4 \text{ mg L}^{-1} \text{ CaSO}_4 \cdot 2\text{H}_2\text{O}$ ,  $60 \text{ mg L}^{-1} \text{ MgSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $4 \text{ mg L}^{-1} \text{ KCl}$ ,  $19 \text{ mg L}^{-1} \text{ Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$  and  $6.4 \text{ mg L}^{-1} (\text{CH}_3\text{CO}_2)_2\text{Ca} \cdot \text{H}_2\text{O}$ ;  $\text{pH}=7.5$ ; US EPA, 1991) using a peristaltic pump controlling a constant infiltration flow rate of  $0.5 \text{ mL min}^{-1}$ . Supplied water was aerated to maintain concentrations of dissolved oxygen between 7.5 and  $8.5 \text{ mg L}^{-1}$  at the inlet of the columns. Dissolved acetate  $[(\text{CH}_3\text{CO}_2)_2\text{Ca} \cdot \text{H}_2\text{O}]$  supplied to all columns was enriched with 0.2% of  $^{13}\text{C}$ -marked acetate ( $^{13}\text{C}_2\text{H}_4\text{O}_2$ , 99 atom%  $^{13}\text{C}$ ; Sigma-Aldrich, Saint-Quentin Fallavier, France) to determine the assimilation of dissolved organic carbon (DOC) in attached bacteria and tubificid worms. About 10 cm of water was left above the sediment surface. During the experiment, the sediment layer was kept in the dark (using 3 layers of black adhesive tape) to suppress photoautotrophic processes in sediments located on inner wall of slow filtration column. Openings at different depths on each column allowed sampling water at centimetric scale and different times during the experiment. Seven days after the set up of the sedimentary columns (time necessary to allow the physico-chemical stabilization of the system), 100 individuals of *T. tubifex* were added to half of the columns ( $n=3$  for each benzo(a)pyrene treatment). The tubificid worms came from a commercial breeding (GREBIL & Fils, Arry, France) and were maintained for 3 months in the laboratory for acclimation to experimental conditions (at  $15^\circ\text{C}$  in aquariums filled with the uncontaminated sand and chemically-controlled water used during the experiment). Tubificid worms used were 15- to 20-mm in length and 0.5-mm in diameter. The density of tubificid worms in the experimental units ( $12,800 \text{ individuals m}^{-2}$ ) was in accordance with densities reported from natural sandy sediments (Fruget, 1989; Martinet, 1993).

During the experiment, water was sampled every 7 days at 4 depths (see below) to determine  $\text{O}_2$ ,  $\text{NH}_4^+$ ,  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ ,  $\text{PO}_4^{3-}$ , and DOC concentrations in all columns. At the end of the experiment, columns were dismantled and sediment was cut into 0.5 cm thick slices. From each slice, sediment subsamples were collected for sediment reworking analyses (see below). The remaining sediments obtained from slices 0–0.5 cm, 0.5–1 cm, 1–1.5 cm and 1.5–2 cm were pooled for each column to reconstitute a sediment sample of the layer 0–2 cm. The same procedure was followed for the layers 2–4 cm and 5–7 cm. The resulting three samples of sediments per column (0–2 cm, 2–4 cm and 5–7 cm) were used to i) realize microbial analyses (abundances of bacteria and active eubacteria, hydrolytic and dehydrogenase activities, bacterial community structure), ii) quantify the assimilation of DOC by bacteria using  $^{13}\text{C}/^{12}\text{C}$  ratios and iii) determine the benzo(a)pyrene concentrations in sediments. Moreover, tubificid worms were collected and counted during column dismantling to assess worm mortality during the experiment. These intact worms were then maintained during 24 h in glass bowls with artificially reconstituted river water to purge their guts before stable isotope analysis and benzo(a)pyrene measurements in their tissues.

## 2.2. Sediment reworking analyses

Particle redistribution induced by tubificid worms in the sedimentary matrix was estimated by the luminophore tracer technique (Gerino, 1990). In each column, 0.5 g of natural sediment particles (150–300  $\mu\text{m}$ ) dyed with pink luminescent paint (Partrac Ltd, UK) was deposited uniformly at the top of the sedimentary matrix a few hours after the introduction of *T. tubifex*. The grain size of luminophores was chosen to represent a significant fraction of the sandy sediment used in columns (23% of sand particles ranged between 150 and 300  $\mu\text{m}$ ). During column dismantling, the top 8 cm of sediment were cut into 0.5 cm thick slices. Each slice of fresh sediment was homogenized and a sub-sample was freeze-dried and deposited in a well of a 24-well microplate. Detection of luminophores was performed with a Synergy Mx microplate reader (Biotek, USA) according to a

protocol adapted from Lagauzère et al. (2011): fluorescence bottom reading;  $\lambda_{\text{em}}$ : 565 nm;  $\lambda_{\text{ex}}$ : 602 nm; and area scan:  $9 \times 9$ .

## 2.3. Physico-chemical analyses of water samples

From day 0 (before worm introduction) to day 28 (4 weeks after worm introduction), water was sampled every 7 days at 2 cm above and 1 cm, 3 cm and 6 cm below the water–sediment interface. An oxygen micro-sensor probe fitted in a glass tube (OX 500, Unisense, Aarhus, Denmark) was used to determine  $\text{O}_2$  concentration without contact with atmospheric oxygen during water sampling. Water samples were filtered through Whatman GF/F filters (pore size: 0.7  $\mu\text{m}$ ; Millipore, Billerica, MA, U.S.A.) and  $\text{N-NH}_4^+$ ,  $\text{N-NO}_3^-$ ,  $\text{N-NO}_2^-$ ,  $\text{P-PO}_4^{3-}$ ,  $\text{SO}_4^{2-}$  concentrations were measured following standard colorimetric methods (Grashoff et al., 1983) by using an automatic analyzer (Easychem Plus, Systea, Anagni, Italia). For DOC measurements, water samples were filtered through Whatman HAWP filters (pore size: 0.45  $\mu\text{m}$ ; Millipore, Billerica, MA, U.S.A.) and acidified with 3 drops of HCl (35%). The DOC concentration in water samples was measured with a total carbon analyzer (multi N/C 3100, Analytik Jena, Jena, Germany) based on combustion at  $900^\circ\text{C}$  after removal of dissolved inorganic C with HCl and  $\text{CO}_2$  stripping under  $\text{O}_2$  flow.

Average  $\text{O}_2$ ,  $\text{N-NO}_3^-$  and DOC uptake rates (UR, expressed in  $\text{mg L}^{-1} \text{ h}^{-1}$ ) between overlying water and 6 cm below the water–sediment interface (i.e. between +2 cm in the overlying water and –6 cm in sediments) were calculated by using the following formula:

$$\text{UR} = \Delta\text{C} \cdot \text{Q} / (\text{V} \cdot \text{P})$$

where  $\Delta\text{C}$  was the difference in  $\text{O}_2$  (or  $\text{N-NO}_3^-$  or DOC) concentration ( $\text{mg L}^{-1}$ ) between sampling points, Q was the hydraulic discharge rate in the column ( $0.03 \text{ L h}^{-1}$ ) and V and P were the volume (L) and the porosity of the sediment layer between the two sampling points (+2 and –6 cm), respectively.

## 2.4. Physico-chemical analyses of sediment and worms

### 2.4.1. Stable isotope analysis

Sediment samples (with attached bacteria) collected at three depths in columns and tubificid worms were freeze-dried for at least 48 h and then crushed using a mortar and pestle. About 500 mg of dry sediments were placed in pre-cleaned Oakridge centrifugation tubes and 2 M HCl was added for 12 h at room temperature to remove calcite. After centrifugation at 4000 g during 10 min, the supernatant was discarded; sediments were rinsed three times with ultrapure water and oven dried at  $50^\circ\text{C}$ . An amount of 10 mg of dry sediments were weighted in tin capsules for stable isotope analysis. A total of 0.25 to 0.5  $\mu\text{g}$  of dry tubificid worms were weighted in tin capsules for stable isotope analysis.

Carbon isotope ratios ( $^{13}\text{C}/^{12}\text{C}$ ) were measured by continuous flow stable isotope ratio mass spectrometer (CF-IRMS) using a GVI Isoprime mass spectrometer interfaced with a Eurovector Euro-EA3028-HT elemental analyzer.  $^{13}\text{C}/^{12}\text{C}$  ratios were expressed as  $\delta$  (‰) and referenced to V-PDB standard. The analytical precision achieved for tyrosine standards analyzed along with the samples was better than 0.2‰ ( $\pm$  standard deviation).

### 2.4.2. Analysis of benzo(a)pyrene

Sediment samples collected at three depths in columns and tubificid worms were dried at  $50^\circ\text{C}$  and ground before benzo(a)pyrene extraction. For each sediment sample, 50 g (equivalent dry soil) were transferred into a stainless steel extraction cell and PAHs were extracted using an accelerated solvent extraction system (Dionex ASE 200™) with 5 mL of dichloromethane at  $50^\circ\text{C}$  and 1500 psi during a 5 min heating phase followed by a 10 min static extraction. Cells were then rinsed with 17 mL of dichloromethane and the sediment

solutions containing the extracted compound were flushed from the extraction cell into a collection vial using  $N_2$  at 1500 psi. The sample was extracted again using fresh solvent and flushed into the same collection vial. Extracts were dried using a speedvac concentrator (Labconco) and then re-suspended with 300  $\mu$ L of ethyl acetate for HPLC analyses.

Worm samples (ca. 30 mg) were extracted 3 times with an ultrasonic probe (Sonicator XL 2020; Misonix Inc., Farmingale, NY, U.S.A.) using 25 mL of dichloromethane/methanol (2:1 v/v). After evaporation of the solvent using a rotary evaporator, the total extract was dried under a  $N_2$  flow and dissolved in 300  $\mu$ L of ethyl acetate for HPLC analyses.

Quantification of benzo(a)pyrene in each sample was carried out using a HPLC HP 1100 (Agilent Technologies) with a C-18 Kromasil® reversed-phase column (250×4.6 mm, 5  $\mu$ m, AkzoNobel). Twenty microliters of each extract were injected and eluted at 1 mL  $min^{-1}$  using solvent A (formic acid, 0.4% in water, v/v) and solvent B (formic acid 0.4% in acetonitrile, v/v) in isocratic condition (85% of solvent B) for 35 min. HPLC was coupled with a fluorescence detector (Agilent Technologies) set to 296 nm for excitation and 406 nm for emission to detect benzo(a)pyrene and diode array detection (DAD; 200 to 600 nm, Agilent Technologies). Benzo(a)pyrene was identified on the basis of its specific retention time and the detection by fluorescence compared to the standard. Quantification of BaP was performed using DAD detection at 254 nm. Few measurements performed on sediment collected at depths below 8 cm in columns treated with 5 mg  $kg^{-1}$  of benzo(a)pyrene showed that no contamination occurred at these depths (concentrations were between 0.002 and 0.004 mg  $kg^{-1}$  and comparable to the uncontaminated columns). Therefore, we assessed the quantity of benzo(a)pyrene recovered in columns at the end of the experiment ( $Q_{BaP}$ ) using benzo(a)pyrene concentrations measured in 0–2 cm, 2–4 cm, and 5–7 cm layers by the following formula:

$$Q_{BaP} = [BaP]_{0-2cm} \cdot M_{0-2cm} + [BaP]_{2-4cm} \cdot M_{2-4.5cm} + [BaP]_{5-7cm} \cdot M_{4.5-7.5cm}$$

where  $[BaP]_{0-2cm}$ ,  $[BaP]_{2-4cm}$ ,  $[BaP]_{5-7cm}$  were the benzo(a)pyrene concentrations measured on sediments collected at the three sampled layers and  $M_{0-2cm}$ ,  $M_{2-4.5cm}$  and  $M_{4.5-7.5cm}$  were the mass of dry sediment of layers 0–2 cm, 2–4.5 cm and 4.5–7.5 cm, respectively.

## 2.5. Microbial analyses

### 2.5.1. Bacterial abundances

For each sampled layers (0–2 cm, 2–4 cm and 5–7 cm), 1 g of fresh sand was collected and immediately fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS; 0.13 M NaCl, 7 mM  $NaH_2PO_4$ , 3 mM  $NaH_2PO_4$ , pH = 7.2) for 10 h. Fixed samples were subsequently washed twice in PBS and stored in ethanol and PBS (50:50) at  $-20^\circ C$ . After storage (2 weeks), 0.5 g of fixed sediment was homogenized in 20 mL of 0.1% pyrophosphate in PBS using a sonicator with a 2-mm-diameter probe at 50 W for two periods of 60 s. All homogenized samples were finally supplemented with the detergent NP-40 (Flucka, Buchs, Switzerland) to a final concentration of 0.01%. Aliquots (10  $\mu$ L) of homogenized samples were spotted onto gelatine-coated slides and were hybridized with Cy3-labeled oligonucleotide probe (mix of EUB 338, EUB 338 II and EUB 338 III, eubacteria) and concomitantly stained with the DNA intercalating dye DAPI (200 ng  $\mu$ L $^{-1}$ ; Sigma, Buchs, Switzerland) according to Navel et al. (2011). Slides were mounted with Citifluor solution (Citifluor Ltd, London, U.K.), and the preparations were examined at 1000× magnification with a BH2-RFCA Olympus microscope fitted for epifluorescence with a high-pressure mercury bulb (50 W) and

filter sets BP 405 (for DAPI) and BP 545 (for Cy3). Bacteria from the samples were analyzed in 20 fields per sample with up to 30 cells per field. Numbers of DAPI and Cy3 bacteria were counted separately from the same field to determine the percentages of active eubacteria (% Cy3-bacteria/DAPI-bacteria, Karner and Fuhrman, 1997). Total numbers of bacteria (DAPI-bacteria) were expressed as number of cells  $g^{-1}$  dry weight (DW) of sediment.

### 2.5.2. Microbial activities

The 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) was used to measure dehydrogenase activity (Hourri-Davignon et al., 1989). For each column and sampled layer, 3 replicates of 1 g of fresh sand were incubated into a 0.02% INT solution (final solution) for 2 h at  $15^\circ C$  and then filtered on a nylon membrane (0.22  $\mu$ m, MSI, Westboro, MA, U.S.A.). In parallel, controls were prepared by adding formaldehyde (2% final) in INT solution. Extraction of INT formazan was made in vials containing 5 mL of methanol. Each vial was sonicated at 100 W during two periods of 60 s using a sonicator fitted with a 2-mm-diameter probe (Sonicator XL 2020; Misonix Inc., Farmingale, NY, U.S.A.) to increase solvent extraction yield (Maurines-Carboneill et al., 1998). The INT formazan extract was measured by a spectrophotometer adjusted at 480 nm against control blank. The quantity of INT formazan was computed by using the molar extinction coefficient of 18,000  $M^{-1} cm^{-1}$  at 480 nm and was expressed as  $\mu$ mol of INT  $h^{-1} g^{-1}$  DW of sediment.

Microbial hydrolytic activity was estimated using the fluorescein diacetate (FDA) hydrolysis method (Jørgensen et al., 1992). For each column and sampled layer, 3 replicates of 1 g of fresh sand were placed into 3 mL of a pH 7.6 phosphate buffer solution with 0.15 mL of 4.8 mM FDA solution. The incubation was maintained for 1 to 3 h until a green coloration of fluorescein appeared. At the same time, following Battin (1997), we prepared controls treated with 1.5 mL of acetone and 1.5 mL of phosphate buffer 40 min prior to the addition of the FDA solution. The reaction was stopped by freezing samples and controls after the addition of 3 mL of acetone. Fluorescein concentration was estimated from the absorbance of the filtered supernatant (0.45  $\mu$ m, Millipore, Billerica, MA, U.S.A.) measured at 490 nm and was expressed as  $\mu$ mol of FDA  $h^{-1} g^{-1}$  DW of sediment.

### 2.5.3. Bacterial community structure

Genomic DNA was extracted from 0.5 g of sediment using the Fast DNA Spin Kit for soil (QBIogene, Illkirch, France) according to the manufacturer's instructions. Modifications in bacterial community structure were assessed by Automated Ribosomal Intergenic Spacer Analysis (ARISA). The 16S–23S intergenic spacer region from the bacterial rRNA operon was amplified by PCR as described in Ranjard et al. (2001). Amplification was performed using the primers S-D-Bact-1522-b-S-20 (eubacterial rRNA small subunit, 5'-TGC GGC TGG ATC CCC TCC TT-3') and L-DBact-132-a-A-18 (eubacterial rRNA large subunit, 5'-CCG GGT TTC CCC ATT CGG-3'). PCR reactions were carried out in a total volume of 25  $\mu$ L containing a 10× Taq reaction buffer (Eurobio), 1.5 mM  $MgCl_2$ , 120  $\mu$ M of each deoxynucleotide, 1  $\mu$ M of each primer, bovine serum albumin (Sigma, 0.5 mg  $mL^{-1}$  final concentration), 1.25 U Taq DNA polymerase (Eurobio) and 2  $\mu$ L of template DNA. PCR reactions were run in a Thermal Cycler Tpersonal (Biometra, Göttingen, Germany) under the following conditions: an initial denaturation at  $94^\circ C$  for 3 min, followed by 25 cycles of denaturation (1 min at  $94^\circ C$ ), annealing (30 s at  $55^\circ C$ ), and extension (1 min at  $72^\circ C$ ), and a final extension at  $72^\circ C$  for 5 min. Amplified fragments were separated by capillary electrophoresis on an Agilent 2100 bioanalyzer using a DNA 1000 kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. Fluorescence data were converted in 2-dimensional gel image using 2100 Expert Software (Agilent Technologies, Santa Clara, CA, USA). Image analysis was then performed using the GelCompar II version 4.6 software (Applied Maths, Ghent, Belgium). Sample profiles

were normalized with an internal standard and external ladder included in each gel and DNA fragments of different size were classified into operational taxonomic units (OTUs). A matrix (with samples as rows and OTUs as columns) based on the presence/absence of a given OTU in each sample was then constructed. Dissimilarity based on OTU composition was calculated between all pairs of samples using the Dice coefficient.

## 2.6. Data treatment

The quantification of luminophore redistribution following sediment reworking by *T. tubifex* was realized using the bioadvection–biodiffusion model (Officier and Lynch, 1982; Gerino et al., 1994). This model was applied under non-steady-state treatments after a pulse input of tracers at the sediment surface at the beginning of the experiment. This model allows the estimation of suitable values for the two parameters *Db* (the biodiffusive rate that quantifies omnidirectional dispersion) and *V* (the bioadvective rate that quantifies the burial velocity of tracers). This model is especially useful in order to estimate sediment mixing by conveyor species like *T. tubifex* (i.e. sediment bioadvective process with sediment ingestion at depth and fecal pellet accumulation at the sediment surface) (Ciutat et al., 2005). Biodiffusion and bioadvection coefficients of particles obtained with tubificid worms were compared among benzo(a)pyrene treatments using a one-way analysis of variance (one-way ANOVA).

The influences of benzo(a)pyrene treatments and tubificid worms on water chemistry (concentrations of dissolved oxygen, DOC, N-NH<sub>4</sub><sup>+</sup>, N-NO<sub>3</sub><sup>-</sup>, N-NO<sub>2</sub><sup>-</sup>, P-PO<sub>4</sub><sup>3-</sup> and SO<sub>4</sub><sup>2-</sup>) were analyzed on data obtained the two last sampling dates of the experimentation (days 21 and 28). In this way, we used a three-way repeated measures analysis of variance (RM-ANOVA3) with “benzo(a)pyrene treatment”, “depth” and “worm” as main factors and time (n=2, days 21 and 28) as repeated factor. Microbial characteristics (bacterial abundance, ratio active/total bacteria, hydrolytic activity, dehydrogenase activity), <sup>13</sup>C/<sup>12</sup>C ratios and benzo(a)pyrene concentrations measured on sediment were analyzed using three-way analysis of variance (3-way ANOVA) with “benzo(a)pyrene treatment”, “depth” and “worm” as main factors. When significant differences (α<0.05) were detected among treatments, Tukey post hoc tests were performed to identify significant pair-wise differences between treatments. The <sup>13</sup>C/<sup>12</sup>C ratios and benzo(a)pyrene concentrations in tubificid worms were compared among benzo(a)pyrene treatments using a one-way analysis of variance (one-way ANOVA).

When necessary, data were log-transformed, and data expressed as percentages (% of active bacteria) were arcsine-transformed before statistical analysis, to satisfy the assumptions of homoscedasticity and normality. Statistical analyses were performed using Statistica 6 TM (Statsoft, Tulsa, OK, USA).

The significance of differences in bacterial community structure that could be accounted for by the effects of “benzo(a)pyrene treatment”, “depth”, and “worm”, and their interactions were estimated using permutational multivariate analysis of variance (PERMANOVA, Anderson, 2001; McArdle and Anderson, 2001). Statistical tests were based on 9999 permutations of the Dice dissimilarity matrix and significant differences among treatments were assessed through pair-wise post hoc multiple comparisons using the PERMANOVA program (Anderson, 2005). Significance for these statistical tests was accepted at α<0.05. Bacterial community structure pattern was visualized using non-metric multidimensional scaling (NMDS) based on Dice dissimilarity matrix. A stress function (which ranges from 0 to 1) was used to assess the goodness of fit between the ordination and the original data. Stress values below 0.2 suggest that the ordination accurately represents the dissimilarity among samples. NMDS and graphical displays were performed using functions in the vegan (Oksanen et al., 2011) package in R (R Development Core Team, 2008).

## 3. Results

### 3.1. Survival of tubificid worms and bioturbation activity

The survival rates of tubificid worms were comparable among the three benzo(a)pyrene treatments with values of 84.3±12%, 79.7±6% and 83±10% for benzo(a)pyrene treatments of 0, 1 and 5 mg kg<sup>-1</sup>, respectively. In treatments without worms, no sediment reworking was measured and all luminophores remained at the sediment surface at the end of the experiment (Table 1). With tubificid worms, the biodiffusive mixing quantified by the *Db* coefficient was the same in all treatments. Due to the high variability among replicated columns, we did not detect statistical difference in bioadvective rates induced by worms among the 3 benzo(a)pyrene treatments (one-way ANOVA, benzo(a)pyrene effect, p>0.2). Despite this lack of statistical significance, it is however important to note a clear tendency of the average values of bioadvective rates to decrease with benzo(a)pyrene concentration, from 4.5 cm year<sup>-1</sup> in unpolluted systems to 2.7 cm year<sup>-1</sup> in columns with 5 mg kg<sup>-1</sup> of benzo(a)pyrene (Table 1).

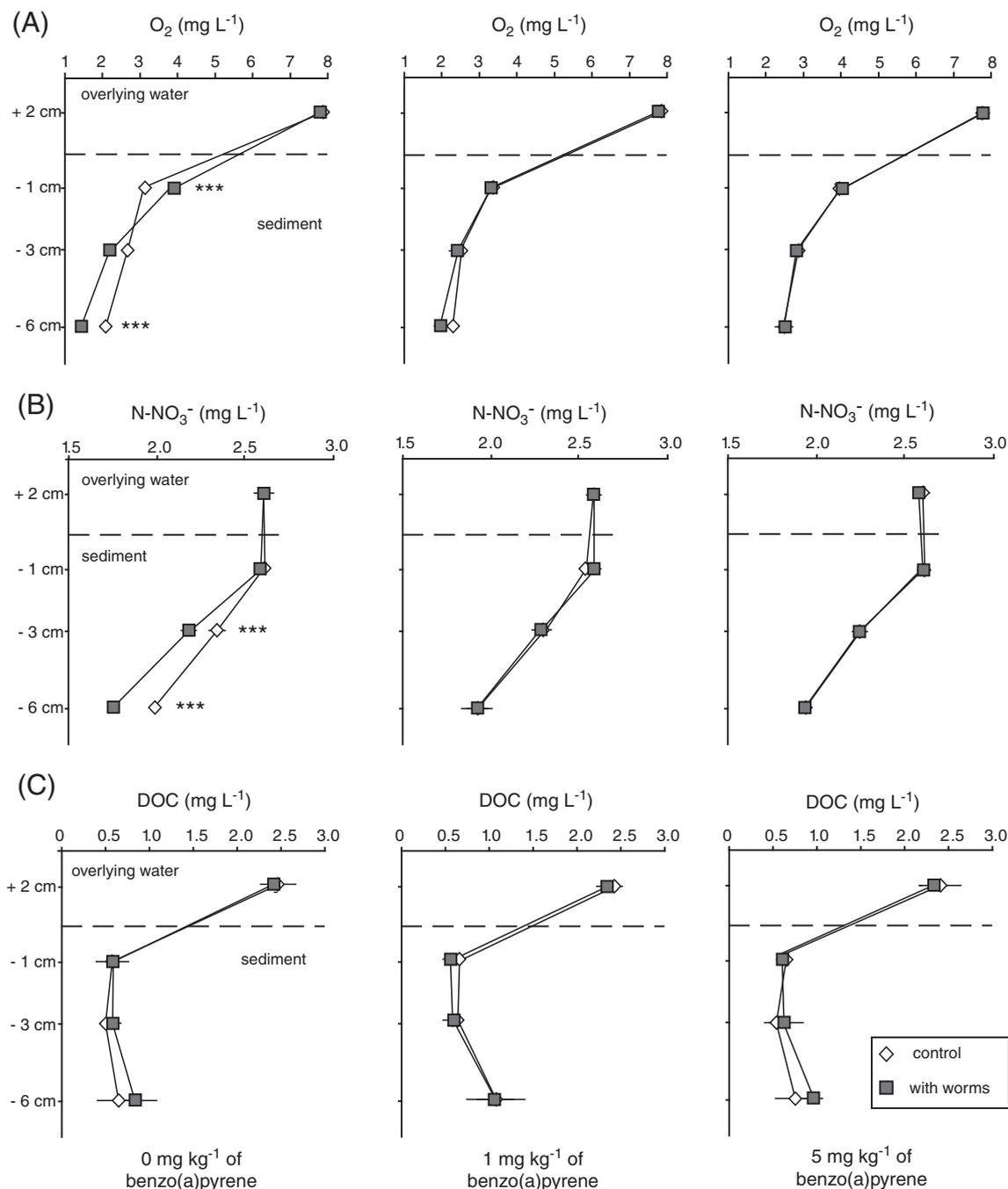
### 3.2. Water chemistry

P-PO<sub>4</sub><sup>3-</sup>, N-NH<sub>4</sub><sup>+</sup> and N-NO<sub>2</sub><sup>-</sup> concentrations remained low (<30 µg L<sup>-1</sup> for both) at all depths throughout the experiment (data not shown). The concentration of SO<sub>4</sub><sup>2-</sup> varied between 73 and 78 mg L<sup>-1</sup> in columns without any significant influence of depth, worms or benzo(a)pyrene treatments (RM-ANOVA3, p>0.25 for the three effects). In contrast, O<sub>2</sub> and N-NO<sub>3</sub><sup>-</sup> concentrations significantly decreased with depth in columns owing to microbial activities (Fig. 1A and B, RM-ANOVA3, depth effect, p<0.0001 for the two variables). Significant influences of benzo(a)pyrene treatments were detected on O<sub>2</sub> and N-NO<sub>3</sub><sup>-</sup> concentrations (RM-ANOVA3, benzo(a)pyrene effect, p<0.01 for the two variables). These effects resulted from slightly higher concentrations of O<sub>2</sub> (+0.35 mg L<sup>-1</sup>) and N-NO<sub>3</sub><sup>-</sup> (+0.04 mg L<sup>-1</sup>) measured in the columns contaminated with 5 mg of benzo(a)pyrene per kg of surface sand compared with data collected in uncontaminated columns. The influence of tubificid worms on vertical profiles of O<sub>2</sub> and N-NO<sub>3</sub><sup>-</sup> strongly depended on the depth and the benzo(a)pyrene treatment considered (RM-ANOVA3, interaction “benzo(a)pyrene\*depth\*fauna”, p<0.0001 for the two variables). In contaminated treatments (with 1 or 5 mg kg<sup>-1</sup> of benzo(a)pyrene), tubificid worms did not influence O<sub>2</sub> and N-NO<sub>3</sub><sup>-</sup> concentrations (Tukey post-hoc tests, p>0.3 for the two variables at the 4 depths for the 2 contaminated treatments). In contrast, the presence of worms significantly increased oxygen concentration at 1-cm depth (Fig. 1A, Tukey post-hoc test, p<0.001) and led to a significant decrease of oxygen concentrations at 6-cm depth (Tukey post-hoc test, p<0.001) in the uncontaminated columns. Consequently, O<sub>2</sub> consumption rates measured between overlying water and 6-cm depth were stimulated (+12%) by the presence of worms (Table 2, Tukey post-hoc test, worm effect in uncontaminated

**Table 1**

Biodiffusion (*Db*) and bioadvection (*V*) coefficients calculated repartition of luminophores initially deposited at the sediment surface. Values are means±SD (n=3 columns per treatment).

Benzo(a)pyrene treatment	Animal treatment	<i>Db</i> (cm <sup>2</sup> year <sup>-1</sup> )	<i>V</i> (cm year <sup>-1</sup> )
0 mg kg <sup>-1</sup>	Control	0.0±0.0	0.0±0.0
	<i>T. tubifex</i>	0.3±0.0	4.5±1.2
1 mg kg <sup>-1</sup>	Control	0.0±0.0	0.0±0.0
	<i>T. tubifex</i>	0.3±0.0	3.6±0.8
5 mg kg <sup>-1</sup>	Control	0.0±0.0	0.0±0.0
	<i>T. tubifex</i>	0.3±0.0	2.7±0.7



**Fig 1.** Vertical profiles of (A) O<sub>2</sub>, (B) N-NO<sub>3</sub><sup>-</sup> and (C) dissolved organic carbon (DOC) measured in slow filtration columns with or without tubificid worms for each benzo(a)pyrene treatment. Values are means ± SD (n = 3). Left column: 0 mg kg<sup>-1</sup> of benzo(a)pyrene; central column: 1 mg kg<sup>-1</sup> of benzo(a)pyrene; and right column: 5 mg kg<sup>-1</sup> of benzo(a)pyrene. Significant influences of tubificid worms determined by post-hoc tests were indicated by asterisks (\*\*\*p < 0.001).

treatment, p < 0.01). The occurrence of *T. tubifex* also significantly reduced the concentrations of N-NO<sub>3</sub><sup>-</sup> at depth in the uncontaminated treatment (Fig. 1B, Tukey post-hoc tests, p < 0.001 at depths of -3 and -6 cm). The consumption rates of nitrate were increased by 49% with worms in this treatment (Table 2, Tukey post-hoc test, “worm” effect in unpolluted treatment, p < 0.01) whereas no influence of worms was detected in contaminated treatments (Tukey post-hoc tests, “worm” effect in the 2 contaminated treatments, p > 0.9). Vertical profiles of DOC concentrations presented two phases: a decrease in the top first cm of the sediments and a slight increase below this depth (Fig. 1C, RM-ANOVA3, depth effect, p < 0.0001). Benzo(a)pyrene concentrations and tubificid worms did not have a

**Table 2**

Average consumption rates ± SD (n = 3) of O<sub>2</sub> (respiration), N-NO<sub>3</sub><sup>-</sup> (denitrification) and dissolved organic carbon (DOC) measured in the 6 treatments (calculated for each column with data obtained on days 20 and 27). Values are expressed in mg of O<sub>2</sub> (N-NO<sub>3</sub><sup>-</sup> or DOC) h<sup>-1</sup> L<sup>-1</sup> sediment.

Benzo(a)pyrene treatment	Animal treatment	O <sub>2</sub>	N-NO <sub>3</sub> <sup>-</sup>	DOC
0 mg kg <sup>-1</sup>	Control	1.87 ± 0.03	0.17 ± 0.01	0.50 ± 0.08
	<i>T. tubifex</i>	2.09 ± 0.04	0.25 ± 0.01	0.53 ± 0.06
1 mg kg <sup>-1</sup>	Control	1.83 ± 0.01	0.20 ± 0.01	0.42 ± 0.01
	<i>T. tubifex</i>	1.91 ± 0.04	0.19 ± 0.03	0.44 ± 0.07
5 mg kg <sup>-1</sup>	Control	1.78 ± 0.05	0.19 ± 0.01	0.54 ± 0.04
	<i>T. tubifex</i>	1.79 ± 0.06	0.19 ± 0.01	0.50 ± 0.04

marked effect on the vertical profiles of DOC and then they did not significantly influence DOC uptake rate (Table 2, two-way ANOVA, “worm” effect and “benzo(a)pyrene” effect,  $p > 0.1$ ).

### 3.3. Benzo(a)pyrene

The concentrations of benzo(a)pyrene measured in columns at the end of the experiment are presented in Table 3. Benzo(a)pyrene was detected down to the 5–7 cm layer whereas the spiked sediments were originally deposited at the sediment surface at the start of the experiment. This redistribution of benzo(a)pyrene was not significantly affected by the occurrence of tubificid worms (3-way ANOVA, worm effect alone or in interaction with depth or/and benzo(a)pyrene concentrations,  $p > 0.4$ ). Using the concentrations measured at the different sediment layers, the amount of total recovered benzo(a)pyrene calculated on the entire columns was significantly lower than the amount of benzo(a)pyrene added to columns with recovery rates fluctuating between 35 and 48%. Variations in recovery rates were however not related to initial benzo(a)pyrene concentrations and/or to the occurrence of tubificid worms.

The incorporation of benzo(a)pyrene in worm tissues led to average concentrations of  $0.09 \text{ mg g}^{-1}$  and  $0.51 \text{ mg g}^{-1}$  in surface sediments contaminated with  $1.08 \text{ mg kg}^{-1}$  and  $5.08 \text{ mg kg}^{-1}$  of benzo(a)pyrene, respectively. Therefore, the ratio of the concentration of benzo(a)pyrene in worms over sediment contamination (i.e. bioaccumulation/bioconcentration factor, Bott and Standley, 2000) gave comparable values (between 83 and 102) in the two contaminated conditions.

### 3.4. $\delta^{13}\text{C}$ on sediments and tubificid worms

The enrichments in  $^{13}\text{C}$  of organic carbon attached to sediments were measured between the start and the end of the experiment for all columns. They significantly decreased with depth in sedimentary columns (3-way ANOVA, depth effect,  $p < 0.0001$ ). The increase in  $\delta^{13}\text{C}$  of total organic carbon attached to sediment during the course of the experiment was the highest in the top sediment layer (from  $-30\%$  to  $+20.5\%$ ). At deeper layers,  $\delta^{13}\text{C}$  values measured at the end of the experiment were comparable ( $-28.7\%$  for the layer 2–4 cm and  $-29.6\%$  for the layer 5–7 cm) to those measured at the start of the experiment ( $-30\%$ ). *T. tubifex* did not significantly influence the carbon isotope composition of total organic carbon attached to the sediment (3-way ANOVA, worm effect alone or in interaction with depth or/and benzo(a)pyrene concentrations,  $p > 0.12$ ).

**Table 3**

Concentrations of benzo(a)pyrene measured at the end of the experiment at three sediment layers, for the 6 treatments. Values are means  $\pm$  SD ( $n = 3$  columns per treatment).

Benzo(a)pyrene treatment	Animal treatment	Sediment layer	BaP ( $\mu\text{g kg}^{-1}$ )
0 $\text{mg kg}^{-1}$	Control	0–2 cm	$2 \pm 1$
	<i>T. tubifex</i>		$3 \pm 4$
	Control	2–4 cm	$3 \pm 2$
	<i>T. tubifex</i>		$3 \pm 2$
	Control	5–7 cm	$2 \pm 1$
	<i>T. tubifex</i>		$3 \pm 1$
1 $\text{mg kg}^{-1}$	Control	0–2 cm	$206 \pm 24$
	<i>T. tubifex</i>		$162 \pm 46$
	Control	2–4 cm	$14 \pm 8$
	<i>T. tubifex</i>		$12 \pm 2$
	Control	5–7 cm	$5 \pm 2$
	<i>T. tubifex</i>		$8 \pm 5$
5 $\text{mg kg}^{-1}$	Control	0–2 cm	$725 \pm 105$
	<i>T. tubifex</i>		$772 \pm 45$
	Control	2–4 cm	$38 \pm 18$
	<i>T. tubifex</i>		$70 \pm 14$
	Control	5–7 cm	$11 \pm 1$
	<i>T. tubifex</i>		$28 \pm 10$

The  $\delta^{13}\text{C}$  values of worms suggest a comparable incorporation of organic carbon in their tissues for the three benzo(a)pyrene treatments (one-way ANOVA,  $p > 0.15$ ): mean increases in  $\delta^{13}\text{C}$  of worms were 140%, 117% and 158% in the treatments with 0, 1 and  $5 \text{ mg kg}^{-1}$  of benzo(a)pyrene, respectively.

### 3.5. Bacterial abundances and microbial activities

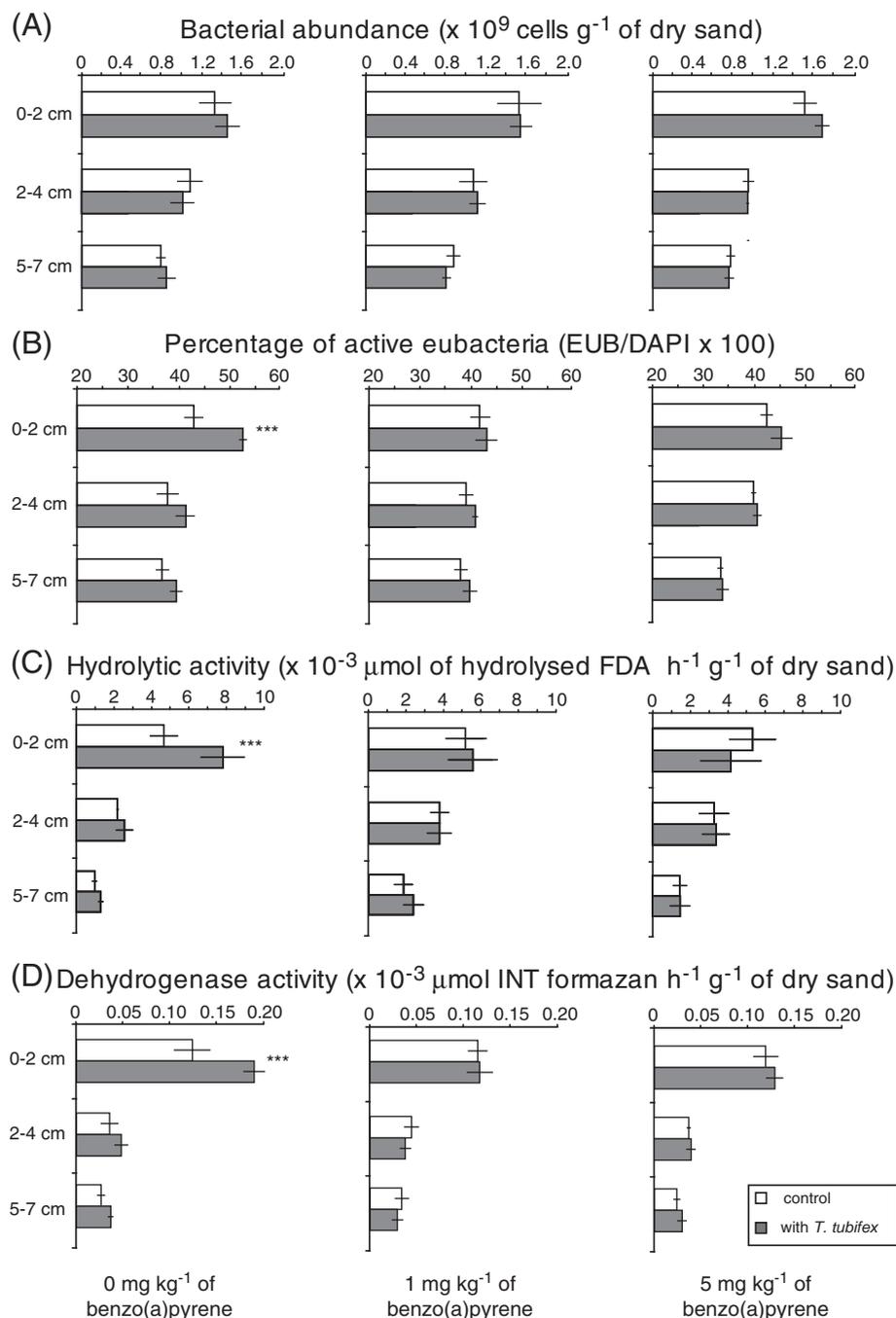
At the end of the experiment, the bacterial abundance, the percentage of active eubacteria and the microbial activities (dehydrogenase and hydrolytic activities) presented a significant decrease with depth in the sedimentary system (Fig. 2, 3-way ANOVA, depth effect,  $p < 0.0001$  for the four variables). The total abundance of bacteria was neither influenced by benzo(a)pyrene concentrations nor by tubificid worms (3-way ANOVA, benzo(a)pyrene and worm effects,  $p > 0.1$ ). Benzo(a)pyrene concentrations significantly influenced the percentage of active bacteria and the dehydrogenase activity (3-way ANOVA, benzo(a)pyrene effect,  $p < 0.001$ ): regardless of its concentration, the presence of benzo(a)pyrene negatively affected these two microbial variables in comparison with the uncontaminated treatment (Tukey post-hoc tests, comparison between unpolluted and polluted conditions,  $p < 0.01$  for the two variables). A significant effect of tubificid worms was detected on percentages of active bacteria and microbial activities depending on the benzo(a)pyrene contamination (3-way ANOVA, interaction between benzo(a)pyrene and worm effects,  $p < 0.01$ ). More precisely, the presence of worms stimulated the percentage of active bacteria, hydrolytic and dehydrogenase activities in the top layer of sediment (0–2 cm) of the unpolluted columns (Fig. 2, Tukey post-hoc tests,  $p < 0.01$ ), whereas these influences were not observed in polluted systems (Tukey post-hoc tests,  $p > 0.2$ ).

### 3.6. Bacterial community structure

A total of 29 OTUs was identified and the number of OTUs averaged  $17 \pm 2$  per sample ( $n = 54$ ). Average dissimilarity between samples based on Dice index was  $0.57 \pm 0.09$ . A significant change in bacterial community structure was observed among sediment depth (PERMANOVA, depth effect,  $p < 0.001$ ). NMDS plot (Fig. 3) and post-hoc tests indicated that bacterial community structure in the top layer of sediment (0–2 cm) was significantly different from that found in deeper sediment layers (2–4 and 5–7 cm, post-hoc test,  $p < 0.001$ ). Benzo(a)pyrene had a significant impact on bacterial community structure but only 10% of the total variation in bacterial community structure could be explained by benzo(a)pyrene treatments. Post-hoc tests revealed a significant difference in bacterial community structure between the uncontaminated treatment and the treatment contaminated with  $1 \text{ mg kg}^{-1}$  of benzo(a)pyrene ( $p = 0.001$ ) whereas no difference was found between the uncontaminated treatment and the treatment contaminated with  $5 \text{ mg kg}^{-1}$  of benzo(a)pyrene ( $p > 0.05$ ). The impact of tubificid worms on bacterial community structure depended on the depth considered (worm\*depth,  $p < 0.05$ ). Tubificid worms significantly modified the bacterial community structure in the 0–2 cm layer (post-hoc test,  $p < 0.05$ ) but had no effect on deeper sediment layers ( $p > 0.05$ ). PERMANOVA indicated that the impact of tubificid worms on bacterial community structure was not dependent on the benzo(a)pyrene contamination level (benzo(a)pyrene\*fauna or benzo(a)pyrene\*fauna\*depth,  $p > 0.05$ ).

## 4. Discussion

Chemical ( $\text{O}_2$  and  $\text{NO}_3^-$  vertical profiles) and microbiological (hydrolytic and dehydrogenase activities, percentage of active bacteria) analyses confirmed our hypothesis stating that benzo(a)pyrene contamination influences the role played by *T. tubifex* in biogeochemical processes. Indeed, tubificid worms stimulated aerobic respiration,

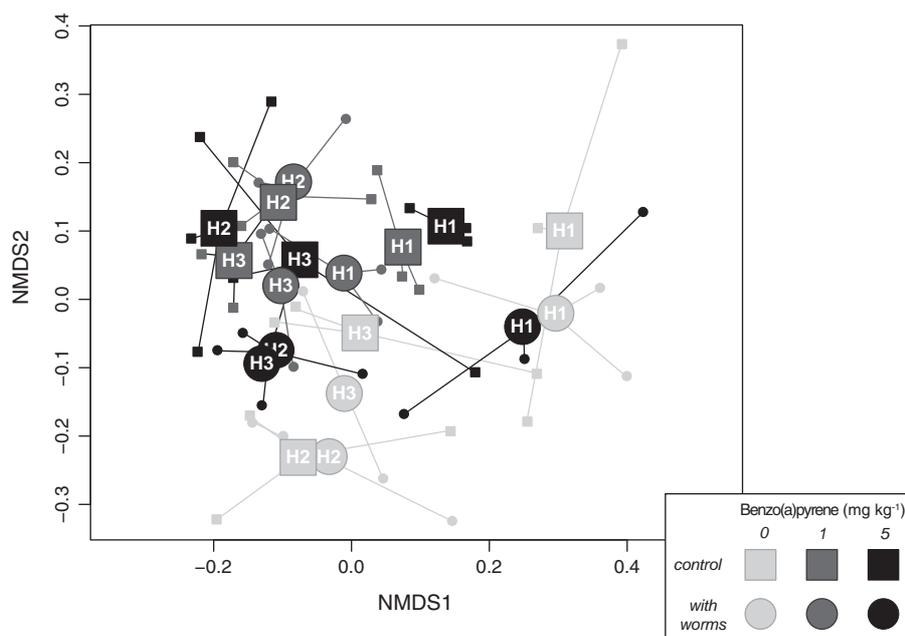


**Fig. 2.** (A) Total abundance of bacteria, (B) percentage of active eubacteria, (C) hydrolytic activity and (D) dehydrogenase activity measured at three layers in slow filtration columns with or without tubificid worms for each benzo(a)pyrene treatment. Values are means  $\pm$  SD ( $n=3$ ). Left column: 0 mg kg<sup>-1</sup> of benzo(a)pyrene; central column: 1 mg kg<sup>-1</sup> of benzo(a)pyrene; and right column: 5 mg kg<sup>-1</sup> of benzo(a)pyrene. Significant influences of tubificid worms determined by post-hoc tests were indicated by asterisks (\*\*\*)  $p < 0.001$ .

denitrification and micro-organisms in uncontaminated sediments whereas such effects were inhibited in sediments treated with benzo(a)pyrene.

The positive influence of tubificid worms on microbial processes measured in uncontaminated sediments was in agreement with previous studies performed in freshwater sediments (Chatarpaul et al., 1979; Mermillod-Blondin et al., 2001; Nogaro et al., 2007; Lagauzère et al., 2009a). *T. tubifex* probably increased dissolved oxygen concentration in subsurface sediments (1 cm below the water-sediment interface) through the construction and irrigation of burrows (Mermillod-Blondin and Lemoine, 2010). Such bioturbation activity of tubificid worms, which increased solute exchanges at the water-sediment

interface, has been recognized as a major factor influencing biogeochemistry in freshwater sediments (Pelegrí and Blackburn, 1995; Svensson et al., 2001; Nogaro et al., 2007). As described by Delmotte et al. (2007), the significant bioadvection of luminophores measured in treatments with *T. tubifex* was linked to the deposition of fecal pellets at the sediment surface by tubificid worms. Burrowing and production of fecal pellets that often act as hot spots for micro-organisms (Quéric and Soltwedel, 2007) were the two main activities of worms stimulating microbial activities in the top 2 cm of the sediment. Such stimulation increased oxygen and nitrate consumptions in the top 6 cm of the sedimentary infiltration column, showing a net positive effect of worms on organic matter processing through aerobic (+12% of aerobic



**Fig. 3.** The first two axes from the NMDS ordination of ARISA community profiles from sediment samples collected at three depths in all columns (stress value: 0.17). Dots represent the factorial scores of sediment samples and labels (circles and squares) are mapped at the gravity center of the three replicates per treatment (depth\*worms\*benzo(a)pyrene treatment). H1: layer 0–2 cm, H2: layer 2–4 cm and H3: layer 5–7 cm.

respiration) and anaerobic (+49% of  $\text{NO}_3^-$  consumption by denitrification) processes.

The presence of benzo(a)pyrene totally inhibited the positive influence of *T. tubifex* on micro-organisms. Such impact was not linked to an increased mortality of animals by contamination because survival rates of worms were comparable in all benzo(a)pyrene treatments (between 79.7% and 85.3% of survival). The impact was rather due to a contaminant-induced change in the activities (bioturbation and/or metabolism) of worms and, consequently, in microbial processes. The fact that worms did not increase  $\text{O}_2$  concentration at 1 cm below the sediment surface in the presence of benzo(a)pyrene indicates that the bioirrigation activity of tubificid worms was affected by this PAH. As observed with a metallic contamination with uranium (Lagauzère et al., 2009c), benzo(a)pyrene might have reduced the density of burrows produced by *T. tubifex*, consequently affecting the transfer of dissolved oxygen through burrows. The impact of benzo(a)pyrene on surface sediment irrigation by tubificid worms could explain why *T. tubifex* tended to rework less surface sediment when the concentration of contaminants increased. The reduction of 40% in bioadvection rates (from 4.5 to 2.7  $\text{cm year}^{-1}$ ) due to the contamination of surface sediment with 5  $\text{mg kg}^{-1}$  of benzo(a)pyrene also supposed a pollution-induced reduction of worm feeding activity associated with the production and deposition of fecal pellets at the sediment surface. However, the  $^{13}\text{C}/^{12}\text{C}$  ratios of worm tissues collected at the end of the experiment were comparable in contaminated and uncontaminated conditions, suggesting that *T. tubifex* fed at the same rate on surface sediments enriched with  $^{13}\text{C}$  whatever the benzo(a)pyrene contamination. The analyses of benzo(a)pyrene in worm tissues also showed similar bioaccumulation factors (between 83 and 102) for the two concentrations of benzo(a)pyrene tested. Because the major pathway for PAH accumulation in aquatic worms results from food ingestion (Bott and Standley, 2000; Leppänen and Kukkonen, 2000), our data suggest a low influence of benzo(a)pyrene on worm feeding activity. Consequently, in the present study, benzo(a)pyrene had a major impact on the bioirrigation activity of *T. tubifex* without affecting significantly its feeding activities. As observed in many studies (e.g. Aller, 1994; Kristensen, 2000; Stief et al.,

2004; Mermillod-Blondin, 2011), the increase of solute fluxes at the water–sediment interface was the main process by which bioturbators influenced microbial processes in our experiment.

Our results also showed that tubificid worms had a significant effect on the bacterial community structure living in the top sediment layer (0–2 cm). In contrast with microbial activities, this influence of tubificid worms on micro-organisms was observed in all contamination treatments (with or without benzo(a)pyrene), suggesting a decoupling between community structure and bacterial activity in our experiment. This could be explained by the upward conveyor mode of tubificid worms. As *T. tubifex* selects fine sediment particles when feeding at depth (Rodríguez et al., 2001), this tubificid worm might have produced a transport of fine sand from deep layers to sediment surface, creating specific colonizable area for micro-organisms in the top sediment. Such sediment transport likely induced a transport of bacteria (which could have been affected during gut transit as observed in the lugworm *Arenicola marina*, Grossi et al., 2006) and, consequently, a change in bacterial communities occurring in the top layer of sediments contaminated with benzo(a)pyrene. It has been shown that the production of irrigated burrows and subsequent oxygenation could modify bacterial communities in deep anaerobic marine sediments (Papaspyrou et al., 2005; Bertics and Ziebis, 2009; Laverock et al., 2010). In our experimental conditions, we did not detect such an effect in sedimentary layers below 2 cm because anaerobic conditions did not occur in the whole sedimentary column. As commonly measured in river sediments (e.g. Ingendahl et al., 2009), the physical interstitial flow rate applied to columns maintained dissolved oxygen concentrations higher than 1  $\text{mg L}^{-1}$  in sediments (Fig. 1) and, in these conditions, bioturbators could not drastically modify aerobic/anaerobic boundaries in sediments. It is also interesting to note that benzo(a)pyrene contamination did not have a clear effect on bacterial community structure as samples obtained with 0  $\text{mg kg}^{-1}$  and 5  $\text{mg kg}^{-1}$  of benzo(a)pyrene exhibited comparable bacterial communities. In sediments, the occurrence of PAH can have both negative (toxic) and positive effects (through organic matter enrichment) on micro-organisms (e.g., Bauer and Capone, 1985a; Verrhiest et al., 2002). However, these effects were essentially

observed with high concentrations of PAHs ( $>100 \text{ mg kg}^{-1}$  of sediment) that are only encountered in heavily contaminated sites (Juhász and Naidu, 2000). By focusing on low contaminated sediments (few  $\text{mg kg}^{-1}$ , WHO, 1982), our experiment clearly demonstrated that, in freshwater ecosystems, microbial processes could be more affected by contaminant-induced changes in microbe–invertebrate interactions rather than by a direct effect of contaminants on micro-organisms.

Although our study was not designed to determine the fate of the PAH in experimental systems, analyses showed that more than 50% of the benzo(a)pyrene was lost during the 30 days of the experiment. The loss rates obtained from the present study varied from 17 to  $105 \mu\text{g}$  of benzo(a)pyrene  $\text{kg}^{-1}$  of sediment  $\text{day}^{-1}$ . Several environmental factors (e.g., oxygen availability, temperature, nutrients, sediment structure) influence the fate and the degradation of PAHs in sediments (Bauer and Capone, 1985b; Lei et al., 2005; Toyama et al., 2011). They can explain the wide range of benzo(a)pyrene degradation rates reported by Shiaris (1989) in estuarine sediments ( $4\text{--}1190 \mu\text{g}$  of benzo(a)pyrene transformed  $\text{kg}^{-1}$  of sediment  $\text{day}^{-1}$ ). It has been demonstrated that oxygen concentrations have a predominant role on the fate of PAHs since the degradation of PAH by micro-organisms is much more efficient under aerobic conditions than under anaerobiosis (Boyd et al., 2005; Quantin et al., 2005; Haritash and Kaushik, 2009). Therefore, bioirrigation of sediments by benthic fauna can stimulate PAH degradation through continuous or periodical increase of oxygen availability in anaerobic sediments (Granberg et al., 2005; Timmermann et al., 2008; Montgomery et al., 2008; Cuny et al., 2011). In the present study, we did not detect a stimulation of benzo(a)pyrene loss due to the bioirrigation activity of *T. tubifex*. This lack of effect is easily explainable because (1) the bioirrigation behavior of tubificid worms was inhibited by benzo(a)pyrene contamination and (2) tubificid worms could not much modify oxygen availability in a sedimentary system where aerobic conditions prevailed. Extending the present experiment to sedimentary systems characterized by aerobic and anaerobic zones would be of great interest to evaluate the role of interactions between bioturbators and environmental conditions on contaminant degradation.

## 5. Conclusion

The present study reveals the importance of sublethal concentrations of a contaminant on ecological processes in river sediments through affecting bioturbator–microbe interactions. A concentration of  $1 \text{ mg kg}^{-1}$  of benzo(a)pyrene in the top layer of sediment was large enough to inhibit the stimulation of micro-organisms by tubificid worms and resulted in the reduction of the capacity of the sedimentary habitat to process organic matter by aerobic respiration ( $-12\%$ ) and denitrification ( $-49\%$ ). In literature, the significant impacts of pollutants on invertebrates and associated ecological processes in streams have been essentially demonstrated in systems where invertebrate communities were severely affected. For instance, it has been shown that the leaf litter breakdown rates were strongly reduced when contaminants decreased the abundances of key functional species such as gammarids (e.g., Dangles et al., 2004; Piscart et al., 2011). Our results further demonstrate that sublethal concentrations of pollutants also need to be considered in ecosystem health assessment as they can affect self-purification processes

## Conflict of interest

The authors declare no conflict of interest in the present study.

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