# THE EFFECTS OF SEDIMENT DEPTH AND OXYGEN CONCENTRATION ON THE USE OF ORGANIC MATTER: AN EXPERIMENTAL STUDY USING AN INFILTRATION SEDIMENT TANK

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**ABSTRACT**

Water flowing through hyporheic river sediments or artificial recharge facilities promotes the development of microbial communities with sediment depth. We performed an 83-day mesocosm infiltration experiment, to study how microbial functions (e.g., extracellular enzyme activities and carbon substrate utilization) are affected by sediment depth (up to 50 cm) and different oxygen concentrations. Results indicated that surface sediment layers were mainly colonized by microorganisms capable of using a wide range of substrates (although they preferred to degrade carbon polymeric compounds, as indicated by the higher β-glucosidase activity). In contrast, at a depth of 50 cm, the microbial community became specialized in using fewer carbon substrates, showing decreased functional richness and diversity. At this depth, microorganisms picked nitrogenous compounds, including amino acids and carboxyl acids. After the 83-day experiment, the sediment at the bottom of the tank became anoxic, inhibiting phosphatase activity. Coexistence of aerobic and anaerobic communities, promoted by greater physicochemical heterogeneity, was also observed in deeper sediments. The presence of specific metabolic fingerprints under oxic and anoxic conditions indicated that the microbial community was adapted to use organic matter under different oxygen conditions. Overall the heterogeneity of oxygen concentrations with depth and in time would influence organic matter metabolism in the sediment tank.

**Keywords**: carbon substrate use, extracellular enzyme activities, bacteria, anoxia.

## 1. INTRODUCTION

The connection between surface water, groundwater and the processes occurring in this interface (i.e., the hyporheic sediment) are important for river ecosystem metabolism (Brunke and Gonser, 1997; Nogaro et al., 2013). The hyporheic zone promotes the exchange of water, nutrients, and biota between alluvial groundwater and stream water (Boulton et al., 1998). This exchange, in turn, influences stream water quality (Sobczak and Findlay, 2002). Microbial communities in sediments are principally composed of heterotrophic microorganisms including bacteria, fungi, and small metazoans which are attached to sand grains and assembled in a polymeric matrix (Pusch et al., 1998) that plays key roles in biogeochemical processes (Findlay et al., 1993; Mermillod-Blondin et al., 2005). Microbial communities are responsible for most of the metabolic activity in hyporheic sediments (Storey et al., 1999), including the degradation of organic matter and the reduction of electron acceptors (e.g., oxygen, nitrate and sulphate) (Ghiorse and Wilson, 1988; Hedin et al., 1998). These processes act as water purification processes, ultimately impacting the water quality of river and aquifer systems. Similarly, when water flows through sediments in the vadose zone, microbial activity enhance the quality of surface water as in slow infiltration bed or in managed artificial recharge facilities (Greskowiak et al., 2005) removing organic carbon content, nutrients and trace organic chemicals (Li et al., 2012; Regnery et al., 2015).

Decomposition of organic matter is one of the main metabolic roles of microorganisms in soils and sediments. Extracellular enzymes released by microbes promote organic carbon cycling, by transforming polymeric material into soluble monomers that can be assimilated by microbes. These actions constitute a limiting step in the entrance of organic matter to the food web (Allison et al., 2007; Romaní et al., 2012). Although many studies have analyzed enzyme activities in surface sediments (Romaní and Sabater, 2001), much less is known

about how these activities change according to depth. For instance, in the upper 12 cm of river sediment, extracellular enzyme activities involved in the degradation of cellulose, hemicellulose, and organic phosphorus compounds decreased together with bacterial density (Romaní et al., 1998). Changes in the utilization of organic matter at different sediment depths may be linked to microbial colonization. Indeed, microorganisms are found in largest quantities at the soil surface, and their abundance declines rapidly with increasing depth (Taylor et al., 2002). Microbially active zones are often limited to the top sediment layer (<60 cm) where bacterial biomass and exchange rates between the river and the hyporheic zone are the highest (Taylor et al., 2002). Bacteria in deeper sediments are more sensitive to physical and chemical changes compared to those in surface layers (Fierer et al., 2003) due to the relatively more stable conditions (Fischer et al., 2005). In deeper sediments, organic matter use may be further affected by physical and chemical changes in oxygen, pH, temperature and nutrient availability (Douterelo et al., 2011). Moreover, the sediment biofilm structure reduces the water infiltration capacity by pore clogging (e.g. Or et al. 2007), also decreasing the soil porosity, stream bed permeability, and thus the water exchange between river and vadose zone (Brunke and Gonser, 1997; Descloux et al., 2010).

Physicochemical conditions appear to be highly heterogeneous at different sediment depths (Storey et al., 1999), and this heterogeneity promotes the coexistence of aerobic and anaerobic microbial communities in sediments (Harvey et al., 1995; Storey et al., 1999). Previous publications showed vertical oxygen consumption in sediments (Glud et al., 2005; Revsbech et al., 1986), but they did not focus on how to link this oxygen gradient to the decomposition of organic matter along the sediment's profile. This is in spite of oxygen and organic matter being known to play key roles in nutrient cycles (Hedin et al., 1998; Nogaro et al., 2013; Rubol et al., 2012). Low oxygen content and redox potential in deeper sediments may cause shifts in microbial metabolism. Indeed, decomposition of organic matter is more

rapid and efficient in oxygenic conditions (Storey et al., 1999) and some extracellular enzymatic activities are inhibited in anoxic conditions (Goel et al., 1998).

The objective of this study was to analyze changes in microbial organic matter use at different sediment depths under continuous infiltration conditions. We hypothesized that microbial activity and biomass would be higher at the sediment surface and decline with depth. At that deeper layer gradients would be more pronounced at the end of the experiment consistent with a vertical oxygen gradient. Specifically, the experiment aims at: i) analyzing organic matter decomposition capabilities and microbial functional diversity of the community developed in depth as a result of a colonization sequence; and ii) investigating the vertical changes of organic matter use due to different oxygenic conditions.

To reach these objectives, a 1- meter sediment tank with continuous infiltration of synthetic water was used to monitor several physical and chemical parameters, including oxygen, temperature, conductivity, inorganic nutrients, dissolved organic carbon, and microbial metabolism. Activities of β-glucosidase, leucine-aminopeptidase and phosphatase were assessed to monitor the hydrolysis of organic compounds containing carbon, nitrogen, and phosphorus (Romaní et al., 2012). Functional diversity and functional fingerprints of sediment microbial communities were analyzed on the community-level using Biolog Ecoplates (Salomo et al., 2009). A meso-scale was chosen to produce biogeochemical and microbial parameters under controlled interstitial flow conditions, similar to those experimental studies using sediment columns (Battin et al., 1999; Mermillod-Blondin et al.,2005) or a sediment tank (Weber and Legge, 2011).

## 2. MATERIAL AND METHODS

### 2.1 Experimental design

An infiltration (flow-through) experiment was conducted in a vertical intermediate-scale tank reconstructed with a heterogeneous sediment porous media. The dimensions of the sediment tank were 1.20 m high × 0.45 m long × 0.15 m wide. The base of the tank was filled with a 15 cm layer of silicic sand (0.7 to 1.8 mm diameter, supplied by Triturados Barcelona, Inc.) covered with a permeable geo-synthetic fabric membrane to prevent soil flowing through. Sediments were collected from a managed aquifer recharge facility site located in the Llobregat River near Barcelona (UTM coordinates 418446.63 N, 4581658.18 E). Dry sediments were sieved at 0.5 cm and packed in the tank by a repeating series of wetting and drying cycles, see Rubol et al. (2014) for details. The top 20 cm of the tank were left free of sediment to allow ponding. A concentrated synthetic solution of 10L mixture of inorganic and organic compounds was prepared in a carboy. This concentrated solution was diluted with deionized water prior to its injection into the infiltration pond of the tank. The carboy solution was continuously mixed with a magnetic stirrer (AREX 230v/50Hz, VELP Scientific) and supplied at the surface of the tank with no recirculation. The carboy was replaced every 4-7 days (depending on water consumption). The chemical composition of the mixture mimics the typical Llobregat River water reported by Fernández-Turiel et al. (2003) which is characterized by high nutrient content ($NO_3$ ~ 8mg/L, $NH_4$~ 1.2mg/L , $PO_4$~ 0.5mg/L and DOC~ 7 mg/L, Table 1).

The upper layer in the tank was exposed to natural light (sun light reaching the tank from the laboratory windows), while the lateral walls were covered with dark plastic to prevent photoautotrophic activity. To stimulate biofilm colonization, an inoculum prepared from sediment collected at the pristine riverbed nearby the site, was added to the top of the tank at the beginning of the experiment. This inoculum contained $2.27 \pm 0.41 \times 10^6$ bacterial cells/mL (mean value ± standard error).

The tank was equipped with duplicate liquid ports (located in the middle and on the left part of the sediment tank) at depths 5, 15, 30, 45 and 58 cm (all distances are measured from the surface of the sediment). Sediment sampling ports consisting of 1.5 cm horizontal holes tapped with cork caps located at 20 and 50 cm depth on the right part of sediment tank. Samples at 20 and 50cm depth were collected with a methacrylate corer (1.5 cm in diameter, 12 cm long) displayed horizontally and samples from the surface were collected vertically. Despite sampling collection led to local changes in hydraulic conductivity right after sampling, the system used minimizes the overall impact as it readjusts quickly to fill the gap created. Then, three subsamples of 0.5 mL of sediment from the beginning, middle and edge of each core were collected in triplicate with an uncapped syringe for each analysis.

**2.2 Physical and chemical analysis**

Measured values of temperature and volumetric water content were recorded continuously by using capacitance sensors (5TE, Decagon Devices, Pullman, WA) placed at 3 different depths. A handheld multiparameter instrument (YSI Professional Plus) recorded temperature, electrical conductivity, dissolved oxygen and pH in continuous at the tank outlet. Dissolved oxygen concentrations were measured continuously with optical fibers (FiboxPresens, Germany) and corrected for temperature. The evolution of infiltration rate (R(t)) with time was determined from the water balance accounting for the infiltration rate and temporal changes in the ponding water level. Direct evaporation was estimated, and found negligible to the overall balance.

Inorganic nutrients were measured from the water samples collected at days 0, 3, 8, 13, 16, 20, 24, 28, 33, 36, 40, 43, 49, 53 and 83 at 5 depths (5, 15, 30, 45 and 58 cm measured from the surface). Water samples were collected in 9 mL vacuum vials and filtered at 0.2 μm (Whatman). Analysis for $NO_3^-$, $NH_4^+$ and Cl- were performed by High Performance Liquid

Chromatography (HPLC). Measurements of dissolved organic carbon (DOC) were obtained at the same depths as those of nutrients from the water samples collected at days 13, 16, 20, 24, 28, 33, 36, 40, 43, 49, 53 and 83. Samples were filtered (Whatman GF/F), conditioned with 2M HCl and stored at 5°C until analyses were performed. DOC was measured using a total organic carbon analyzer (Shimadzu TOC-V-CSH 230V, Tokyo, Japan). Three replicates were used for each sample. Due to technical problems phosphate ($PO_4$) were analyzed only at days 3, 13, 49 and 83 at 3 different depths (5, 15, and 45 cm). Phosphate was analyzed spectrophotometrically as described by Murphy and Riley (1962).

**2.3 Biological analysis**

Microbial activity and bacterial abundance were analyzed from sediment samples and were processed during the same sampling day. Samples for extracellular enzyme activities were collected on days 0, 3, 6, 9, 14, 21, 34, 50 and 83. Bacterial abundance and viability and sole-carbon-source utilization profiles (Biolog Ecoplates) were estimated on days 3, 14, 34, 50 and 83.

*2.3.1 Bacterial abundance and viability*

Live and dead bacteria in sediment were counted using Live/Dead bacterial viability kit (Invitrogen Molecular Probes, Inc.). On each sampling day, each collected sediment subsample (1 mL of sand volume) from each depth (3 replicates per depth) was placed in a sterile vial with 10 mL of Ringer solution (Scharlau S.L). Bacteria were detached from sediment after sonication for 1 min using an ultrasonic bath (Selecta, 40 W and 40 kHz). The extract was diluted (20 times at the beginning of the experiment, 50 times from day 14) with Ringer solution. The diluted sediment extract was then used for bacterial density and viability analysis and also as the inoculum for the Biolog Ecoplates incubations (see below). The

extract dilution was determined in advance following the recommendations for Ecoplates incubations with bacterial density values around $10^6$ cell $mL^{-1}$ (Garland et al., 2001).

For each diluted sediment extract, 2 mL were stained by a 1:1 mixture of Syto9 and Propidium Iodide and incubated for 15 min in dark conditions. Samples were filtered through a 0.2 µm pore-size black polycarbonate filters (GE Water and Process Technologies) and then mounted on a microscope slide. Twenty randomly chosen fields were counted for each slide for live and dead bacteria (Nikon E600 epifluorescence microscope, 1000X, Nikon Corporation, Tokyo, Japan). Results are expressed as cells/ g DW (dry weight) of sediment.

*2.3.2 Extracellular enzyme activities*

Three extracellular enzyme activities were analyzed in the sediment, linked to the capacity to decompose cellobiose (β-glucosidase activity, EC 3.2.1.21, BG), peptides (leucine-aminopeptidase activity, EC 3.4.11.1, LEU) and phosphomonoesters (phosphatase activity, EC 3.1.3.1, PHO).

Extracellular enzyme activities were determined with a spectrofluorometer using artificial fluorescent substrates 4-methylumbelliferone (MUF)-β-D-glucoside, MUF-phosphate, and L-leucine-4-7-methylcoumarylamide (AMC), for BG, PHO, and LEU, respectively in triplicate for each time and depth. Sediment samples were placed in vials filled with 4 mL of filtered water from the tank (0.2 µm nylon, Whatman). Samples were incubated at saturating conditions (final concentration of 300 µM) at 20ºC under continuous shaking (150 rpm) during 1 h in dark conditions. Blanks (with 0.2 µm filtered water from the tank) were also incubated to eliminate the background signals and water fluorescence. At the end of the incubation period, 4 mL of glycine buffer (pH 10.4) solution was added, and fluorescence was measured at 365/455 nm excitation/emission wave lengths for MUF and at 364/445 nm excitation/emission wave lengths for AMC (Kontron SFM 25, Munich, Germany). Standard

curves (0-200 nmol/L) were prepared for MUF and AMC, separately. Activity values are expressed as nmol of AMC or MUF released per g DW of sediment per hour.

*2.3.3 Carbon substrate utilization profiles*

Biolog Ecoplates (Biolog Inc., Hayward, California, USA) were used in order to determine the differences in the metabolic fingerprint in time and depth of the sediment tank based on carbon source utilization.

Each sampling day, the diluted sediment extracts from each depth (3 replicates per depth, see extraction procedure in Bacterial abundance and viability section) were incubated in the Ecoplates immediately after sampling (maximum 5 h). Ecoplates were inoculated with 130 µL of each sediment extract under sterile conditions and incubated at 20ºC in dark conditions for 6 days. Optical density (OD) in the plates was read every 24 h at 590 nm using a microplate reader (SynergyTM 4, BioTek, Winooski, VT, USA). After 6 days (144 h) most wells had achieved sigmoid color development saturation and the AWCD (Average Well Color Development) was close to 0.6 (Insam and Goberna, 2004). Raw absorbance data obtained from Biolog Ecoplates were corrected by the mean absorbance of the control wells (3 wells with no substrate) in each plate. Values < 0.05 (or negative) were set to zero. Data from each Ecoplate were analyzed by calculating the AWCD, Shannon diversity index (H') and substrate richness (S) to evaluate microbial community functional diversity and functional richness (Garland and Mills, 1991). Substrate richness is the number of different substrates used by the community (counting all positive OD readings, i.e., positive wells). Moreover, kinetic analysis was carried out for AWCD for each time and depth. Three kinetic parameters (a, 1/b and x0) were estimated by fitting the curve of color development on plates to a sigmoid equation (Freixa and Romaní, 2014; Lindstrom et al., 1998) where a is the

maximum absorbance in the event of color saturation, 1/b is the slope of the maximum rate of color development and x0 is the time when maximum color development rate is achieved. The three kinetic parameters (a, 1/b and x0) are invariant with respect to inoculums density (Lindstrom et al., 1998). To evaluate utilization of dissolved organic nitrogen compounds, the nitrogen use (NUSE) index was calculated as the proportion (expressed as percentage) of the summed absorbance of those substrates that have C and N over the total absorbance measured in each Ecoplate (Sala et al., 2006).

*2.3.4 Extracellular enzymes and carbon substrate utilization profiles under anoxic conditions in the 50 cm depth sediment*

Vertical variability in oxygen concentrations was observed during the experiment. For this reason, we performed a test to analyze possible differences in microbial functioning under oxic and anoxic conditions for samples collected at 50 cm depth.

To test the potential effect of oxygen conditions on sediment microbial metabolism, an extra set of samples from days 14, 34, 50 and 83 at 50 cm depth were collected for Biolog Ecoplates and extracellular enzyme activity measurements under anoxic conditions. The analytical protocols were the same as those described above, except that the incubations were performed under an anoxic atmosphere and the collected samples and sediment extracts were purged with nitrogen gas at the moment of collection. The incubations for Biolog Ecoplates and extracellular enzyme activities were performed within a hermetic bottle with anoxic conditions already created inside (AnaeroGen system, Oxoid, UK). For the Biolog Ecoplates incubations, plates were further covered with silicone sealing film (Sigma). Oxygen values were measured before and after incubations (WTW oxygen meter).

**2.4 Data analysis**

Differences among depths and days for temperature, oxygen, extracellular enzymes, bacterial density and viability, and parameters obtained from carbon substrate utilization profiles (AWCD, Shannon diversity index, Richness, NUSE index and kinetic parameters) were tested using repeated measures analysis of variance (RM-ANOVA, depth and days as factors). All variables were logarithmically transformed, except for AWCD and Shannon index and kinetic parameters to render symmetric variables. Differences between depths observed on day 83 were further analyzed using a one-way analysis of variance (ANOVA, depth as a factor) between enzyme activities, Biolog parameters (Shannon diversity index, Richness, NUSE index) and live and bacterial density. Also, the differences between oxic and anoxic incubations for enzyme activities and Biolog Ecoplates were tested by analysis of variance (ANOVA, oxygen as a factor). Nutrients ($NO_3$, $NH_4$, $PO_4$, DOC and Cl) for each day and depth were analyzed using a two-way analysis of variance (ANOVA, depth and time as factors). All data were previously logarithmically transformed. All of these statistical analyses were performed using the program SPSS v.15.0 (SPSS, Inc., Chicago, IL, USA) and differences were considered to be significant at $p < 0.05$.

The ratios between carbon, nitrogen and phosphorus degrading enzymes (BG: LEU, BG: PHO, and LEU: PHO, as indicators of C:N, C:P and N:P nutrient needs and nutrient acquisition capabilities relationships, respectively) obtained under oxic and anoxic conditions were calculated in order to estimate potential imbalances in nutrient needs and capabilities. These enzyme ratios were estimated based on linear regression analysis of the natural log transformed enzyme activities. Results were expressed in terms of the slope and 95% confidence interval (as proposed by Sinsabaugh et al. (2011, 2009)). This analysis was performed with Sigmaplot 11.0 (Systat software, Inc, CA, USA).

Non-metric multi-dimensional scaling (NMDS) ordination plots were performed to visualize the spatial distribution pattern of the metabolic profiles in time and depth obtained from the Biolog Ecoplates of the 31 carbon substrates as well as to distinguish between oxic and anoxic metabolic profiles obtained at 50 cm depth. A previous distance matrix with Bray-Curtis similarity was created. NMDS is based on the rank order relation of dissimilarities where the largest distance between samples denotes the most different microbial functional profile. In addition, as suggested by Choi and Dobbs (1999), the 31 carbon sources in the plate were grouped in six functional categories including polymers (n=4), carbohydrates (n=10), carboxylic acids (n=7), phenolic compounds (n=2), amines (n=2) and amino acids (n=6). Data for all substrates and group of substrates from Biolog were previously standardized by sampling dates and then were fitted to the ordination plot using the "envfit" function of the "vegan" package in R software. This function was used to identify the correlations ($p<0.05$) with the ordination space to identify the groups of substrates mostly responsible for the spatial distribution of the samples in the NMDS plot (Blanchet et al., 2008; Legendre and Legendre, 1998). Based on these data, ANOSIM (analysis of similarity) (Clarke, 1993) were performed using the "vegan" package in R software to test for differences between functional profiles in depth and time.

## 3. RESULTS

### 3.1 Physicochemical parameters

Dissolved oxygen concentrations decreased at all depths after the start of the experiment, approaching values below 2 mg/L after day 34. Significant differences were observed among depths indicating lower oxygen concentration at the bottom of the tank ($p < 0.01$, Fig. 1).

Based on oxygen data, three time periods were used for analyses of nutrient content and enzyme ratios.

- **Period 1 (P1**): From day 1 to 28, defined by the development of a clear oxygen gradient ranging from 9.5 mg/L at the sediment surface to 4.5 mg/L at 50 cm depth.
- **Period 2 (P2)**: From day 33 to 53, defined by a reduction of the oxygen gradient, with small differences between the different depths and values close to 4 mg/L.
- **Period 3 (P3)**: From day 64 to 83, defined by a decrease in oxygen concentrations, ranged from 4 mg/L to 0.5 mg/L at the sediment surface and from 2.7 to 0.02 (anoxic conditions) at the bottom (50 cm in depth).

Water temperature increased from 18.14 ± 0.10 ºC to 25.18 ± 0.14 ºC (mean ± standard error) during the experiment, although no significant differences in temperature were observed among depths, indicating rapid re-equilibration with atmospheric conditions. The infiltration rate changed dynamically throughout the experiment, ranging from an initial value of 40 L/day to 15 L/day at day 83 (Fig. 1).

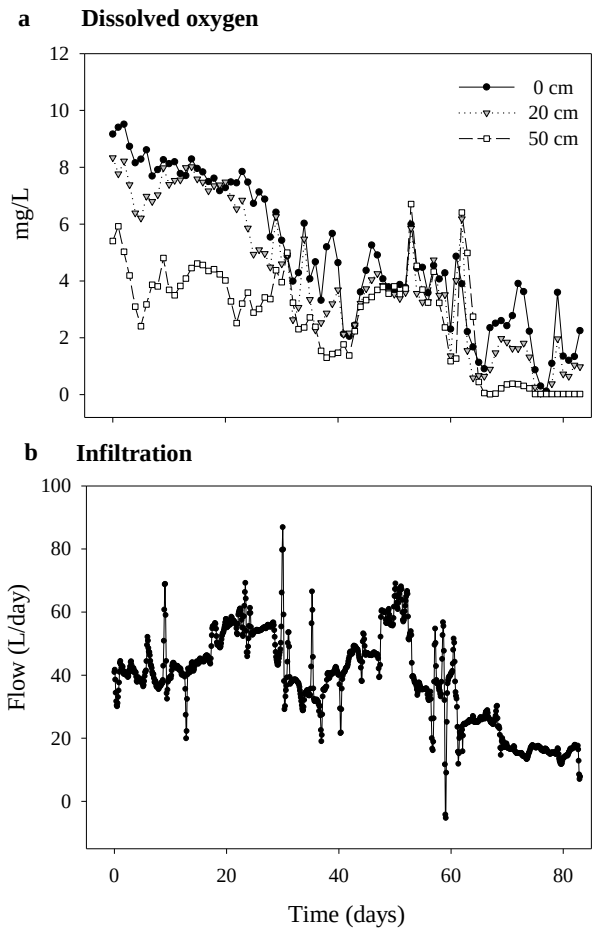



**Fig. 1** Temporal evolution of dissolved oxygen concentration at three different depths (a) and infiltration rates (b). a) Point values represent daily means and had been corrected for the drift of the instruments due to varied with temperature.

The chemical composition of the interstitial water varied according to time and depth (Fig. 2), whereas the pH values remained relatively stable throughout the experiment (pH 7.6–8). Dissolved $NO_3^-$ varied from 3.5 to 28.2 mg/L over time ($p = 0.01$). $NH_4^+$ also varied according to depth ($p = 0.043$) and time ($p < 0.01$), peaking at 1.5 mg/L during P1 and remaining below 0.05 mg/L after day 33. Dissolved organic carbon (DOC) concentration values diminished over time ($p < 0.01$), but did not differ by depth ($p > 0.05$). Inorganic phosphorous did not show any trend with depth, however a decrease of phosphate was

observed at the end of the experiment. Chloride concentration remained stable over time and depth, ranging from 186 to 227 mg/L ($p > 0.05$; Fig. 2).

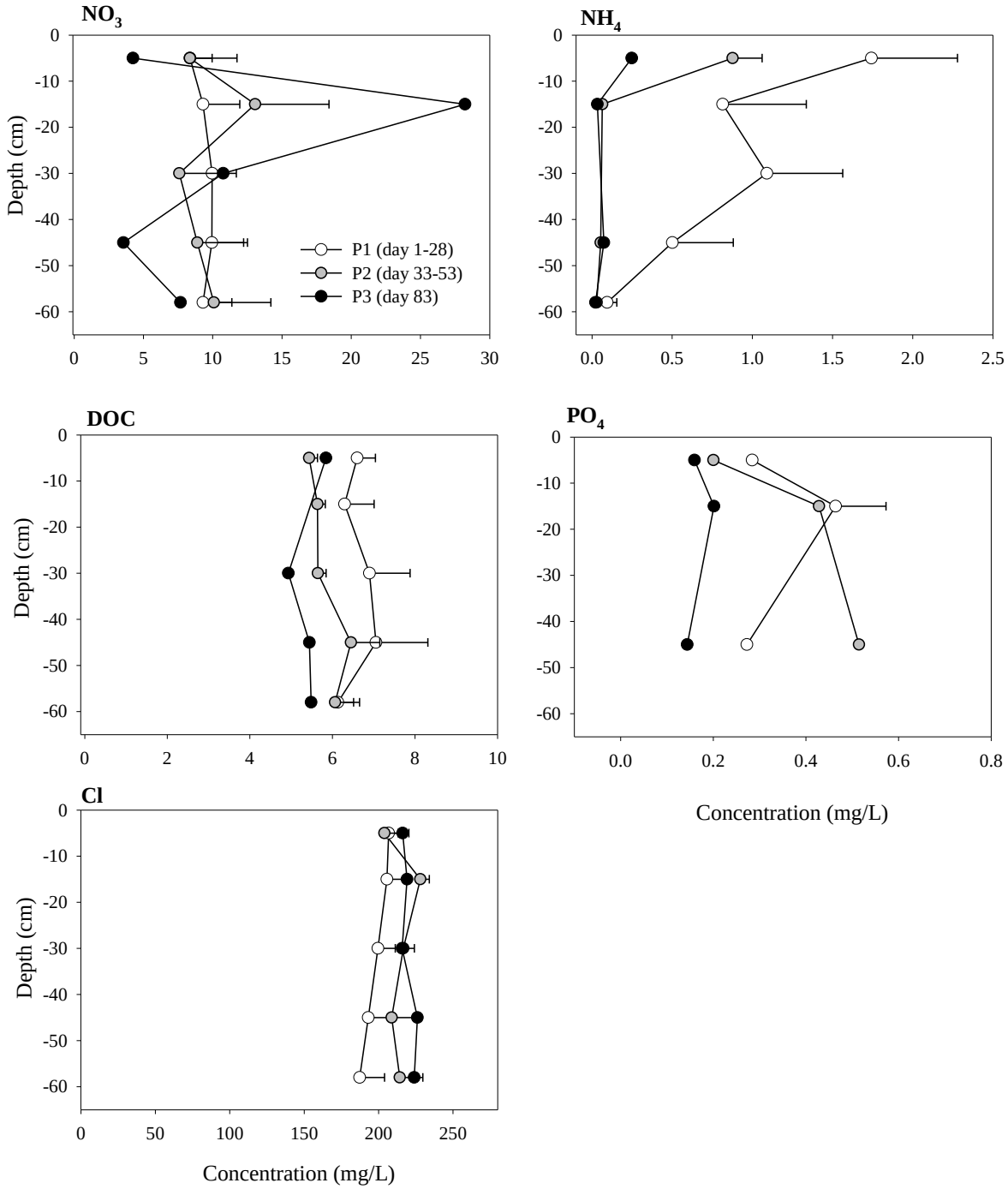



**Fig. 2** Mean values of nitrate ($NO_3$), ammonia ($NH_4$), dissolved organic carbon (DOC) phosphate ($PO_4$) and Chloride (Cl) at the three selected periods. Periods are defined as P1

(days 1 to 28), P2 (days 33 to 53) and P3 (day 83) as a function of depth. $PO_4$ data were only collected at days 3, 13 (P1), 49 (P2) and 83 (P3) at 3 different depths.

### 3.2 Biological parameters

#### *3.2.1 Bacterial abundance and viability*

Bacterial density increased rapidly during the colonization process, with a mean maximum of $1.20 \times 10^9$ cells/g dry weight on day 83. The maximum value was $1.96 \times 10^9$ cells/g dry weight obtained at day 34 in surface sediment (Table 2). Live bacteria accounted for 44.5% ± 7.1% of the average total bacteria for the whole experiment. No significant differences in bacterial density and viability at different depths were observed ($p > 0.05$, Table 3).

#### 3.2.2 *Extracellular enzyme activities*

Leu-aminopeptidase (LEU) and phosphatase (PHO) activities increased significantly during the experiment (Fig. 3, Table 3). LEU activity increased from the beginning of the experiment, and the highest values were depicted on day 83. In contrast, PHO activity increased slowly until day 21 and was stable until the end of the experiment (Fig. 3). At the end of the experiment, PHO activity was the highest, followed by LEU and β-glucosidase (BG) activities. Significant increases in phosphatase activity were observed at day 83 at the bottom of the tank (Table 3). BG was significantly higher in surface sediment and decreased with increasing depth for the whole experiment (Table 3, Fig. 3).

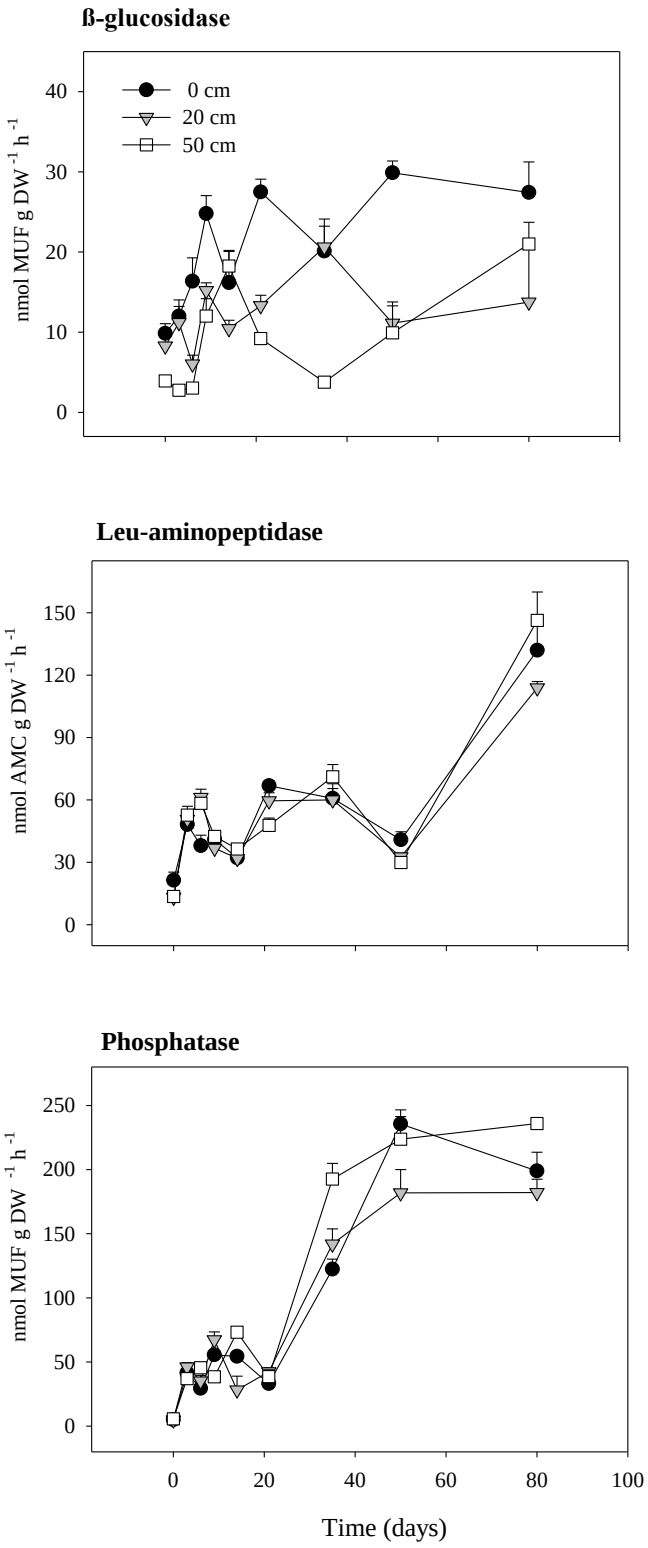



**Fig. 3** Temporal changes in extracellular enzymatic activities at 3 different depths, after incubating samples under oxic conditions. Values are means ± standard error (from 3 replicates).

Differences in extracellular enzyme activities were observed under different oxygenic conditions (Fig. 4). PHO and LEU activities were significantly reduced in anoxic conditions, mainly PHO activity was reduced 82% compared to oxic conditions. In contrast, BG activity was not significantly affected by oxygen concentrations (Fig. 4).

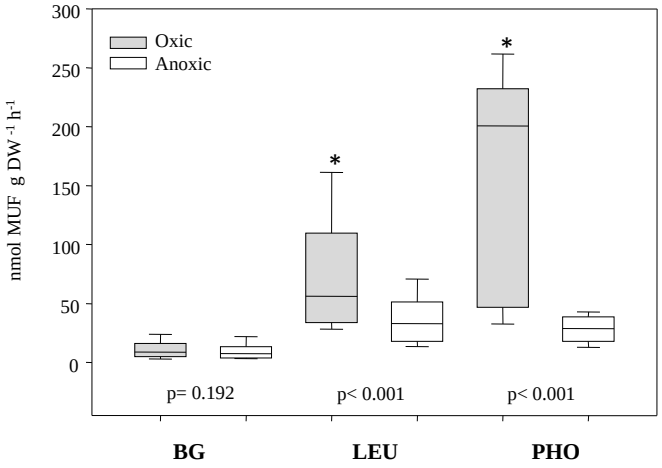



**Fig. 4** Box plot of extracellular enzymes (BG, LEU, PHO) comparing activities measured under oxic conditions versus activities measured under anoxic conditions for 50 cm depth sediment samples (n=12, including data from days 14, 34, 50 and 83). The asterisk indicates significant different values respect to anoxic data.

The ratio of Ln BG: Ln LEU activities (obtained as the slope of the linear regression between Ln LEU vs. Ln BG, Fig. 5) was used as an indicator of the greater or lower capacity of the microbial community to degrade organic matter, containing carbon in contrast to the its capacity to degrade organic matter containing both carbon and nitrogen. Results from these activities measured under oxic conditions showed a slope of 0.90, a value close to the equilibrium (1:1 ratio, 95% confidence interval was 0.71-1.10, Fig. 5). In contrast, slopes for Ln BG: Ln PHO and Ln LEU: Ln PHO ratios were 0.56 (95% confidence interval was 0.41-0.71) and 0.53 (95% confidence interval was 0.44-0.63) respectively, indicating enhanced

ability to degrade organic compounds containing phosphorus to that containing carbon and/or nitrogen. The largest increase in PHO relative to BG and LEU was observed during P3, indicating that the microbial communities first acquired more carbon and nitrogen, while more phosphorous was assimilated during the third period of the experiment (Fig. 5). Slopes close to 1 were measured in anoxic conditions at a depth of 50 cm. No differences between depths were observed for enzyme activity ratios (data not shown).

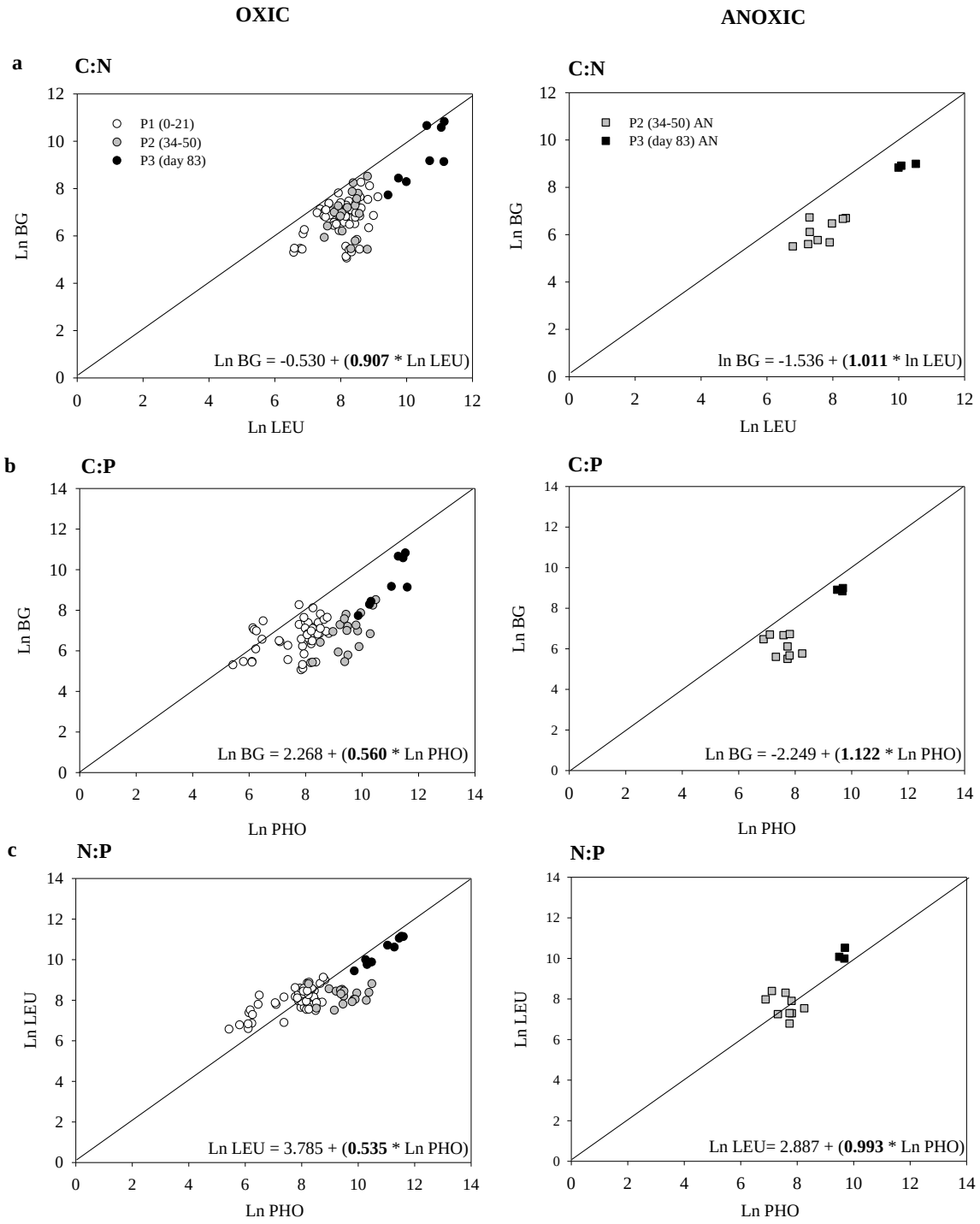



**Fig. 5** Relationships (in ln space) between BG:LEU (a) BG:PHO (b) LEU:PHO (c) organic matter acquisition enzymes. Data are values from each sampling day grouped by periods (P1, P2 and P3). Relationships on the left are those from oxic measurements while those on the right are those from anoxic measurements. The solid line indicates a 1:1 relationship. Linear regressions are included for each chart and the slopes are highlighted in bold.

*3.2.3 Carbon substrate utilization profiles*

Biolog Ecoplates were used to characterize the functional diversity and metabolic fingerprint of the sediment tank communities according to depth and time (Fig. 6). The percentage of positive wells (richness) ranged between 65 - 100%, with lower values being measured at the end of the experiment. Consistently, functional diversity (Shannon index) and functional richness (positive wells) also decreased significantly through time (Table 3, Fig. 6). Significant differences were found at different sediment depths; functional richness was highest at the surface and decreased with depth over time (Fig. 6, Table 3). However, at 50 cm, measurements under oxic and anoxic conditions were not significantly different ($p = 0.92$, Shannon index; $p = 0.59$, functional richness). Moreover, differences in the use of nitrogen compounds (NUSE index) at different depths were detected. High NUSE index values were found at 50 cm on day 83 (Fig. 6, Table 3). The NUSE index at 50 cm was not significantly different between oxic and anoxic conditions ($p = 0.54$).

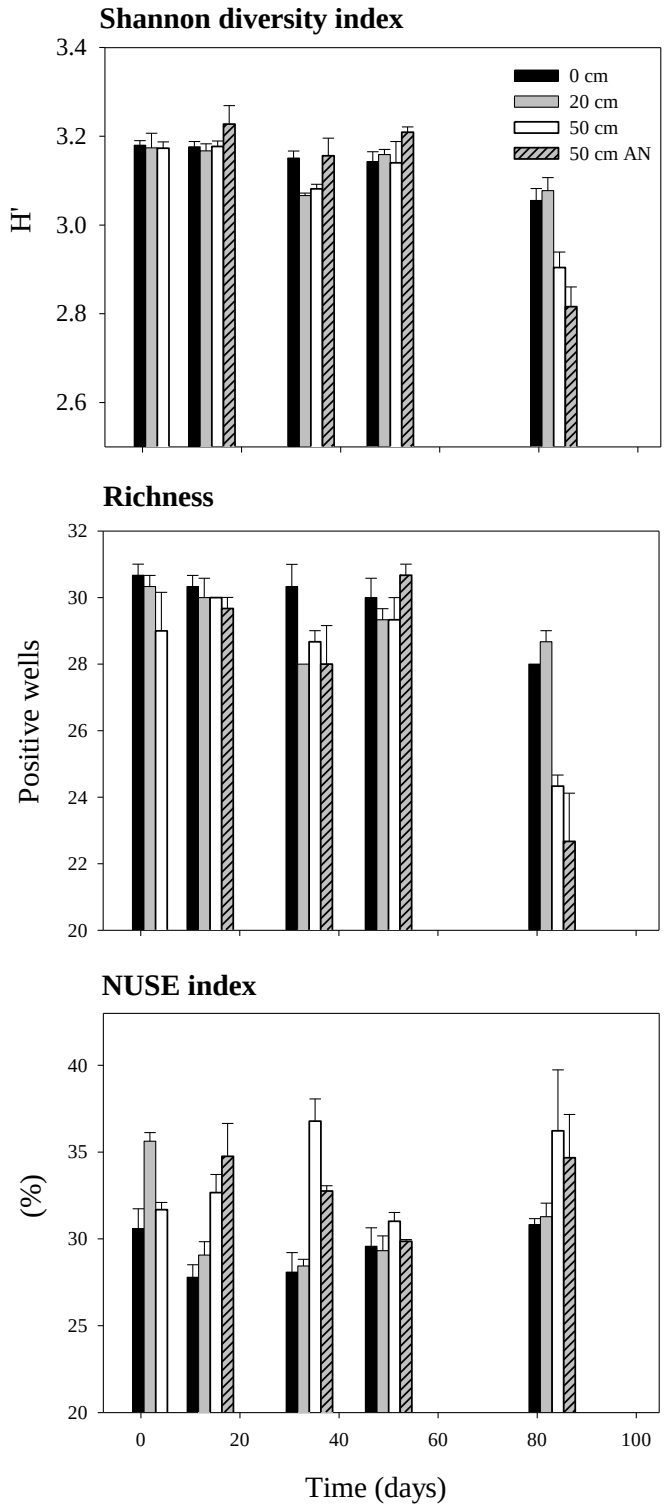
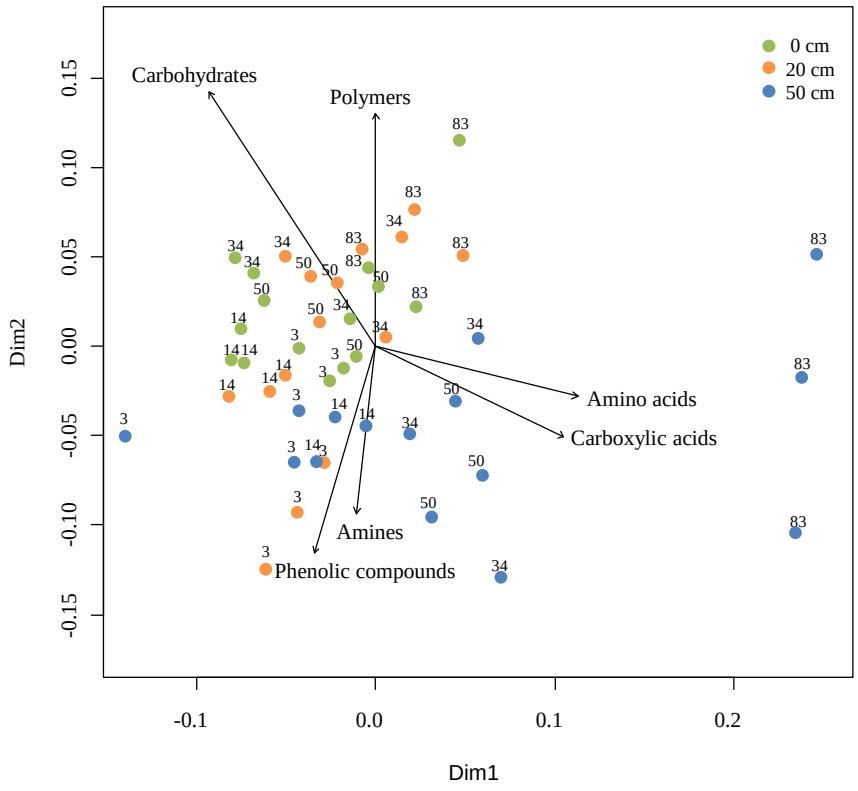



**Fig. 6** Shannon diversity index, Richness (positive wells) and NUSE index at different depth (0, 20 and 50 cm) for 5 different sampling dates. Values at 50 cm in anaerobic incubations are also included after day 14. Values are means and standard deviation (from 3 replicates).

The change in the metabolic fingerprint with depth was also remarkable. The community present at depth 50 cm was clearly distinct from that of the surface and the first 20 cm, as shown in the NMDS plot (Fig. 7a) and in the ANOSIM analysis (depth factor, R= 0.228, p=0.001 between surface - 50cm: R= 0.114, p=0.009 between 20cm -50 cm). At 50 cm, microbial communities were able to degrade amino acids and carboxylic acids, including L-asparagine and pyruvic acid, whereas surface and 20 cm communities principally degraded polymers (Tween 80) and carbohydrates (α-D-lactose and D-xylose) (Fig. 7a). At 50 cm, high dispersion in the ordination analysis (NMDS) was found, indicating larger heterogeneity between samples; this finding was especially relevant on day 83. Metabolic fingerprints of oxic and anoxic communities at 50 cm were different (ANOSIM analysis, Global R= 0.232, p = 0.007, Fig. 7b). However, anoxic samples from day 14 were similar to oxic samples from days 14, 34, and 50, whereas clear differences were observed between oxic and anoxic conditions for other sampling dates (Fig. 7b). Under anoxic conditions, decomposition of carboxylic acids and amino acids were enhanced, whereas phenolic compounds and amines were degraded in the presence of oxygen.

a



b

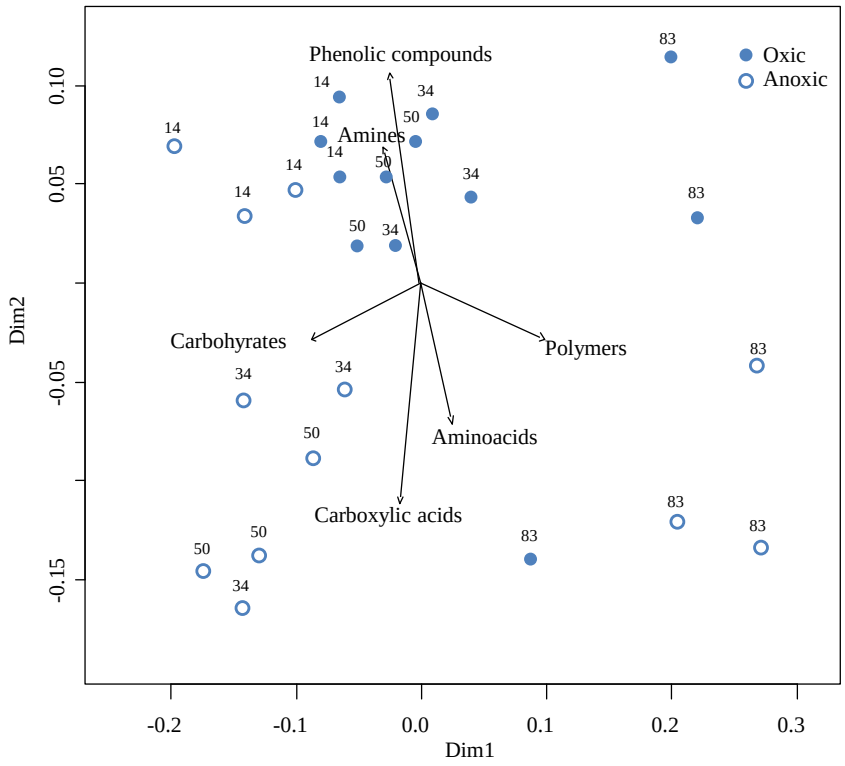



**Fig. 7** NMDS ordination plots based on Bray-Curtis distances according to 31 substrates of Biolog Ecoplates after 144 hours of incubation. a) Data include all depths. Color indicates different depths and numbers the sampling date. b) Data for 50 cm depth after incubation at different oxygen conditions. Color indicates oxic / anoxic incubated samples, and numbers the sampling date. The six groups of carbon substrates are fitted on the ordination plot $p<0.05$. Kruskal 2D stress is equal to 0.15 and 0.11, respectively.

Average well color development (AWCD) values revealed significant differences in kinetic parameters $a$ and $x_0$ between oxic and anoxic incubation conditions at 50 cm ($p < 0.001$, $a$; $p= 0.038$, $x_0$). Under aerobic conditions, metabolic activity took longer (higher $x_0$ values) to achieve maximum color development and higher maximum metabolic capacity ($a$) was

observed on all sampling dates. In contrast, no differences were observed between oxic and anoxic conditions at day 83 ($p > 0.05$ for $a$, $1/b$, and $x_0$).

## 4. DISCUSSION

Changes in microbial community metabolism and functional diversity at selected depths were found to occur in a controlled porous media subjected to continuous infiltration. Previous studies described the structure and activity of microbial communities with soils depth being driven by physicochemical factors (e.g., grain size, oxygen, pH, temperature, and redox potential (Bundt et al., 2001; Fierer et al., 2003). Here we complement this knowledge by reporting changes in microbial metabolism as a function of sediment depth and oxygen conditions.

Our findings indicate that bacteria colonizing the sediment tank had different capacities to decompose organic compounds depending on depth. At the surface, bacteria used simple polysaccharides through β-glucosidase activity, and this activity decreased with depth. Previous studies documented the decrease of β-glucosidase activity in sand filters (Hendel et al., 2001) and deep-sea sediments (Boetius et al., 2000) as the result of reduced availability of simple polysaccharides and low bacterial densities in deeper sediments (Fischer et al., 2002; Romani et al., 1998). In our study, no significant differences in alive or total bacteria density were found. The latter is consistent with the work of Franken et al. (2001), that also reported no differences in bacterial densities until 60 cm in a hyporheic zone temperate stream. Additional studies linked the decrease values of temperature, pH and organic matter content to the reduction in enzyme activities with increasing depth (Douterelo et al., 2011). However, in our experimental conditions, no significant differences in temperature or pH were observed. Although significant reduction of oxygen content was measured, the decrease in β-

glucosidase activity along the vertical profile could not be explained by low oxygen content, as BG activity was not affected by incubation under different oxygen conditions (Fig. 4). According to Kristensen et al. (1995), the availability of labile organic matter limits bacterial heterotrophic activity in various aquatic ecosystems, regardless of oxygen concentration. Therefore, the decrease in simple polysaccharide use with depth may be explained by the presence of easy-to-decompose material (more labile) and first degraded compounds at the tank surface. This first degradation in the surface sediment might determine a reduction of labile organic matter in depth, and more resistant material were accumulated in deeper sediments (Costa et al., 2007a). Moreover, our results indicated that metabolic changes should be mainly linked to DOC quality than to DOC quantity, since we did not observe differences in DOC with depth. Nevertheless, high DOC values at depth (50 cm), as well as constant pH and temperature were likely promoted by the high infiltration rates measured during the experiment, which were in the upper range of the ones measured in the hyporheic zone, but similar to those observed in artificial recharge areas (Pedretti et al., 2011).

Microbial functional diversity was also depth dependent, and differences were more evident by the end of the experiment. Biolog Ecoplates incubations were used to characterize carbon source utilization in the sediment tank. Despite the limitations associated to this method (Konopka et al., 1998; Smalla et al., 1998; Verschuere et al., 1997), robust information to describe microbial functional diversity can be obtained after the data normalization and protocol standardization (i.e., use of similar inoculum size and incubation conditions) (Stefanowicz, 2006). At the end of the experiment, the microbial community became more specialized and used a narrower range of carbon substrates, as indicated by the lower Shannon diversity and richness scores. These data suggested that the microorganisms had assembled to those better adapted to the environmental conditions of the sediment tank. This ecological specialization was defined by Devictor et al. (2010) as an adaptation process to the

diversity of resources used by a species in different environments. Decreased use of available substrates was also observed at 50 cm, similar to results reported by Griffiths et al. (2003), that in substrate utilization decreased at 20 cm compared to surface samples. Likewise, the microbial community showed different functional fingerprints depending on depth (Fig. 7). Carbohydrates and polymers were used readily at the surface (0 and 20 cm depth, Fig. 7a). Similarly, concentrated use of carbohydrates and polymers was observed at the seawater surface (Sala et al., 2008). These compounds are considered to be the largest bioavailable source of carbon in sediments (Oliveira et al., 2010) and greater use of them at the surface is consistent with high surface labile compounds as β-glucosidase activity. On the other hand, the metabolic fingerprint at 50 cm was distinct from fingerprints at 0 and 20 cm (Fig. 7a). The former was mainly characterized by the use of nitrogen compounds, as shown by higher NUSE index values. Until day 50, microbial communities used amines and phenolic compounds and from day 50 until the end of the experiment, amino acids and carboxylic acids were used (Fig. 7a). These results indicate the significant use of nitrogen-containing organic compounds at the bottom of the tank, consistent with the maintenance of leu-aminopeptidase activity.

Differences in microbial metabolism with depth may have been affected by oxygen availability, most significantly by the end of the experiment. Bacterial colonization and biofilm formation may have contributed to pore clogging, providing a substantial decrease in permeability and infiltration flow rate, and an increase in anoxia with increasing depth (Rubol et al., 2014). In our experiment, decrease in dissolved oxygen and infiltration rate followed a similar pattern during the experiment, but temperature increased. Although a temperature increase determines a lower viscosity of water and thus would increase water infiltration, our results suggest that microbial colonization and metabolism are the main responsible for changes in water infiltration, determining its decrease in time. In river ecosystems, decreases

in dissolved oxygen along the vertical profile correlates with microbial respiration, interstitial flow, and water residence time (Brunke and Gonser, 1997; Fischer et al., 2003). Indeed, oxygen plays an important role in microbial metabolism and diversity (Brune et al., 2000; Rubol et al., 2013). In our study, a significant reduction in the degradation of organic nitrogen and phosphorus compounds was found under anoxic conditions, whereas no polysaccharide degradation reduction was detected (Fig. 4). This result suggests that inactivation rates of the hydrolytic enzymes vary for different enzymes (Freeman et al., 2004; Goel et al., 1998). Christy et al. (2014) reported that during anaerobic and aerobic decomposition, polysaccharides are hydrolyzed by secreted enzymes, such as cellulase and cellobiase, and cellulose-hydrolyzing enzymes, including β-glucosidase, can be released under different oxygen conditions. In contrast, hydrolysis of organic phosphorus compounds was inhibited by anoxic conditions. The differential effects of anoxia on extracellular enzyme activities at different depths affected the balance between carbon, nitrogen, and phosphorus acquisition. Sinsabaugh et al. (2009) suggested that C:N:P activity ratios of 1:1:1 indicate equilibrium between organic matter composition, nutrient availability, and microbial metabolism. Specifically, extracellular enzyme activities and carbon, nitrogen, and phosphorus acquisition might be correlated with water and sediment chemistries (Hill et al., 2012). In our study, oxic conditions led to greater degradation of phosphorus compounds compared to carbon and nitrogen over time. Although the extracellular enzymes measured do not account for the degradation of all C, N and P available organic compounds, these enzymes describe the last steps of the decomposition process for a broad range of organic compounds. This wide-ranging justify the comparison of different ratios to the potential nutrient imbalances and needs of microbial community present inside the tank. In these sense, equilibrium was observed between C:N acquiring enzymes, but it remained imbalanced for C:P and N:P acquiring enzymes (Fig. 5). These data suggest that the sediment community

inside the tank was phosphorus limited, especially at the end of the experiment, as we observed a reduction of available inorganic phosphorus in interstitial water. Phosphorus limitation in sediment may affect bacterial growth rates and microbial nutrient assimilation (Sinsabaugh et al., 2011); similar imbalances in sediments were recently reported by Hill et al. (2012) and Romaní et al. (2013). However, due to inhibition of phosphatase activity under anoxic conditions, the equilibrium between phosphorus-acquiring enzymes and carbon- and nitrogen-acquiring enzymes was re-established (Fig. 5). Reduction of phosphatase activity in deep anoxic sediment was also reported by Steenbergh et al. (2011), who suggested the presence of lower biological phosphorus retention efficiency under anoxic conditions in Baltic Sea sediments.

In our study, a different functional fingerprint was obtained for communities incubated in oxic and anoxic conditions at 50 cm (Fig. 7b) where carboxylic acids and amino acids were used preferentially under anoxic conditions. Tiquia (2011) reported that low oxygen conditions promoted the use of carboxylic acids and amino acids in an urbanized river. In our sediment tank, distinct metabolic fingerprints were observed due to depth and anoxia occurred gradually over time; e.g., results for day 14 in anoxia were still similar to those found under oxic conditions (Fig. 7b). The gradual change in oxygen conditions suggests that both aerobic and anaerobic processes may have taken place simultaneously. Indeed, nitrification and denitrification processes might also have occurred with time, as shown by $NH_4^+$ consumption and $NO_3^-$ production in the first 20 cm of the sediment tank. Toward the bottom of the tank, $NO_3^-$ was consumed and no ammonium was present, suggesting that nitrogen had to be acquired from complex nitrogen compounds. These data hints the spatially coexistence of nitrification and denitrification in the sediment profile, already reported in marine sediments (Bonin et al., 1998). The gradual change in the metabolic fingerprint with increasing depth may be related to changes in the quality of the available organic matter and

changes in metabolic processes that occurred due to depleted oxygen concentrations. However, although not measured in this study, changes in the composition of bacterial communities through the sediment tank may also have occurred. Adaptation of the communities to anoxic conditions was shown by the presence of active bacteria at all depths, including the transition zone from oxic to anoxic conditions. For instance facultative bacteria, capable to live in sediments with fluctuating oxygen concentrations, may have colonized the tank. Indeed, microorganisms responsible for oxidation of organic matter are not only aerobic bacteria (Brune et al., 2000). In areas of low oxygen, components of anaerobic respiration (e.g., nitrifiers, sulfate reducers, and methanogenic bacteria) can metabolize organic carbon (Kristensen et al., 1995; Storey et al., 1999). Maintenance of live bacteria at monitored depths was shown by similarities in kinetic parameters ($a$, $b$, and $x_0$) under oxic and anoxic conditions. These data indicate that aerobic and anaerobic communities metabolized substrates in the plate with similar velocities, suggesting that the microbial communities adapted to the environmental conditions after the lag phase (Kristensen et al., 1995). In this context, it is known that oxic and anoxic bacteria can hydrolyze particulate material or to mineralize dissolved organic matter equally fast in sea sediments (Hulthe et al., 1998).

Our experiments revealed higher heterogeneity between replicates at greater depths and under anoxic conditions, especially at the end of the experiment, indicating larger spatial heterogeneity combined with lower functional richness and diversity. Functional heterogeneity may be linked to physicochemical conditions in sediments, which also appear to have high spatial and temporal heterogeneity at greater depths (Storey et al., 1999).

Functional approaches, including measurements of extracellular enzyme activities and Biolog Ecoplate incubations, provided complementary information on the microbial community in the sediment tank. Previous authors analyzed results from extracellular enzymes and Biolog

Ecoplates, but found no direct correlations for bacterioplankton (Sinsabaugh and Foreman, 2001) and salt marsh sediments (Costa et al., 2007b), and slight correlations in river biofilms (Ylla et al., 2014). These studies suggest that extracellular enzyme activities reflect the inherent activity of the resident community, whereas Biolog Ecoplates assess the potential functional diversity of microbial communities. In our study, extracellular enzyme activities showed larger differences over time compared to Biolog Ecoplates, which were more sensitive to spatial differences. Altogether, these data indicate that while biogeochemical processes changed over time, the functional diversity characteristics changed with depth.

The laboratory experiment used allowed the simulation and monitoring of sediment conditions subject to continuous infiltration at the meso-scale study. Although extrapolation of these results to natural aquatic ecosystems must be done with caution, meso-scale studies can be seen as a first step to better understand the biogeochemical processes occurring at the hyporheic zone and artificial recharge facilities. The transferability of these results at larger scales should be through a directly field experiment, as a solution to validate the laboratory experiment, simulating the same conditions in the field, by using fine scale hydrology tools (such as the monitoring of hydraulic conductivity by temperature sensors) and intensive and extensive core sampling and interstitial water sampling for microbiological and biogeochemical analysis.

## 5 CONCLUSIONS

We conclude that the microbial community showed different abilities to degrade organic matter at different sediment depths. Greater decomposition of carbon compounds occurred in surface sediments, and greater use of nitrogen compounds occurred at greater depths. Under anoxic conditions at increased depths, phosphatase activity was inhibited, limiting phosphorus availability. Milder effects of anoxia were found for peptidase activity, and

glucosidase activity was not affected. Coexistence of aerobic and anaerobic communities, promoted by greater physicochemical heterogeneity, was also observed in deeper sediments. Bacteria (including living bacteria) occurred at all sediment depths and were able to adapt to different oxygen concentrations. These factors may affect the biogeochemical potential of deep sediment tanks for water purification processes.

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