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Microbial responses to polycyclic aromatic hydrocarbon contamination in temporary river sediments: Experimental insights

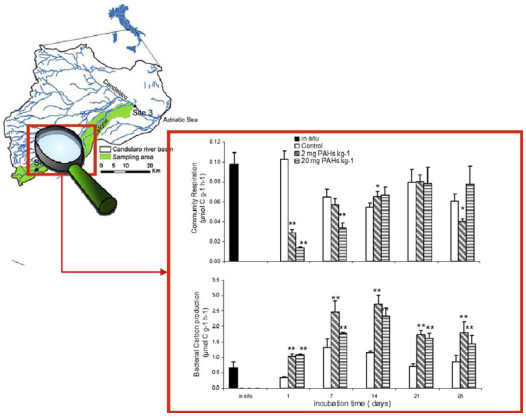
Annamaria Zoppini [a,*], Nicoletta Ademollo [a], Stefano Amalfitano [a], Silvio Capri [a], Patrizia Casella [a], Stefano Fazi [a], Juergen Marxsen [b], Luisa Patrolecco [a]

[a] Istituto di Ricerca Sulle Acque, Consiglio Nazionale delle Ricerche (IRSA-CNR), Via Salaria km 29.300, CP10, 00015 Monterotondo, Roma, Italy
[b] Limnologische Fluss-Station des Max-Planck-Instituts für Limnologie, Schlitz, Germany and Institut für Allgemeine und Spezielle Zoologie, Tierökologie, Justus-Liebig-Universität, Gießen, Germany

HIGHLIGHTS

- Microbes of temporary river sediments showed low potential for PAH degradation
- PAH contamination altered the structure and function of microbial communities
- Putative toxic effects detrimentally affected oxidative phosphorylation processes
- Impoverished cell energetic resources may alter the entire river functioning.

GRAPHICAL ABSTRACT



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ABSTRACT

Temporary rivers are characterized by dry–wet phases and represent an important water resource in semi-arid regions worldwide. The fate and effect of contaminants have not been firmly established in temporary rivers such as in other aquatic environments. In this study, we assessed the effects of sediment amendment with Polycyclic Aromatic Hydrocarbons (PAHs) on benthic microbial communities. Experimental microcosms containing natural (Control) and amended sediments (2 and 20 mg PAHs kg^{-1}) were incubated for 28 days. The PAH concentrations in sediments were monitored weekly together with microbial community structural (biomass and phylogenetic composition by TGGE and CARD-FISH) and functional parameters (ATP concentration, community respiration rate, bacterial carbon production rate, extracellular enzyme activities). The concentration of the PAH isomers did not change significantly with the exception of phenanthrene. No changes were observed in the TGGE profiles, whereas the occurrence of Alpha- and Beta-Proteobacteria was significantly affected by the treatments. In the amended sediments, the rates of carbon production were stimulated together with aminopeptidase enzyme activity. The community respiration rates showed values significantly lower than the Control after 1 day

* Corresponding author.
E-mail address: zoppini@irsa.cnr.it (A. Zoppini).

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from the amendment then recovering the Control values during the incubation. A negative trend between the respiration rates and ATP concentration was observed only in the amended sediments. This result indicates a potential toxic effect on the oxidative phosphorylation processes. The impoverishment of the energetic resources that follows the PAH impact may act as a domino on the flux of energy from prokaryotes to the upper level of the trophic chain, with the potential to alter the temporary river functioning.

1. Introduction

Temporary rivers are natural water bodies that experience a recurrent dry phase of varying duration and spatial extent (Uys and O'Keeffe, 1997). The climate change is sharpening these features by increasing the flow seasonality with higher floods in the rainy season and extended dry periods. The predictive models that traditionally rely on outputs from permanent rivers, produces inconsistent projections, since they do not consider all the factors influencing the water quality status at the typically recurrent hydrological phases (e.g. Gallart et al., 2011; Benstead and Leigh, 2012). Despite representing crucial resources for water supply in most arid and semi-arid regions worldwide, temporary rivers have only recently been catalogued by environmental studies and monitoring agencies, by recognizing specific policies to improve the efficiency of tools for an integrated water management (Prat et al., 2014). There is an overall indication that fluctuating streamflow conditions and wet–dry dynamics drive sediment biogeochemical processes during drought and nutrient release during flood periods (Tzoraki et al., 2007; Zoppini et al., 2010; Acuña et al., 2015). However, a tenuous knowledge still exists on the linkages between environmental contamination and river ecosystem functioning under fluctuating flow regime. In particular, the effects of contamination and the fate of hazardous substances have not been firmly established in temporary rivers (Ademollo et al., 2011), while this issue was comparatively better explored in sediments from other aquatic environments (e.g. Bihari et al., 2006; Guo et al., 2012; Pratt et al., 2012).

Among the hazardous contaminants, polycyclic aromatic hydrocarbons (PAHs) are of special concern because they persist in the environment with toxic and carcinogenic effects (Haritash and Kaushik, 2009) and spread even in pristine areas (Johnsen and Karlson, 2007). PAHs enter in riverine environments through different processes, including atmospheric deposition, urban run-off, industrial discharges, oil spillage and leakage, thus being mainly originated from anthropic activities (e.g., fossil fuel combustion) rather than from natural events (e.g., forest fires) (Srogi, 2007). In the aquatic environment, these contaminants have higher affinity for streambed sediments and suspended particulate matter owing to low aqueous solubility and high hydrophobicity (Peng et al., 2008). The WFD and the Directive 2008/105/EC have set Environmental Quality Standards (EQS) for surface waters on the base of a list of priority substances that comprises PAHs. Despite PAHs representing a significant constituent of the sediment carbon pool, (Fernandez et al., 2000; Boyd et al., 2005) only fluoranthene and benzo(a)pyrene are retained within standard quality assessments (CCME, 2003).

Under fluctuating streamflow conditions, river sediments can act either as a transient storage compartment for PAHs during drought or as a contamination source through dissolution and resuspension mechanisms during flooding (Katayama et al., 2010; Zoppini et al., 2014). Besides various abiotic transformations affecting PAH contamination levels (e.g., adsorption, volatilization, photo-oxidation and chemical degradation), the microbial metabolism represents the major biodegradation pathway (Haritash and Kaushik, 2009). A conspicuous number of PAH-degrading bacterial species were isolated from contaminated sediments (Haritash and Kaushik, 2009, and references herein). However, most of the experimental studies on PAH biodegradation focused on specific isomers by following the growth dynamics of selected bacterial species (e.g. Yu et al., 2005; Lei et al., 2005).

So far, the potential effects of an interacting-PAH mixture and the changes imposed on the structural and functional characteristics of natural microbial assemblages were rarely taken into account (e.g. Verrhiest et al., 2002). Moreover, microbial communities in temporary river sediments may play a different and unpredictable role in organic matter oxidation, pollutant degradation and energy transfers to higher levels of the detritus-based food web (Reed and Martiny, 2007; Marxsen et al., 2010; Zoppini and Marxsen, 2011; Casella, 2012; Romaní et al., 2013).

Moving a step forward from field observations on organic pollutants and microbial processes performed on a temporary river (Zoppini et al., 2014), we carried out an experimental study by testing the effects of imposed PAH contamination on the benthic microbial communities. A mixture of seven compounds was used to amend the natural sediment at concentrations ten and hundred times higher than those found at the sampling site. The over imposed PAHs contamination levels fall within the lower range of pollution reported for river sediment (i.e. Patrolecco et al., 2010; Mostafa et al., 2009).

In particular, the present study aimed to assess i) the biodegradation patterns in sediments spiked with different concentrations of seven PAH congeners, and ii) the changes in microbial community structure (i.e., abundance, composition) and function (i.e. cell viability, bacterial carbon production, mineralization rates and extracellular enzyme activities).

2. Materials and methods

2.1. Sampling survey and sediment characterization

The sampling survey was performed on the River Celone (N 41° 29′ 44.6″ E 15° 15′ 18.3″, May 2010), flowing through a semi-arid climatic region of Southern Italy. This is a non-permanent stream, included in the Candelaro river basin, considered as mirror basin in the EU Project MIRAGE (Mediterranean Intermittent River ManAGEment, FP7-ENV-2007-1). This site was described in our previous study (Zoppini et al., 2014) as moderately polluted by PAHs (mean concentration 0.20 ± 0.13 mg $\sum$PAHs kg^{-1}). Sediment samples were collected under regular flow conditions (mean 0.35 ± 0.17 m^3 s^{-1}) from the uppermost oxic layers (0.5–2 cm) of three homogeneous patches. Sediments were sieved through 2 mm mesh, then collected in polycarbonate acid washed buckets (3 dm^3), stored at 4 °C and analysed for chemical and biological parameters, within 24 h (values will be indicated such as *in situ*). The sediment granulometry was assessed according to Gerakis and Baer (1999).

2.2. Experimental design and microcosm setup

After determining the concentration of phenanthrene (Phe), fluoranthene (Flu), benzo(b)fluoranthene (B(b)flu), benzo(k)fluoranthene (B(k)flu), benzo(a)pyrene (B(a)pyr), benzo(g,h,i)perylene (B(g,h,i)per), and indeno(1,2,3-c,d)pyrene (In(1,2,3-cd)pyr) in the natural sediment (0.20 mg $\sum$PAH kg^{-1}), a mix solution of these isomers was prepared in acetone starting from individual PAH stock solutions (100 mg L^{-1} in cyclohexane), supplied by Aldrich (98% purity, Steinheim, Germany). In the mixture, the original proportion among individual concentration of the compounds in the natural sediment, was retained. Four different experimental conditions were ran within 24 h

from sampling: i) 15 control microcosms setup with natural sediments without PAH addition (Control), ii) 15 microcosms spiked with a PAH mixture in concentration ten folds higher than the control (2.4 mg $\sum$PAH kg^{-1}), iii) 15 microcosms spiked with a PAH mixture in concentration hundred-folds higher than the control (21.0 mg ΣPAH kg^{-1}), iv) 3 microcosms setup with autoclaved sediment (121 °C for 15 min) successively spiked with 2.4 mg $\sum$PAH kg^{-1} under sterile conditions (sterile control). The treatments will be indicated such as: Control, sterile control, 2 and 20 mg PAHs kg^{-1}.

The PAH mixtures (2 and 20 mg PAHs kg^{-1}) were added into two empty acid-washed Erlenmeyer glass buckets under sterile conditions. Once acetone was completely evaporated (within 2 h), aliquots of sediments (100 g) were added into the 250 mL buckets together with 200 mL of dissolved organic carbon-free sterile synthetic freshwaters (APHA, AWWA, WPCF, 2012). The water was previously amended with inorganic nutrients (NaH_2PO_4 and KNO_3) in the concentration measured in Celone river (0.16 mmol N and 0.016 mmol P) in the site where sediments were sampled.

The microcosms were incubated under controlled temperature (20 ± 2 °C) in the dark to minimize photolytic and thermal PAH degradation. Each bucket was kept aerated by gentle bubbling with filtered air (Millipore Millex, 0.2 μm) through an aeration distribution system fed by an air diaphragm pump (Serra air 550R, Italy) 550 Lh^{-1}. After an equilibration time of 1 day, the sediments were characterized for physical, chemical and microbial parameters (day 1) and then repeated at intervals of 7 days (days 7, 14, 21 and 28). At each time three buckets per treatment were randomly collected and sacrificed for physical, chemical and microbial measurements, as described hereafter. Sterile sediments were analysed only at the end of the experiment to exclude abiotic changes of PAH concentrations. Before sacrificial sampling, each microcosm was monitored for oxygen saturation, pH and conductivity. All chemical and microbiological analyses were performed in triplicate, and values normalized by dry weight (*w*/*w*).

2.3. Chemical analysis

At the scheduled times, 30 g of homogenized sediment from each replicate microcosm were collected and kept in an oven at 30 °C until complete dryness. PAHs were then extracted from 5 g in three replicates of dried sediments, by sonication in hexane/acetone (1:1, *v*/*v*) (Patrolecco et al., 2010). The extracts were evaporated and reconstituted in acetonitrile/water (60:40, *v*/*v*) to a final volume of 0.5–1 mL.

All sediments were extracted and analysed at least in triplicate, until the relative standard deviation of replicates did not exceed 20%. Final extracts (50 μL) were injected in duplicate in a RP-HPLC (Varian 9012) coupled to a fluorescence detector (PerkinElmer LS4) using a Supelco LC18-PAH column, 5 μm, 250 × 4.6 mm I.D preceded by a guard column (4 × 3 mm) of the same packing material. The elution profile utilized a mobile phase with acetonitrile/water in gradient mode as described in Patrolecco et al. (2010). The detection limits were in the range 0.1–0.3 ng g^{-1} (dry weight) for all PAHs in the sediments. Acetonitrile, hexane and acetone at HPLC grade were from Merck (Darmstadt, Germany). Water for chromatography was ultrapure (Milli-Q system, Millipore).

2.4. Microbial community structure

The prokaryotic cell abundances were determined by epifluorescence microscopy (DAPI staining) and biomass estimated by considering a per-cell C content of 40 fg, as described elsewhere (Zoppini et al., 2014). At least 300 cells were counted in > 10 microscopic fields randomly selected across the filter sections.

For Temperature Gradient Gel Electrophoresis analysis (TGGE), sediment was stored at −20 ± 1 °C without further treatment. Extraction of DNA, amplification of bacterial 16S rRNA genes, separation of gene fragments and evaluation of gels were performed as described earlier (Beier et al., 2008) by using samples from 1, 14 and 28 days of the experiment only. The TGGE bands were treated as operational taxonomic units (OTUs), a surrogate for bacterial species. The relative intensities of the bands (as a measure of abundance) were calculated for each lane and used for further statistical treatment (XLSTAT 2013.4.03, Addinsoft SARL, Andernach, Germany).

Community composition was analysed by Catalysed Reported Deposition Fluorescence *in situ* Hybridization (CARD-FISH) as described by Fazi et al. (2013). The following rRNA-target HRP-labelled probes (Biomers, Ulm, Germany) were used at the beginning (day 1) and the end of the incubation (day 28): ALF968, BET42a, GAM42a and DELTA495a,b,c respectively targeting sequence types affiliated with four classes of Proteobacteria (alpha-, beta-, gamma, delta-Proteobacteria); CF319a for Bacteroidetes (formerly Cytophaga-*Flavobacterium-Bacteroides*); HGC69a for Actinobacteria and LGC354a for bacteria from various genera within the class Bacilli (Firmicutes), (Loy et al., 2003). The stained filter sections were inspected on a Leica DM LB 30 epifluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany) at 1000× magnification.

2.5. Microbial functional parameters

The ATP (Adenosine triphosphate) concentration was determined according to Karl (1993) by extraction in 5 mL of cold 1 M H_3PO_4. Six replicates of wet sediments (1 g) in sterile tubes were heat-killed on a heater block (100 °C for 1 h). Four of those were amended with known concentrations of ATP (internal standard), to estimate adsorptive and other losses of extracted ATP, whereas the other two were utilized as control. TRIS buffer (0.02 M, pH 7.8) was added to all tubes and centrifuged at 4 °C. After removing the supernatant (4 mL), EDTA (1 mL, 48 mM) was added to each tube and pH adjusted to 7.8 with NaOH. The extract was stored at −20 °C and thawed immediately prior to analysis. The ATP extracts were then assayed by using the firefly luciferase–luciferin enzyme (FLE-250, SIGMA) bioluminescence assay utilizing a luminometer Victor X3 multiplate reader 2030 (PerkinElmer). ATP concentrations were converted to carbon equivalents by using the factor 250 mg of C per mg ATP (Karl, 1993).

The bacterial carbon production (BCP) was estimated by measuring the [^{3}H]-leucine incorporation rates as described elsewhere in Amalfitano et al. (2008).

Community respiration (CR) was estimated by measuring the Electron Transport System activity (ETS) by the reduction of the electron transport acceptor INT (2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride) to INTformazan (iodonitrotetrazolium formazan) (Blenkinsopp and Lock, 1990, Romaní et al., 1998). INT was added to 6 aliquots of sediment (1 mg g^{-1} *w*/*w*) at saturating concentration. Two of those aliquots were previously heat-killed (100 °C for 1 h), kept in filtered formaldehyde solution (2% final concentration) and utilized as controls. These sediments were amended with INT-formazan and utilized for the standard curve. Incubations were performed in a shaker at 20 °C for 8–10 h in the dark. The reaction was stopped by the addition of formaldehyde solution. INT-formazan was extracted with cold methanol for at least 1 h at 4 °C in the dark. Successively the samples were sonicated for 2 min at 40 W. The extracts were then centrifuged (3200 rcf) for 5 min at 4 °C. The absorbance was measured spectrophotometrically (Victor X3 multiplate reader 2030 (PerkinElmer) at 480 nm. Conversion factors were then utilized to transform INT-F to O_2 (2:1) and to C units, assuming a respiratory quotient (RQ) of 1.

The extracellular enzyme activities, leucine aminopeptidase (AMA) and alkaline phosphatase (APA), were determined fluorometrically (multiplate reader 2030 Victor X3, PerkinElmer) as described elsewhere (Zoppini et al., 2010).

2.6. Statistical analysis

For TGGE banding, the Shannon–Wiener diversity index H_S was calculated as

$$H_S = -\sum_{i=1}^{S} P_i \ln P_i \quad (1)$$

where P_i is the proportion of OTU i ($\Sigma P_i = 1$) and S is the total number of OTUs for each lane. The evenness J was determined as: $J = H_S/H_{max}$.

Moreover, the Correspondence Analysis (CA) was used as an approach that enabled the analysis of major tendencies of the variance of the bacterial community structures on the basis of TGGE profiles (Fromin et al., 2002).

Three-way ANOVA among the three experimental conditions and the two sampling time points (day 1 and day 28) for the seven bacterial clusters was performed in order to evaluate differences in community composition. In order to evaluate the significant interactions, a pairwise comparison procedure (Student–Newman–Keuls) was applied.

Bivariate relationship between different parameters was examined using the Pearson correlation (r) and the regression analyses (r^2). Two-way ANOVA was performed examining the difference in the mean values among the different levels of time and treatments (Control, 2 and 20 mg PAHs kg^{-1}) to evaluate differences in the structural and functional parameters of the microbial community.

3. Results

3.1. Sediment characteristics and changes in PAH concentrations

The sediment utilized in this experiment resulted composed by 53% silt–clay (<0.063 mm), 15% very fine sand (0.125–0.063 mm), 14% fine sand (0.25–0.125 mm), 15% medium sand (0.5–0.25 mm) and 3% coarse sand (>0.5 mm). Moreover it contained 3.8% AFDW, 0.2 ± 0.01% total N and 1.0 ± 0.05% total organic C and 0.20 mg PAHs kg^{-1}, as the sum of the isomers analysed.

The concentrations of the single PAH isomers over the incubation time are shown in Fig. 1. No changes were observed in the concentration of the single isomers in the control whereas a significant decrease of phenanthrene (Phe) was observed with time ($r = -0.96$, $p < 0.01$) in sediments amended with 2 mg PAHs kg^{-1}. Phe concentration decreased from 0.046 to 0.023 mg kg^{-1} after 28 days, corresponding to a consumption of the 49%. In the sediment amended with 20 mg PAHs kg^{-1} the concentration of PAH isomers were not statistically different during the incubations, with relative standard deviation not exceeding the 20%. No significant changes in the concentrations of the single PAH isomers were observed in the sterile control, thus excluding any potential interference of abiotic factors.

3.2. Changes in the microbial community structure and abundance of dominant taxa

The natural sediment utilized in this experiment showed prokaryotic biomass (mean ± sd, 4.48 ± 0.33 μmol C g^{-1}) and metabolic rates in the range of values previously reported for this ecosystem (Zoppini et al., 2014).

During the experiment the prokaryotic biomass did not change significantly as a consequence of the treatments with respect to the control (ANOVA, $p > 0.05$), with average values 2.15 ± 0.86 μmol C g^{-1}.

The total number of TGGE bands of identical position between samples was 11, deviating between 8 and 11 per sample. No trends were identified from the profiles for diversity (Shannon–Wiener diversity H_s, ranging between 1.76 and 2.23) and evenness (J, ranging between 0.77 and 0.93). Community patterns achieved as TGGE profiles were summarized in a correspondence analysis plot (Fig. 2). Axes 1 and 2 explained 75% of variation (60% and 15%, respectively). No significant

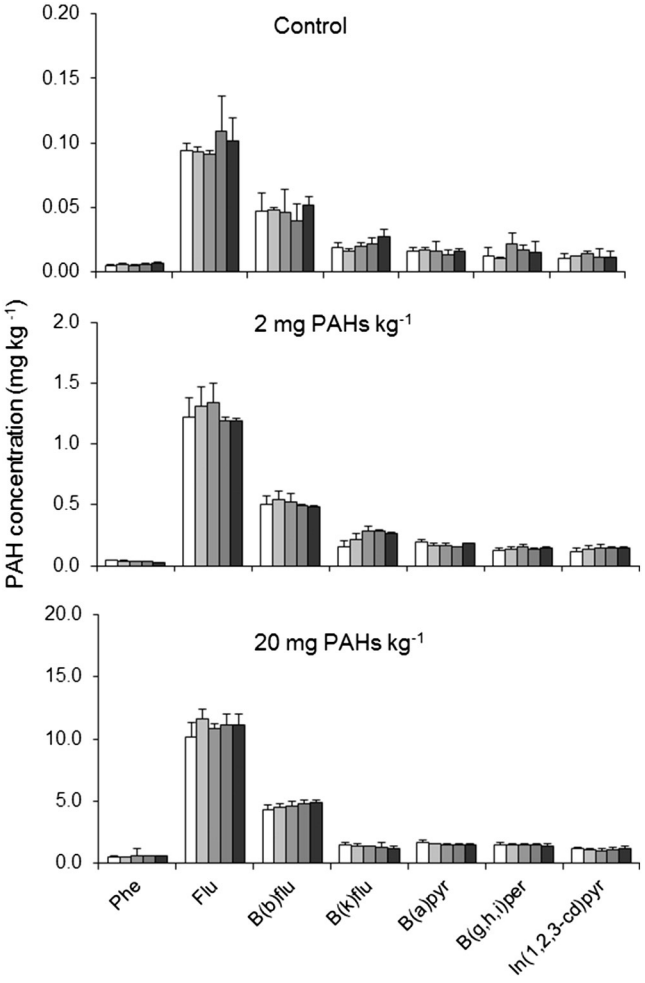

Fig. 1. Concentrations of PAH isomers (mg kg^{-1}) measured in the control and in the spiked sediment (2 and 20 mg PAHs kg^{-1}) during the incubation time at the sampling times (days 1, 7, 14, 21, 28 in an increasing scale of grey). Phe = phenanthrene, Flu = fluoranthene, B(b)flu = benzo(b)fluoranthene, B(k)flu = benzo(k)fluoranthene, B(a)Pyr = benzo(a)pyrene, B(g,h,i)per = benzo(g,h,i)perylene, In(1,2,3-cd)pyr = indeno(1,2,3-c,d)pyrene. Error bars indicate standard deviations.

relationships between occurrence of OTUs and samples were found. Visual inspection of the plot (Fig. 2) allows no clear estimation of any trends between treatments or during time course of the experiment.

Overall, the bacterial cells, hybridized by EUB 338 probes, represented 74.3 ± 10.1% of total DAPI stained cells (prokaryotes) and were all affiliated to the seven analysed groups (Fig. 3). The seven probes covered 77.2 ± 7.0% of prokaryotes.

The community was mainly dominated by Alpha- and Beta-Proteobacteria that represented on average 20.7 ± 4.8% and 27.0 ± 4.8% respectively. Delta-Proteobacteria and Bacteroidetes accounted for 10.6 ± 1.9% and 6.0 ± 1.0% while the other three analysed clusters (Gamma-Proteobacteria, Actinobacteria and Bacilli) were below 5% (Fig. 3). Three-way ANOVA showed a significant interaction among the three experimental conditions and the two sampling time points for the seven bacterial clusters ($F = 8.9$; $P < 0.001$), thus an unambiguous interpretation of the main effects is not possible. All pairwise multiple comparison results, however, showed an overall significant difference between 20 mg PAHs kg^{-1} *vs* Control and 2 mg PAHs kg^{-1} ($q = 9.68$ and 9.49 respectively, $p < 0.001$) with no differences between Control and 2 mg PAHs kg^{-1}. After 28 days of incubation only Alpha- (Control *vs* 2 mg PAHs kg^{-1}: $q = 16.39$, $p < 0.001$; Control *vs* 20 mg PAHs kg^{-1}: $q = 3.83$ $p < 0.05$) and Beta-Proteobacteria (Control *vs* 2 mg PAHs kg^{-1}: $q = 4.76$, $p < 0.001$; Control *vs* 20 mg PAHs kg^{-1}: $q = 6.21$ $p < 0.001$) showed a significant difference between Control

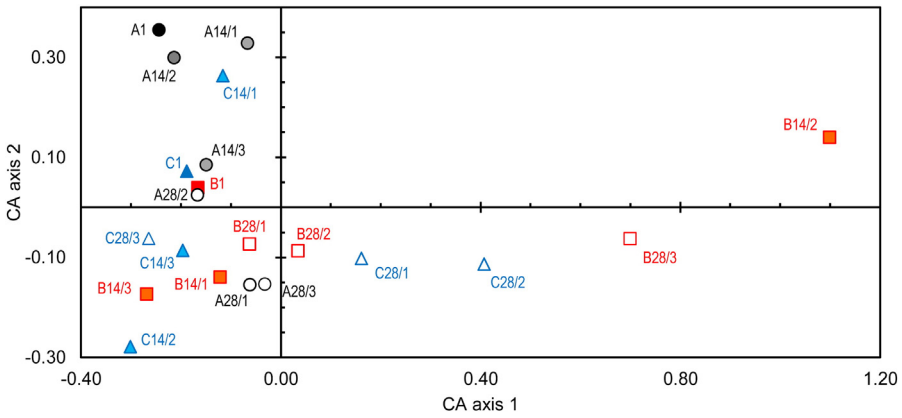

Fig. 2. Correspondence analysis (CA) of bacterial community composition in the control and in the spiked sediments at different incubation time (Full symbols = day 1; pale symbols = day 14; open symbols = day 28), based on TGGE band patterns prepared with 16S rRNA gene fragments. A = control (black circles); B = 2 mg PAHs kg^{-1} (red squares); C = 20 mg PAHs kg^{-1} (blue triangles). The letters are followed by the indications of the incubation time and the number of replicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and the two experimental conditions. In particular, at day 28, Alpha-Proteobacteria showed the highest percentage in the Control (25.7 ± 3.3%) while Beta-Proteobacteria showed the highest percentage at 20 mg PAHs kg^{-1} (26.5 ± 2.3%). No significant differences were observed for all the other bacterial clusters.

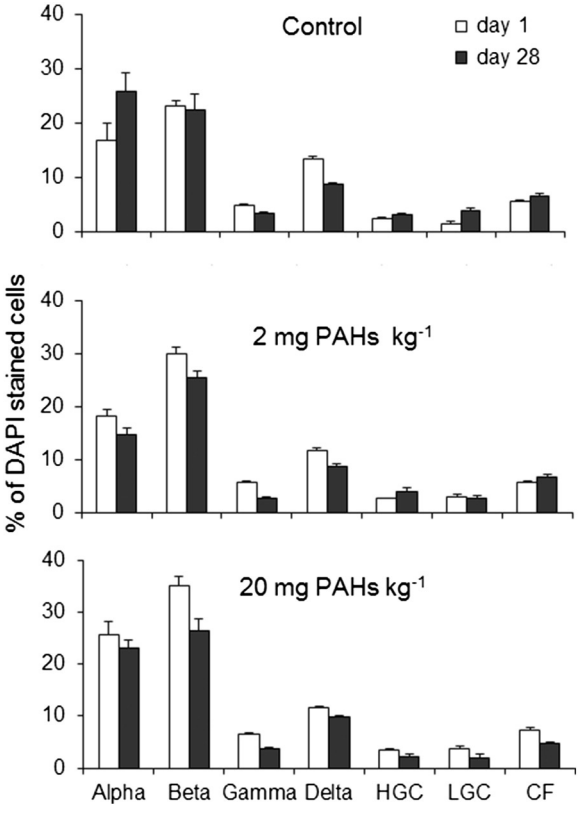

Fig. 3. Taxonomic composition, expressed as percentages of total DAPI-stained cells of seven taxonomic analysed taxa, of bacterial communities at day 1 and day 28 in the control and in the spiked sediments (2 and 20 mg PAHs kg^{-1}). (Alpha = Alpha-Proteobacteria; Beta = Beta-Proteobacteria; Gamma = Gamma-Proteobacteria; Delta = Delta-Proteobacteria; HGC = Actinobacteria; LGC = Bacilli; CF = Bacteroidetes). Error bars indicate standard deviations.

3.3. Changes in the microbial functional properties

The values of the microbial functional properties measured within 24 h from sampling (Fig. 4, *in situ*) did not differ significantly from values measured after 1 day incubation of the unamended sediment (Control).

In the spiked sediments, ATP concentration (Fig. 4a) followed a decreasing trend from the day 7 to day 21 (ATP *vs* time, Pearson correlation, $r = -086$, $p < 0.01$ and $r = -0.84$, $p < 0.05$ in the 2 and 20 mg PAHs kg^{-1} treatment respectively) while an increasing trend was observed in the Control (ATP *vs* time, $r = 0.83$, $p < 0.05$). At day 28 we observed a significant increase of the ATP concentrations in both treated samples ($p < 0.01$), compared to the same treatments at day 21.

In both PAH treatments the community respiration rates (CR) (Fig. 4b) were negatively affected from day 1 in both treatments with respect to the Control (ANOVA, $p < 0.01$) up to recover the control values in the following days (CR vs time, Pearson correlation $r = 0.92$ and $r = 0.91$, $p < 0.001$ respectively). The Control did not show any significant trend over time ($r = 0.33$, $p > 0.05$). A negative correlation was observed between the CR rates and ATP concentration, between days 7 and 21, in both spiked sediments ($r = -0.78$, $p < 0.01$ and $r = -0.75$ $p < 0.05$, in the 2 and 20 mg PAHs kg^{-1} respectively) whereas no correlation was observed in the Control ($r = -0.10$, $p = 0.74$).

The bacterial carbon production rates (BCP, Fig. 4c) were significantly higher in PAH treatments than those assessed in the Control throughout the experiment (ANOVA, $p < 0.01$) (Fig. 4c). The aminopeptidase activity (AMA) showed a similar trend (ANOVA, $p < 0.01$) (Fig. 4d) and was linearly correlated with BCP both in the control and in the 2 mg PAHs kg^{-1} treatment ($r = 0.58$, $p < 0.05$ and $r = 0.67$, $p < 0.01$ respectively). This was not the case in the 20 mg PAHs kg^{-1} treatment ($r = 0.51$, $p = 0.51$).

In both PAH treatments the alkaline phosphatase activity (APA) did not show any significant difference with the control values (ANOVA, $p > 0.05$) (Fig. 4e). A linear positive trend was observed between APA and ATP concentrations in the treated sediments ($r^2 = 0.51$ and 0.84 in the 2 and 20 mg PAHs kg^{-1} respectively, $p < 0.05$), whereas a negative relationship was observed in the control ($r^2 = -0.71$, $p < 0.05$).

4. Discussion

PAH treatments were able to change significantly the functional parameters of the microbial community, although the concentrations of PAH congeners did not change significantly over the incubation time (28 days).

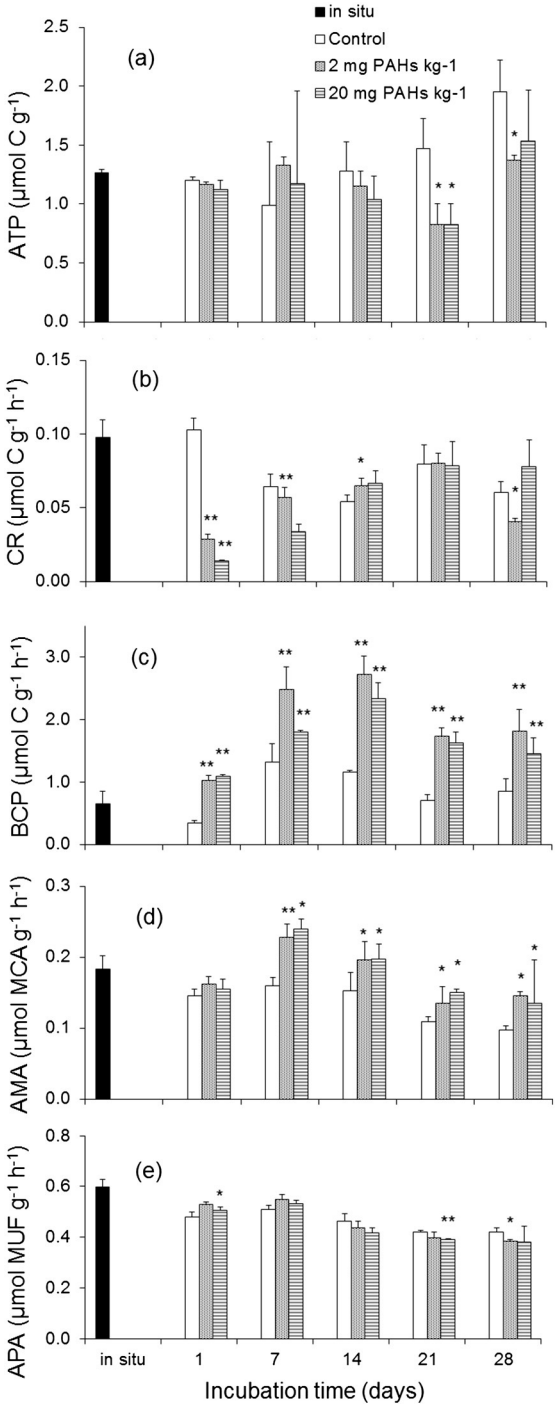

Fig. 4. Functional properties of the benthic microbial community measured *in situ*, in the control and in the amended sediments (2.0 and 20 mg PAHs kg^{-1}) at different incubation times (days). a) Adenosine triphosphate concentrations (ATP); b); Community Respiration rates (CR) c); Bacterial Carbon Production rates (BCP) d) Aminopeptidase Activity (AMA); e) Alkaline Phosphatase Activity (APA). (* = $p < 0.05$; **$p < 0.01$). Error bars indicate standard deviations.

The ability of microbial communities to metabolize and mineralize some PAH compounds depends on the environmental conditions, physical and chemical properties of the contaminants and the contribution of hydrocarbon degrading microbes to the total microbial community (Cerniglia and Heitkamp, 1989; Johnsen and Karlson, 2005; Hilyard et al., 2008). The microbial communities utilized in our study originated from a river site previously described as moderate polluted by PAHs (Zoppini et al., 2014). This may result in a sort of pre-adaptation that, together with the likely occurrence of the co-metabolic reactions (e.g. van Herwijnen et al., 2003), may have favoured the utilization of the phenanthrene in sediments amended with 2 mg PAHs kg^{-1}. Microbial degradation can be considered the major responsible for this result as in the sterile control we did not observe significant changes in PAH concentrations. The observed phenanthrene degradation rates were comparable with those reported for adapted mixed culture strains (Yuan et al., 2000) and natural microbial communities of freshwater sediments (Verrhiest et al., 2002). Specific strains showed high rates in the phenanthrene utilization by adopting different uptake mechanisms (Kallimanis et al., 2007). In sediments with 20 mg PAHs kg^{-1}, the concentration of phenanthrene did not show significant decrease with time, probably indicating toxic effects at this level of contamination. Apart from phenanthrene, in sediments amended with 2 mg PAHs kg^{-1}, the concentrations of PAH congeners did not change significantly over the incubation time (28 days), indicating a scarce microbial degradation potential.

The results obtained by the elaboration of the TGGE profiles showed no trends in the profile of diversity (Shannon-Wiener H_s and the richness S) and evenness, as well no significant relationships between occurrence of dominant OTUs and samples. The total prokaryotic counting and the quantitative results obtained by CARD-FISH showed that Alpha- and Beta-Proteobacteria classes were the only prokaryotic taxa significantly affected by the PAHcontamination at the end of the incubations (day 28): a slight decrease of Alpha- and a slight increase of Beta-Proteobacteria were observed in the amended conditions with respect to the Control. Similar observation was previously reported for PAH enriched cultures of indigenous bacteria of river sediments (Hilyard et al., 2008). The Proteobacteria accounted for the major fraction of the prokaryotes that colonize the benthic substrates in temporary river sediments (Romanì et al., 2013). The Alpha- and Beta-subgroups of this highly diversified phylum were reported to be highly efficient in nutrient assimilation and to lead transformation processes of organic compounds in association with specific extracellular enzyme activities (Zoppini et al., 2010). Therefore, our results could represent a further confirmation that the active players within the river sediment processes should be retrieved within those two classes, even at low PAH contamination levels.

In both PAH treatments the functional parameters of the microbial community showed significant changes with respect to the Control and more prominent modifications than those observed in the community structure at the high phylogenetic level herewith investigated. The bacterial community showed significant increases of the bacterial C production (BCP) rates with respect to the control. Synergies between metabolic activities delegated to anabolic processes, were also observed. Indeed, aminopeptidase extracellular enzyme activity (AMA), important in retrieving labile substrata from the organic matter (i.e. amino acids), correlated with the BCP rates along the incubation time. Moreover, alkaline phosphatase extracellular enzyme activity (APA), whose role is to retrieve inorganic phosphorous from the organic matter, correlated with the ATP concentration, probably in support of the phosphorylation processes. These two key functions of the cellular metabolism decreased together with the incubation time only in the treated sediments, highlighting an increasing cellular alteration. The stimulation of the anabolic functions was accompanied by a steady prokaryotic biomass concentration and by a decrease of the ATP concentration. Such decrease reflects a decrease in the cell viability, being the ATP molecule essential for the energetic purposes of living cells and utilized as an index of the cellular stress and toxicity (e.g. Reid et al., 1998; Romero et al., 2008). Interestingly this scenario is also accompanied by the significant negative correlation between the ATP concentration and respiration rates (CR) in the treated sediments. In viable cells, substrate oxidation creates a proton motive force across the intracellular cytoplasm membrane and this provides the driving force for the phosphorylation of ADP to form high energy covalent bonds in ATP. This process can be influenced by a number of compounds most of which are either uncouplers or inhibitors of oxidative phosphorylation (Haubenstricker

et al., 1990; Low et al., 2000). PAHs are known to inhibit the mitochondrial electron transport system by preventing the ATP-forming mechanism in eukaryotic cells (Tripuranthakam et al., 1999; Falahatpisheh et al. 2007). The uncoupling of oxidative phosphorylation as a specific mechanism of action of chemicals on prokaryotic cells has been matter of debate (Jaworska and Schultz, 1994; Reid et al., 1998; Low et al., 2000).

5. Conclusions

Although microbial communities of temporary rivers are adapted to drought and flood and efficiently utilize the available organic matter pulses (e.g. Zoppini et al., 2014), in this experimental work we have observed a poor efficiency in the decomposition of PAHs and the detrimental effects of these pollutants on cell functional integrity (i.e. detrimental effect on the oxidative phosphorylation processes).

At the high phylogenetic level herewith tested, the community structure (i.e., dominant OTUs, total and class level abundance) did not show significant changes with the PAH amendments, with the only exception of two dominant clusters (Alpha- and Beta-Proteobacteria).

The current study brings evidences of the impact of the PAHs on microbial communities inhabiting temporary rivers, by catching the effects on metabolic processes, usually neglected, occurring within few hours from the impact. The effect of impoverishment of the energetic resources that follows the PAH impact may act as a domino on the flux of energy from prokaryotes to the upper level of the trophic chain, with the potential to alter the temporary river functioning.

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References

Acuña, V., Casellas, M., Corcoll, N., Timoner, X., Sabater, S., 2015. Increasing extent of periods of no flow in intermittent waterways promotes heterotrophy. Freshw. Biol. http://dx.doi.org/10.1111/fwb.12612.

Ademollo, N., Capri, S., Patrolecco, L., Puddu, A., Polesello, S., Rusconi, M., et al., 2011. Fate and monitoring of hazardous substances in temporary rivers. TrAC-Trend. Anal. Chem. 30, 1222–1232.

Amalfitano, S., Fazi, S., Zoppini, A., Caracciolo, A.B., Grenni, P., Puddu, A., 2008. Responses of benthic bacteria to experimental drying in sediments from Mediterranean temporary rivers. Microb. Ecol. 55 (2), 270–279.

APHA, AWWA, WPCF, 2012. Preparing organisms for toxicity tests. In: Rice, EW, Baird, R.B, Eaton, A.D, Clesceri, L.S (Eds.), Standard Methods for the Examination of Water and Wastewater, 22st edn American Public Health Association, Washington, DC, pp. 8/7–8/14.

Beier, S., Witzel, K.P., Marxsen, J., 2008. Bacterial community composition in Central European running waters examined by temperature gradient gel electrophoresis and sequence analysis of 16S rRNA genes. Appl. Environ. Microbiol. 74, 188–199.

Benstead, J.P., Leigh, D.S., 2012. An expanded role for river networks. Nat. Geosci. 5, 678–679.

Bihari, N., Fafand, M., Hamer, B., Kralj-Bilen, B., 2006. PAH content, toxicity and genotoxicity of coastal marine sediments from the Rovinj area, Northern Adriatic, Croatia. Sci. Total Environ. 366 (2), 602–611.

Blenkinsopp, S.A., Lock, M.A., 1990. The measurement of electron transport system activity in river biofilms. Water Res. 24, 441–445.

Boyd, T.J., Montgomery, M.T., Steele, J.K., Pohlman, J.W., Reatherford, S.R., Spargo, B.J., et al., 2005. Dissolved oxygen saturation controls PAH biodegradation in freshwater estuary sediments. Microb. Ecol. 49, 226–235.

Casella, P., 2012. Study of benthic microbial communities in temporary rivers: effect of hydrological conditions on nutrient flux and fate of organic micropollutants (PhD thesis), Department of Ecological and Biological Sciences, University of Tuscia, Viterbo (Ed) (http://dspace.unitus.it/handle/2067/2533).

CCME, 2003. Canadian Council of Ministers of the Environment, Canadian environmental quality guidelines (Winnipeg, Manitoba (USA). Winnipeg, Manitoba (USA)) .

Cerniglia, C.E., Heitkamp, M.A., 1989. Microbial degradation of polycyclic aromatic hydrocarbons (PAH) in the aquatic environment. Metabolism of Polycyclic Aromatic Hydrocarbons in the Aquatic Environment. CRC Press Inc., New York, pp. 41–68.

Directive 2008/105/EC of the European Parliament and the Council of 16 December 2008 on environmental quality standards in the field of water policy.

Falahatpisheh, H., Nanez, A., Montoya-Durango, D., Qian, Y., Tiffany-Castiglioni, E., Ramos, K.S., 2007. Activation profiles of HSPA5 during the glomerular mesangial cell stress response to chemical injury. Cell Stress Chaperones 12, 209.

Fazi, S., Vàzquez, E., Casamayor, E.O., Amalfitano, S., Butturini, A., 2013. Stream hydrological fragmentation drives bacterioplankton community composition. PLoS One 8 (5), e64109.

Fernandez, P., Vilanova, R.M., Martinez, C., Appleby, P., Grimalt, J.O., 2000. The historical record of atmospheric pyrolytic pollution over Europe registered in the sedimentary PAH from remote mountain lakes. Environ. Sci. Technol. 34, 1906–1913.

Fromin, N., Hamelin, J., Tarnawski, S., Roesti, D., Jourdain-Miserez, K., Forestier, N., et al., 2002. Statistical analysis of denaturing gel electrophoresis (DGE) fingerprinting patterns. Environ. Microbiol. 4 (11), 634–643.

Gallart, G.F., Latrón, J., Llorens, P., Barbera, G.G., Quiñonero, J.M., Froebrich, J., 2011. Developing a novel approach to analyse the regimes of temporary streams and their controls on aquatic biota. Hydrol. Earth Syst. Sci. Discuss. 8 (5), 9637–9673.

Gerakis, A., Baer, B., 1999. A computer program for soil textural classification. Soil Sci. Soc. Am. J. 63 (4), 807–808.

Guo, C.L., Ke, L., Dang, Z., Tam, N.F., 2012. Effect of exposure time, contamination level, and type of PAH compound on biodegradation capacity of mangrove sediment. Soil Sediment Contam. 21 (3), 291–304.

Haritash, A.K., Kaushik, C.P., 2009. Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): a review. J. Hazard. Mater. 169, 1–15.

Haubenstricker, M.E., Holodnick, S.E., Mancy, K.H., Brabec, M.J., 1990. Rapid toxicity testing based on mitochondrial respiratory activity. Bull. Environ. Contam. Toxicol. 44, 675–680.

van Herwijnen, R., Wattiau, P., Bastiaens, L., Daal, L., Jonker, L., Springael, D., et al., 2003. Elucidation of the metabolic pathway of fluorene and cometabolic pathways of phenanthrene, fluoranthene, anthracene and dibenzothiophene by *Sphingomonas* sp. LB126. Res. Microbiol. 154 (3), 199–206.

Hilyard, E.J., Jones-Meehan, J.M., Spargo, B.J., Hill, R.T., 2008. Enrichment, isolation, and phylogenetic identification of polycyclic aromatic hydrocarbon-degrading bacteria from Elizabeth River sediments. Appl. Environ. Microbiol. 74 (4), 1176–1182.

Jaworska, J.S., Schultz, T.W., 1994. Mechanism-based comparisons of acute toxicities elicited by industrial organic chemicals in procaryotic and eucaryotic systems. Ecotox. Environ. Safe 29, 200–213.

Johnsen, A.R., Karlson, U., 2005. PAH degradation capacity of soil microbial communities-does it depend on PAH exposure? Microb. Ecol. 50, 488–495.

Johnsen, A.R., Karlson, U., 2007. Diffuse PAH contamination of surface soils: environmental occurrence, bioavailability, and microbial degradation. Appl. Microbiol. Biotechnol. 76, 533–543.

Kallimanis, A., Frillingos, S., Drainas, C., Koukkou, A.I., 2007. Taxonomic identification, phenanthrene uptake activity, and membrane lipid alterations of the PAH degrading *Arthrobacter* sp. strain Sphe3. Appl. Microbiol. Biotechnol. 76 (3), 709–717.

Karl, D.M., 1993. Total microbial biomass estimation derived from the measurement of particulate adenosine-5′-triphosphate. I. In: Kemp, P.F., SB, Cole, J.J. (Eds.), Handbook of Methods in Aquatic Microbial Ecology. Lewis Pub-Publishers, Boca Raton, FL, pp. 359–368.

Katayama, A., Bhula, R., Burns, G.R., Carazo, E., Felsot, A., Hamilton, D., et al., 2010. Bioavailability of xenobiotics in the soil environment. Rev. Environ. Contam. Toxicol. 203, 1–86.

Lei, L., Khodadoust, A.P., Suidan, M.T., Tabak, H.H., 2005. Biodegradation of sediment-bound PAHs in field-contaminated sediment. Water Res. 39 (2), 349–361.

Low, E.W., Chase, H.A., Milner, M.G., Curtis, T.P., 2000. Uncoupling of metabolism to reduce biomass production in the activated sludge process. Water Res. 4 (12), 3204–3212.

Loy, A., Horn, M., Wagner, M., 2003. probeBase: an online resource for rRNA-targeted oligonucleotide probes. Nucleic Acids Res. 31 (1), 514–516.

Marxsen, J., Zoppini, A., Wilczek, S., 2010. Microbial communities in streambed sediments recovering from desiccation. FEMS Microbiol. Ecol. 71 (3), 374–386.

Mostafa, A.R., Wade, T.L., Sweet, S.T., Al-Alimi, A.K.A., Barakat, A.O., 2009. Distribution and characteristics of polycyclic aromatic hydrocarbons (PAHs) in sediments of Hadhramout coastal area, Gulf of Aden, Yemen. J. Mar. Syst. 78 (1), 1–8.

Patrolecco, L., Ademollo, N., Capri, S., Pagnotta, R., Polesello, S., 2010. Occurrence of priority hazardous PAHs in water, suspended particulate matter, sediment and common eels (*Anguilla anguilla*) in the urban stretch of the River Tiber (Italy). Chemosphere 81 (11), 1386–1392.

Peng, R.H., Xiong, A.S., Xue, Y., Fu, X.Y., Gao, F., Zhao, W., et al., 2008. Microbial biodegradation of polyaromatic hydrocarbons. FEMS Microbiol. Rev. 32 (6), 927–955.

Prat, N., Gallart, F., Von Schiller, D., Polesello, S., García, R., Latron, E.M., et al., 2014. The Mirage Toolbox: an integrated assessment tool for temporary streams. River Res. Appl. 30 (10), 1318–1334.

Pratt, B., Riesen, R., Johnston, C.G., 2012. PLFA analyses of microbial communities associated with PAH-contaminated riverbank sediment. Microb. Ecol. 64 (3), 680–691.

Reed, H.E., Martiny, J.B.H., 2007. Testing the functional significance of microbial composition in natural communities. FEMS Microbiol. Ecol. 62, 161–170.

Reid, B.J., Semple, K.T., Macleod, C.J., Weitz, H.J., Paton, G.I., 1998. Feasibility of using prokaryote biosensors to assess acute toxicity of polycyclic aromatic hydrocarbons. FEMS Microbiol. Lett. 169 (2), 27–233.

Romaní, A.M., Amalfitano, S., Artigas, J., Fazi, S., Sabater, S., Timoner, X., et al., 2013. Microbial biofilm structure and organic matter use in Mediterranean streams. Hydrobiologia 719 (1), 43–58.

Romaní, A.M., Butturini, A., Sabater, F., Sabater, S., 1998. Heterotrophic metabolism in a forest stream sediment: surface versus subsurface zones. Aquat. Microb. Ecol. 16, 143–151.

Romero, A., Santos, A., Tojo, J., Rodriguez, A., 2008. Toxicity and biodegradability of imidazolium ionic liquids. J. Hazard. Mater. 151 (1), 268–273.
Srogi, K., 2007. Monitoring of environmental exposure to polycyclic aromatic hydrocarbons: a review. Environ. Chem. Lett. 5, 169–195.
Tripuranthakam, S., Duxbury, C.L., Babu, T.S., Greenberg, B.M., 1999. Development of a mitochondrial respiratory electron transport bioindicator for assessment of aromatic hydrocarbon toxicity. In: Henshel, D.S., Black, M.C., Harrass, M.C. (Eds.), Environmental Toxicology and Risk Assessment: Standardization of Biomarkers for Endocrine Disruption and Environmental Assessment. ASTM STP, Toronto, Canada, pp. 350–361.
Tzoraki, O., Nikolaidis, N.P., Amaxidis, Y., Skoulikidis, N.T., 2007. In-stream biogeochemical processes of a temporary river. Environ. Sci. Technol. 41 (4), 1225–1231.
Uys, M.C., O'keeffe, J.H., 1997. Simple words and fuzzy zones: early directions for temporary river research in South Africa. Environ. Manag. 21 (4), 517–531.
Verrhiest, G.J., Clement, B., Volat, B., Montuelle, B., Perrodin, Y., 2002. Interactions between a polycyclic aromatic hydrocarbon mixture and the microbial communities in a natural freshwater sediment. Chemosphere 46, 187–196.
Yu, K.S.H., Wong, A.H.Y., Yau, K.W.Y., Wong, Y.S., Tam, N.F.Y., 2005. Natural attenuation, biostimulation and bioaugmentation on biodegradation of polycyclic aromatic hydrocarbons (PAHs) in mangrove sediments. Mar. Pollut. Bull. 51, 1071–1077.
Yuan, S.Y., Wei, S.H., Chang, B.V., 2000. Biodegradation of polycyclic aromatic hydrocarbons by a mixed culture. Chemosphere 4, 1463–1468.
Zoppini, A., Marxsen, J., 2011. Importance of extracellular enzymes for biogeochemical processes in temporary river sediments during fluctuating dry–wet conditions. In: Shukla, G., Varma, A. (Eds.), Soil Enzymology, Soli Biology. Springer, Berlin Heidelberg, pp. 103–117.
Zoppini, A., Ademollo, N., Amalfitano, S., Casella, P., Patrolecco, L., Polesello, S., 2014. Organic priority substances and microbial processes in river sediments subject to contrasting hydrological conditions. Sci. Total Environ. 484, 74–83.
Zoppini, A., Amalfitano, S., Fazi, S., Puddu, A., 2010. Dynamics of a benthic microbial community in a riverine environment subject to hydrological fluctuations (Mulargia River, Italy). Hydrobiologia 657, 37–51.