CHANGES IN ORGANIC MATTER USE IN AN INFILTRATION SEDIMENT EXPERIMENT ACCORDING TO DEPTH AND OXYGEN CONCENTRATION

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ABSTRACT

Water flowing through hyporheic river sediments or artificial recharge facilities promotes the development of microbial communities in depth. We performed an 83-day mesocosm infiltration experiment, aiming at analyzing microbial functions (e.g., extracellular enzyme activities and carbon substrate utilization) in the sediment profile and determining organic matter use under different oxygenic conditions. In the experiment, surface sediment layers were 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 and used 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 column showed reduced dissolved oxygen concentrations, which inhibited phosphatase activity. The presence of specific metabolic fingerprints under oxic and anoxic conditions indicated that the microbial community was adapting to use organic matter and adapt to the existing oxygen gradient. The experimental results indicate that heterogeneous oxygen conditions influence organic matter metabolism in a sediment column.

INTRODUCTION

The connection between surface water, groundwater and the processes occurring in this interface (i.e., the hyporheic sediment) is important for river ecosystem metabolism [1, 2]. The hyporheic zone promotes the exchange of water, nutrients, and biota between alluvial groundwater and stream water [3]. This exchange, in turn, influences stream water quality. 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 [4] that plays key roles in biogeochemical processes [5]. Microbial communities are responsible for most of the metabolic activity in hyporheic sediments [6], including the degradation of organic matter and the reduction of electron acceptors (e.g., oxygen, nitrate and sulphate) [7, 8], which are important water purification processes associated with river and aquifer systems. Similar processes occur when water flows through sediments in vadose zones during, for instance, artificial groundwater recharge operations taking place in surface infiltration ponds. Artificial recharge may promote the development of microbial communities at different depths [9]. Percolation through a saturated zone is often used to enhance the quality of surface water [10], although the microbial processes occurring in the hyporheic sediment are not well defined.

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. These actions are a limiting step in the entrance of organic matter to the food web [11, 12]. The capacity of microorganisms to use and recycle organic compounds is also linked to functional diversity; higher functional diversity and richness may be related to higher microbial diversity [13] and eventually to higher availability of organic compounds. Indeed,

Tiquia (2010) found that higher functional diversity was linked to the physicochemical and biological properties of river water samples. Functional diversity promotes ecosystem function and increases the ability to cope with the changing availability of organic matter [15]. Although many studies have analysed enzyme activities in surface sediments [16], much less is known about how these activities change according to depth. For example, 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 [17].

Changes in the utilization of organic matter at different sediment depths may be linked to microbial colonization. Indeed, microbial biomass is not homogeneously distributed throughout the sediment. Microorganisms are found in largest quantities at the soil surface, and their abundance declines rapidly with increasing depth [18]. 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 highest [19]. Bacteria in deeper sediments are more sensitive to physical and chemical changes compared to those in surface layers [20] due to the relatively more stable conditions [21]. In deeper sediments, organic matter use may be further affected by physical and chemical changes in oxygen, pH, temperature and nutrient availability [22]. The sediment biofilm structure reduces the water infiltration capacity by pore clogging (e.g., [23]), also decreasing the soil porosity, stream bed permeability, and thus the water exchange between river and vadose zone [1, 24].

Physicochemical conditions appear to be highly heterogeneous at different sediment depths [6], and this heterogeneity promotes the coexistence of aerobic and anaerobic biogeochemical communities. Biofilms may create microenvironments that allow anaerobic processes to coexist within aerobic sediments [6, 25] indicating that anoxic environments can promote

areas of organic matter preservation with slower rates of carbon decomposition. Previous publications showed vertical oxygen consumption in sediments [26, 27] but limited information is available linking this distribution with the decomposition of organic matter along the sediment's profile [27]. This is in spite of oxygen and organic matter being known to play key roles in nutrient cycles [2, 8, 28] . 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 [6] and some extracellular enzymatic activities are inhibited in anoxic conditions [29].

The objective of this study was to analyse changes in microbial organic matter use at different sediment depths under infiltration conditions. We hypothesized that microbial activity and biomass would be higher at the sediment surface and decline with depth, consistent with a vertical oxygen gradient. Specifically, the experiment aims at: i) analysing 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 this aim, a 1- meter sediment column 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 [12]. Functional diversity and functional fingerprints of sediment communities were analysed on the community-level using Biolog Ecoplates [30]. A meso-scale was chosen to produce biogeochemical and microbial parameters under controlled interstitial flow conditions [31–33]

MATERIAL AND METHODS

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). Sediments were sieved at 0.5 cm and packed in the tank without further treatment (see Rubol et al. [9], 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. A system of two pumps and a connecting valve ensured the proper mixing of the two fluids. 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). Water chemical composition of the mixture mimics the typical Llobregat River water reported by Fernández-Turiel [34] (Table 1). All tubes, valves and carboys were autoclaved and covered with a black foil prior to the start of the experiment.

The upper layer in the tank was exposed to sunlight, while the lateral walls were covered with dark plastic to prevent photoautotrophic activity. At the beginning of the experiment, a

130 microbial inoculum was added to the top of the tank to promote colonization. The inoculum
131 was prepared from sediment collected at the pristine riverbed nearby the site. 20 mL of
132 sediment extract from five core sediment samples (5 cm diameter, 5-10 cm depth) were
133 added into the tank. This inoculum contained 2.27 ± 0.41 x 10^6 bacterial cells/mL (mean
134 value ± standard error).

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

143 **Physical analysis**

144 Measured values of temperature and volumetric water content were recorded continuously by
145 using capacitance sensors (5TE, Decagon Devices, Pullman, WA) placed at 3 different
146 depths. A handheld multiparameter instrument (YSI Professional Plus) recorded temperature,
147 electrical conductivity, dissolved oxygen and pH in continuous at the tank outlet. Sensors
148 were recalibrated and checked daily. Dissolved oxygen concentrations were measured
149 continuously with optical fibers (FiboxPresens, Germany) and corrected for temperature.

150 The evolution of infiltration rate (R) with time was determined from water balance
151 considerations at the pond, resulting in the following equation:

$$R(t) = I(t) - E(t) - \frac{\Delta h}{\Delta t}$$

where I(t) is the pumping rate per unit surface area at time t, E(t) is the evaporation rate, and h(t) is the height of water at the pond. Direct evaporation was estimated, and found negligible to the overall balance.

Chemical analysis

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 analyser (Shimadzu TOC-V-CSH 230V, Tokyo, Japan). Three replicates were used for each sample. Due to technical problems phosphate (PO_4) were analysed only at days 3, 13, 49 and 83 at 3 different depths (5, 15 and 45) . Phosphate was analysed spectrophotometrically as described by Murphy and Riley [35].

Biological analysis

Microbial activity and bacterial abundance were analysed 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. Biolog EcoPlate incubations and bacterial abundance and viability were estimated on days 3, 14, 34, 50 and 83.

2.4.1 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, 40W and 40kHz). 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 (bacterial density $>10^6$ cell mL^{-1} [36]).

For each diluted sediment extract, 2 mL were stained by a 1:1 mixture of Syto9 and PI 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.

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 column 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 for 5 h after sampling. Ecoplates were inoculated with 130 µL of each sediment extract under sterile conditions and incubated at 20ºC in dark conditions for 6 days. Plates

were 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 colour development saturation and the AWCD (Average Well Colour Development) was close to 0.6 [37]. 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 analysed by calculating the AWCD, Shannon diversity index (H') and Substrate richness (S) to evaluate microbial community functional diversity and functional richness [38]. 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 x_0) were estimated by fitting the curve of colour development on plates to a sigmoid equation [39] where a is the maximum absorbance in the event of colour saturation, $1/b$ is the slope of the maximum rate of colour development and x_0 is the time when maximum colour development rate is achieved. The three kinetic parameters (a, $1/b$ and x_0) are invariant with respect to inoculums density [39]. 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 [40].

Extracellular enzyme activities

Three extracellular enzyme activities were analysed 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 µmol/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.

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 analyse 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).

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 a factor). All variables were logarithmically transformed, except for AWCD and Shannon index and kinetic parameters to render symmetric variables. Differences between depth observed on day 83 were further analysed 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 analysis were analysed by analysis of variance (ANOVA, oxygen as a factor). Nutrients (NO^{3-}, NH^{4+}, DOC and CL) for each day and depth were analysed 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 relationships 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 the coefficient of variation (as proposed by Sinsabaugh [41, 42]. 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 [43], 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 [44, 45]. Based on these data, ANOSIM analysis (analysis of similarity) [46] were performed using the "vegan" package in R software to test for differences between functional profiles in depth and time.

RESULTS

Physicochemical parameters

Dissolved oxygen 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 in depth with values of about 8 mg/L at the sediment surface and 4 mg/L at 50 cm in depth.
- **Period 2 (P2)**: From day 33 to 53, defined by an oxygen gradient reduction leading to similar values in depth close to 4 mg/L.
- **Period 3 (P3)**: From day 64 to 83, defined by a decrease in oxygen from values of about 4 mg/L to 2 mg/L in the first 20 cm of the sediment and to 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).

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 6 to 16 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) values diminished over time ($p < 0.01$), but did not differ by depth ($p > 0.05$). Chloride concentration remained stable over time and depth, ranging from 186 to 227 mg/L ($p > 0.05$; Fig. 2). Inorganic phosphorous did not show any trend with depth; however a decrease of phosphate was observed at the end of

316 the experiment (day 3 (0.27±0.01, indicating mean ± standard error), day 13 (0.41±0.09), day
317 49 (0.38±0.06) and day 83(0.16±0.03).

318 **Biological parameters**

319 *Bacterial abundance and viability*

320 Bacterial density increased rapidly during the colonization process, with a mean maximum of
321 1.20×10^9 cells/g dry weight on day 83. No differences in bacterial density with depth were
322 observed ($p > 0.05$, Table 3). Live bacteria accounted for 44.5% ± 7.1% of the average total
323 bacteria for the whole experiment. The maximum value, 51.3%, was obtained at day 34 in
324 surface sediment (Table 2). No differences were observed at different depths (Table 3).

325 *Extracellular enzyme activities*

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

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

The relationship between degradation of organic matter containing carbon and nitrogen (BG:LEU) measured under oxic conditions showed a slope of 0.90, close to the equilibrium value between the enzymes (Fig. 5). In contrast, slopes for ratios of BG:PHO and LEU:PHO were 0.56 and 0.54, respectively, indicating enhanced ability to degrade organic compounds containing phosphorus. 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 in ratios of extracellular enzyme activities were observed at different depths (data not shown).

Carbon substrate utilization profiles

Biolog Ecoplates were used to characterize the functional diversity and metabolic fingerprint of the sediment column communities according to depth and time (Fig. 6). The percentage of positive wells 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 ($p = 0.54$).

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, Global R= 0.118, p=0.002; pairwise test surface-20 cm: R= 0,012, p=0.32). 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, R global = 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 day 83 samples were not similar under oxic and anoxic conditions (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.

Average well colour 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 colour 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).

DISCUSSION

Changes in microbial metabolism and functional diversity with depth were found to occur in a controlled porous media subject to continuous infiltration. To complement previous studies describing the structure and activity of microbial communities driven by physicochemical

386 factors (e.g., water content, grain size, oxygen, pH, temperature, and redox potential [20,
387 47]), we report changes in microbial metabolism as a function of sediment depth and oxygen
388 conditions.

389 Our findings indicated that bacteria colonizing the sediment column had different capacities
390 to decompose organic compounds, depending on depth. At the surface, bacteria used simple
391 polysaccharides through β-glucosidase activity, and this activity decreased with depth.
392 Previous studies documented also the decrease of β-glucosidase activity in a sand filter [48]
393 and deep-sea sediments [49], although other studies linked decreased polysaccharide
394 activities in deeper sediment to low bacterial densities or reduced availability of simple
395 polysaccharides [17, 50]. In our study, no significant differences in alive or total bacteria
396 density were found. Franken et al. [51] also reported no differences in bacterial densities until
397 60 cm in a hyporheic zone temperate stream. Other studies indicate that environmental
398 factors (e.g., temperature, pH, oxygen, and nutrient availability) in the upper soil layers are
399 responsible for the reduced enzyme activities that occur with increasing depth [22]. However,
400 no significant differences in temperature or pH were observed. Although significant oxygen
401 content deplete was measured, the decrease in β-glucosidase activity with depth could not be
402 explained by low oxygen content, as the activity was not affected by incubation with different
403 oxygen conditions (Fig. 4). According to Kristensen et al. [52]Kristensen *et al.* (1995), the
404 availability of labile organic matter limits bacterial heterotrophic activity in various aquatic
405 ecosystems, regardless of oxygen concentration. Therefore, the decrease in simple
406 polysaccharide use with depth may be explained by the accumulation of easy-to-decompose
407 material at the tank surface and more resistant material in deeper sediments [53]. We did not
408 observe a difference in DOC with depth. Nevertheless, results seems to suggest that the high
409 infiltration rates measured during the experiment, which are in the upper range of values

410 compared to those typically found in hyporheic zones, can produce relatively constant high
411 DOC values in depth (at least 50 cm), together with constant pH and temperature.

412 However, degradation of organic compounds containing nitrogen and phosphorus remained
413 approximately constant with depth (Fig. 3). This result may be due to bacterial colonization
414 as indicated before, but also to the availability of organic N and P compounds at different
415 depths. Consistent with this model, higher phosphatase activity has been reported in
416 hyporheic zones, contributing to phosphorus flux in sediments by hydrolyzing
417 phosphomonoesters and making phosphate available [54]. Notably, several authors have
418 reported that phosphatase and leu-aminopeptidase activities decrease across sediment profiles
419 [22, 53]. Moreover, the delayed increase in phosphatase activity may indicate that few (if
420 any) complex- P-compounds were present at the beginning of the experiment, and that
421 bacteria may be producing complex P-compounds during the experiment.

422 Microbial functional diversity was also depth dependent, and differences were more evident
423 by the end of the experiment. Biolog Ecoplates incubations were used to characterize carbon
424 source utilization in the sediment tank. This culture method has several well documented
425 limitations [55–57]. However, when data normalization and protocol standardization (i.e., use
426 of similar inoculum size and incubation conditions) are applied, this method allows obtaining
427 robust data on microbial functional diversity [58]. By the end of the experiment, microbial
428 community had become more specialized and used a narrower range of carbon substrates, as
429 indicated by the lower Shannon diversity and richness scores. These data suggested that the
430 microorganisms had assembled to those better adapted to the environmental conditions of the
431 sediment column. This ecological specialization was defined by Devictor et al. [59] as an
432 adaptation process to the diversity of resources used by a species in different environments.

433 Decreased use of available substrates was observed at 50 cm, similar to results reported by
434 Griffiths et al. [60], who showed decreased substrate utilization in the surface soil at 20 cm.

435 The microbial community also showed different fingerprints depending on depth (Fig. 7).
436 Carbohydrates and polymers were used readily at the surface (0 and 20 cm depth, Fig. 7a).
437 Similarly, concentrated use of carbohydrates and polymers was observed at the seawater
438 surface [61]. These compounds are considered to be the largest bioavailable source of carbon
439 in sediments [62] and greater use of them at the surface is consistent with high surface β-
440 glucosidase activity. The metabolic fingerprint at 50 cm was distinct from fingerprints at 0
441 and 20 cm (Fig. 7a). The former was mainly characterized by the use of nitrogen compounds,
442 as shown by higher NUSE index values. Until day 50, microbial communities used amines
443 and phenolic compounds and from day 50 until the end of the experiment, amino acids and
444 carboxylic acids were used (Fig. 7a). These results indicate the significant use of nitrogen-
445 containing organic compounds at the bottom of the tank, consistent with the maintenance of
446 leu-aminopeptidase activity.

447 Differences in microbial metabolism with depth may have been affected by oxygen
448 availability, most significantly by the end of the experiment. Bacterial colonization and
449 biofilm formation may have contributed to pore clogging, providing a substantial decrease in
450 permeability and infiltration flow rate, and an increase in anoxia with increasing depth [9]. In
451 river ecosystems, decreases in dissolved oxygen along the vertical profile correlates with
452 microbial respiration, interstitial flow, and water residence time [1, 63]. Indeed, oxygen plays
453 an important role in microbial metabolism and diversity [64]. In our study, a significant
454 reduction of degradation of organic nitrogen and phosphorus compounds was found under
455 anoxic conditions, whereas no polysaccharide degradation was detected (Fig. 4), suggesting
456 that inactivation rates of the hydrolytic enzymes vary for different enzymes [29, 65].

Christy et al. [66] reported that during anaerobic and aerobic decomposition, polysaccharides are hydrolysed by secreted enzymes, such as cellulase and cellobiase. Cellulose-hydrolysing 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. [41] 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 [67]. In our study, oxic conditions led to greater degradation of phosphorus compounds compared to carbon and nitrogen over time. Equilibrium was observed between C:N acquiring enzymes, but it remained imbalanced for C:P and N:P acquiring enzymes (Figure 5). These data suggest that the sediment community inside the tank was phosphorus limited, specially at the end of the experiment, as we observed a reduction of available phosphorus in water which a decline of phosphate concentration.

Phosphorus limitation in sediment affects bacterial growth rates and microbial nutrient assimilation [42]; similar imbalances in sediments were recently reported by Hill et al. [67] and Romaní et al.[68] 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. [69], who suggested the presence of lower biological phosphorus retention efficiency under anoxic conditions in Baltic Sea sediments.

480 The significant depth effects observed at the end of the time-course and the effect of anoxia
481 were clearly shown in the functional fingerprint measured at 50 cm (Fig. 7b). A different
482 functional fingerprint was obtained for communities incubated in oxic and anoxic conditions;
483 carboxylic acids and amino acids were used preferentially under anoxic conditions. Tiquia
484 [70] reported that low oxygen conditions promoted the use of carboxylic acids and amino
485 acids in an urbanized river. In our sediment column, the distinct metabolic fingerprints that
486 occurred due to depth and anoxia occurred gradually over time; e.g., results for day 14 in
487 anoxia were still similar to those found under oxic conditions (Fig. 7b). The gradual change
488 in oxygen conditions that occurred in the column suggests that both aerobic and anaerobic
489 processes may have occurred simultaneously. Indeed, nitrification and denitrification
490 processes might also have occurred with time, as shown by NH_4^+ consumption and NO_3^-
491 production in the first 20 cm of the sediment column. Toward the bottom of the tank, NO_3^-
492 was consumed and no ammonium was present, suggesting that nitrogen had to be acquired
493 from complex nitrogen compounds. These data hints the spatially coexistence of nitrification
494 and denitrification in the sediment profile, already reported in marine sediments [71].

495 The gradual change in the metabolic fingerprint with increasing depth may be related to
496 changes in the quality of the available organic matter and changes in metabolic processes that
497 occur due to depleated oxygen concentrations. However, changes in the composition of
498 bacterial communities through the sediment column may also occur. The results from the
499 Biolog Ecoplates indicated significant metabolic changes, supporting the model that changes
500 in the community composition occurred. Adaptation of the communities to anoxic conditions
501 was shown by the presence of active bacteria at all depths, including the transition zone from
502 oxic to anoxic conditions. Facultative bacteria, adapted to live in sediments with changeable
503 oxygen concentrations, may have colonized this column. Indeed, microorganisms responsible
504 for oxidation of organic matter are not only aerobic bacteria [64]. In areas of low oxygen,

components of anaerobic respiration (e.g., nitrifiers, sulfate reducers, and methanogenic bacteria) can metabolize organic carbon [6, 52]. Maintenance of live bacteria throughout the sediment depths was shown by similarities in kinetic parameters (a, b, and x_0) under oxic and anoxic conditions. The 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 [52]. In this context, it is known that oxic and anoxic bacteria can hydrolyse particulate material or to mineralize dissolved organic matter equally fast in sea sediments [72].

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 appear to have high spatial and temporal heterogeneity at greater depths [6].

Functional approaches, including measurements of extracellular enzyme activities and Biolog Ecoplate incubations, provided complementary information on the microbial community in the sediment column. Previous authors analysed results from extracellular enzymes and Biolog Ecoplates, but found no direct correlations for bacterioplankton [73] and salt marsh sediments [74], and slight correlations in river biofilms [75]. 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, this indicated that the biogeochemical processes changed over time, whereas the functional diversity characteristics changed due to sediment depth.

529 The laboratory experiments used simulated natural sediment conditions and allowed the
530 environmental conditions to be controlled, enabling a meso-scale study, and thus can be seen
531 as a first step to give insights about the biogeochemical processes occurring at the hyporheic
532 zone. Extrapolation of these results to natural aquatic ecosystems must be done with caution,
533 and the transferability of these results at larges scales should be assured through a directly
534 field experiment, as a solution to validate the laboratory experiment, simulating the same
535 conditions in the field. We conclude that the microbial community showed different abilities
536 to degrade organic matter at different sediment depths. Greater decomposition of carbon
537 compounds occurred in surface sediments, and greater use of nitrogen compounds occurred at
538 greater depths. Under anoxic conditions at increased depths, phosphatase activity was
539 inhibited, limiting phosphorus availability. Milder effects of anoxia were found for peptidase
540 activity, and glucosidase activity was not affected. During the experiment, the microbial
541 community in the sediment became specialized to use a narrow range of carbon sources,
542 especially at increasing depths, as shown by decreased functional diversity. Coexistence of
543 aerobic and anaerobic communities, promoted by greater physicochemical heterogeneity, was
544 also observed in deeper sediments. Bacteria (including living bacteria) occurred at all
545 sediment depths and were able to adapt to different oxygen concentrations. These factors may
546 affect the biogeochemical potential of deep sediment columns for water purification
547 processes.

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TABLES

Table 1. Synthetic water composition used as input water to the flow-through system during the experiment.

Synthetic water composition

Compound	mg/L
$CHNaO_3$	160
KH_2PO_4	1.2
$MgCl_2\ 6H_2O$	211.7
$Mg(NO_3)_2\ 6H_2O$	20.0
KCl	60.0
$CaCl_2\ 2H_2O$	352.8
Na_2SO_4	240.0
NH_4Cl	4.0
Na_2SiO_3	16.0
Cellobiose	1.2
Leucine-proline	1.2
Humic acid	5.6

764

765

766 Table 2. Bacterial density and live bacteria at different sampling dates and depths. Values are
767 means (from 3 replicates), except for day 3 (n=1) expressed as 10^{9}cell/g of DW of sediment.
768 Standard error is shown.

	Bacterial density			Live bacteria		
Days	**0 cm**	**15 cm**	**50 cm**	**0 cm**	**15 cm**	**50 cm**
3	0.11	0.07	nd	0.03	0.03	nd
14	0.19±0.04	0.36±0.01	0.29±0.03	0.09±0.01	0.15±0.02	0.12±0.01
34	1.96±0.25	1.23±0.07	1.41±0.03	1.17±0.16	0.47±0.04	0.78±0.03
50	1.39±0.18	1.08±0.03	1.11±0.30	0.58±0.11	0.39±0.03	0.41±0.20
83	1.69±0.65	1.87±0.36	1.48±0.36	0.73±0.24	0.78±0.07	0.69±0.15

769
770

Table 3. Results of a repeated measures ANOVA considering two factors (time and depth) for extracellular enzyme activities (BG: β-glucosidase, LEU:Leu-aminopeptidase and PHO: phosphatase), functional diversity indexes from Biolog Ecoplates (H': Shannon index, S: richness (number of positive substrates), and NUSE index (use of nitrogen substrates) and live bacteria and total bacterial density (Live and total). Probabilities are corrected for sphericity by the Greenhouse–Geisser correction. Analysis of variance at day 83 (ANOVA, one factor) for each parameter is added. Significant values are indicated in boldface type.

	BG	LEU	PHO	H'	S'	NUSE	LIVE	TOTAL
Time	0.127	**0.005**	**0.001**	**0.025**	**0.013**	0.150	**0.005**	**0.010**
Depth	**0.012**	0.551	0.213	0.098	**0.007**	**0.019**	0.362	0.509
Time x Depth	0.133	0.156	0.092	0.123	0.080	0.303	**0.043**	0.071
Day 83 Depth	0.317	0.230	**0.044**	0.057	**<0.001**	**0.035**	0.783	0.628

785 **FIGURE LEGENDS**

786 **Fig. 1** Temporal evolution of oxygen concentration and infiltration rates at three different
787 depths. a) Point values represent daily means, and had been corrected for the drift of the
788 instruments due to changes in temperature

789

790 **Fig. 2** Mean values of nitrate (NO_3^-), ammonia (NH_4^+), organic carbon (DOC) and Chloride
791 (Cl) at the three selected periods. Periods are defined as P1 (days 0 to 29), P2 (days 33 to 54)
792 and P3 (day 83) as a function of depth. SE is not shown for clarity.

793

794 **Fig. 3** Temporal changes in extracellular enzymatic activities at 3 different depths. Values
795 presented displays mean ± standard error (from 3 replicates).

796

797 **Fig. 4** Relationships (in ln space) between β-Glucosidase and peptidase (a) β-Glucosidase and
798 phosphates (b), and peptidase and phosphatase (c) organic matter acquisition . Data are
799 values from each sampling data and grouped by periods (P1 white, P2 gray and P3 dark).
800 Anoxic ratios are also added on the right- The solid line indicates a 1:1 relationship

801

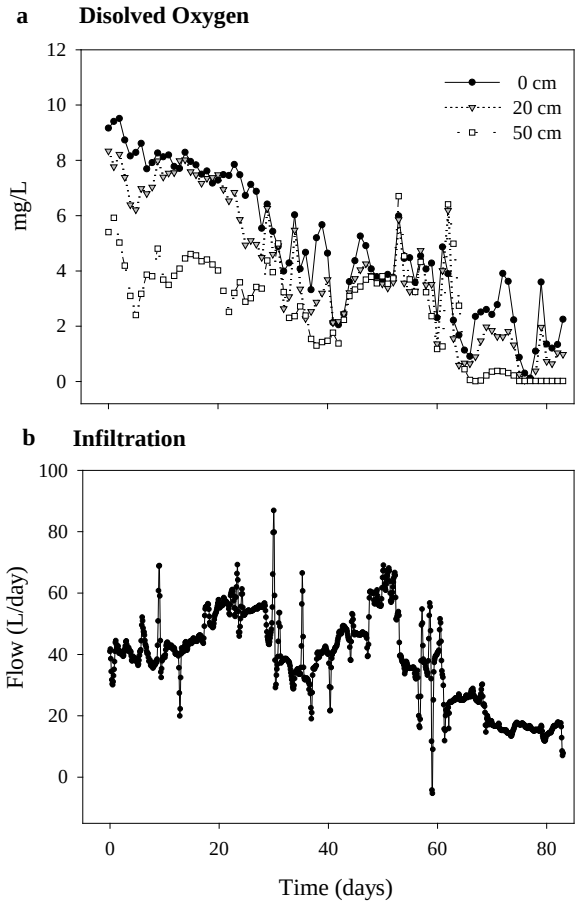
802 **Fig. 5** Box plot of extracellular enzymes activities (BG, AP, LEU) after different oxygen
803 incubations for 50cm depth samples (n=9). Standard deviations are presented as thin lines
804 over the bars, An asterisk indicates that the bar was significant different from the anoxic data.

805

806 **Fig. 6** Shannon diversity index, Richness (positive wells) and NUSE index at different depth
807 (0, 20 and 50cm) for 5 different sampling dates. Values at 50cm in anaerobic incubations are
808 also included after day 14. Values displayed are the calculated means and standard deviation
809 values (from 3 replicates).

Fig. 7 NMDS ordination plots based on Bray-Curtis distances according of 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 separating only the 50cm depth values after different oxygen incubations 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.

818 **Fig. 1**



819

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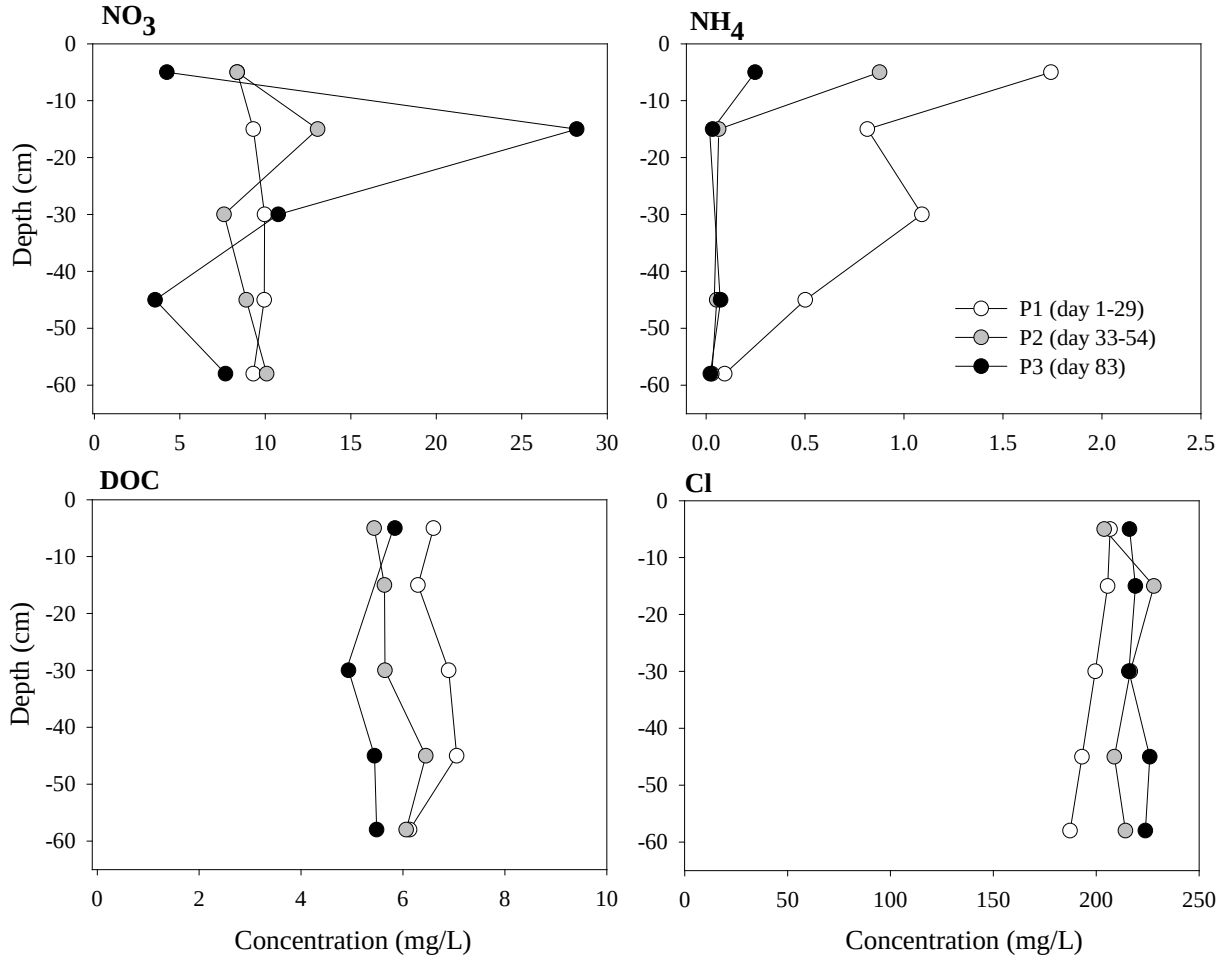
821

822 **Fig. 2**

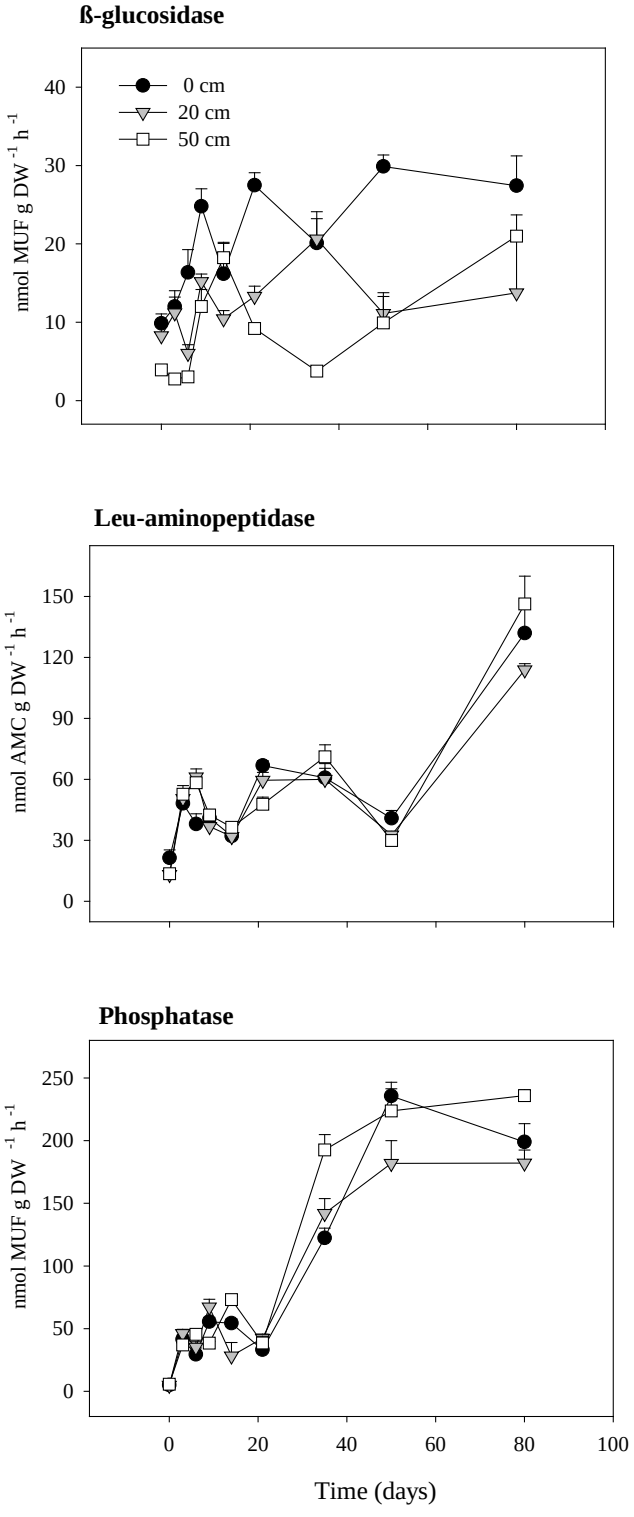
823



824

825

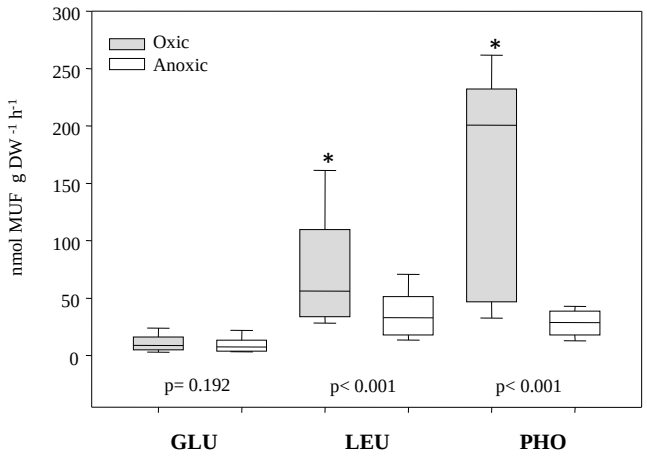
826 **Fig. 3**



827

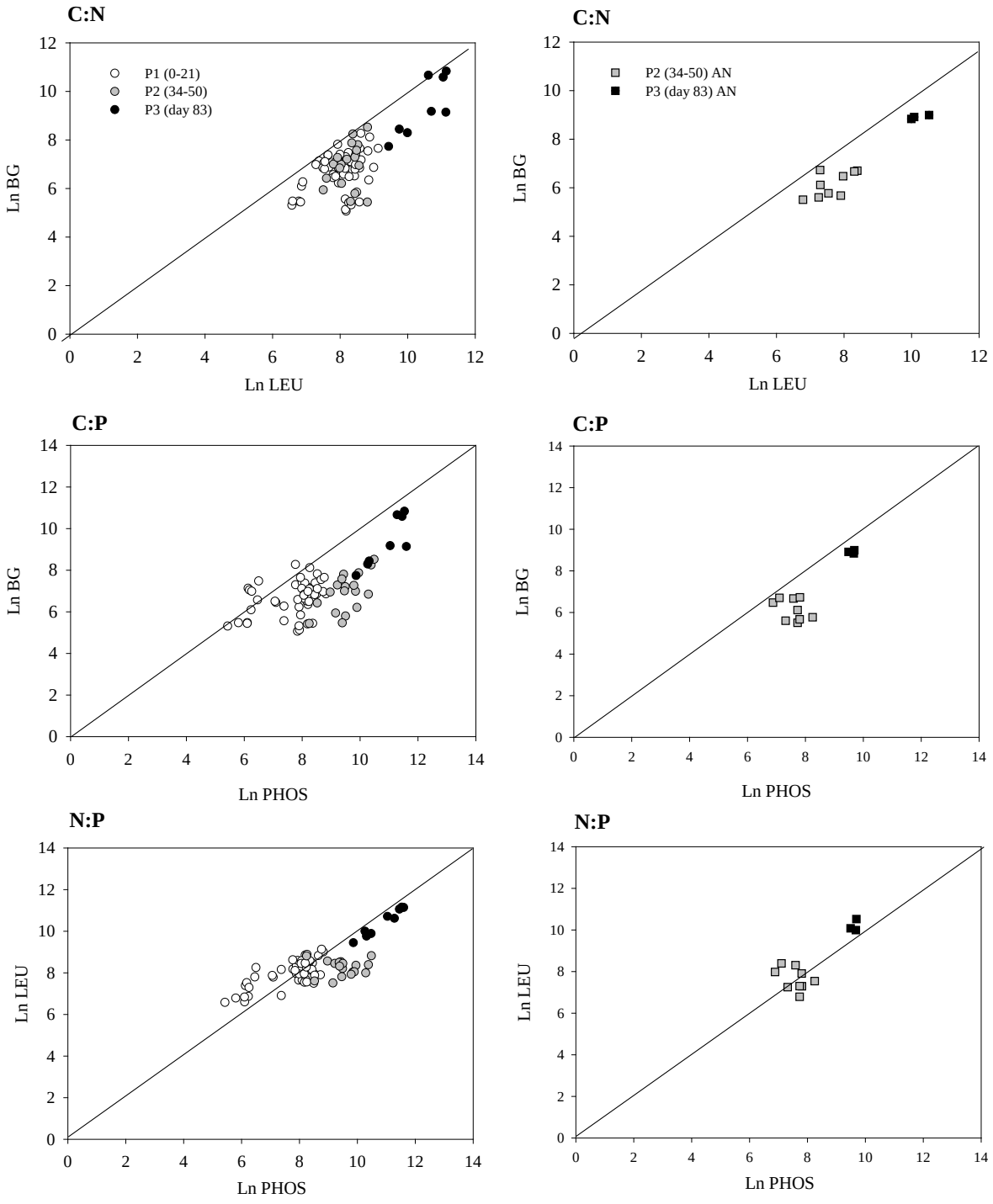
828

Fig. 4


300
250
200
150
100
50
0
Oxic
Anoxic
nmol MUF g DW -1 h-1
*
*
p= 0.192
p< 0.001
p< 0.001
GLU
LEU
PHO

832 **Fig. 5**

833

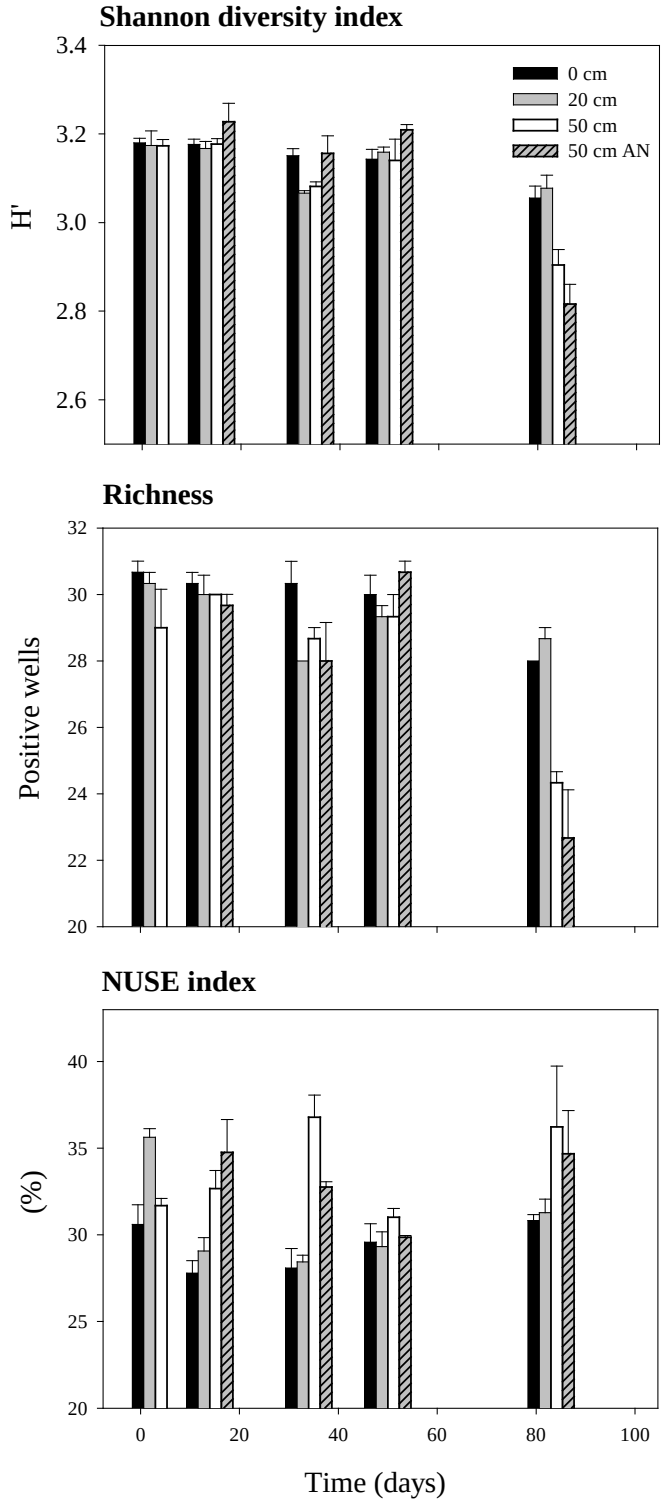


834

835

836 **Fig. 6**

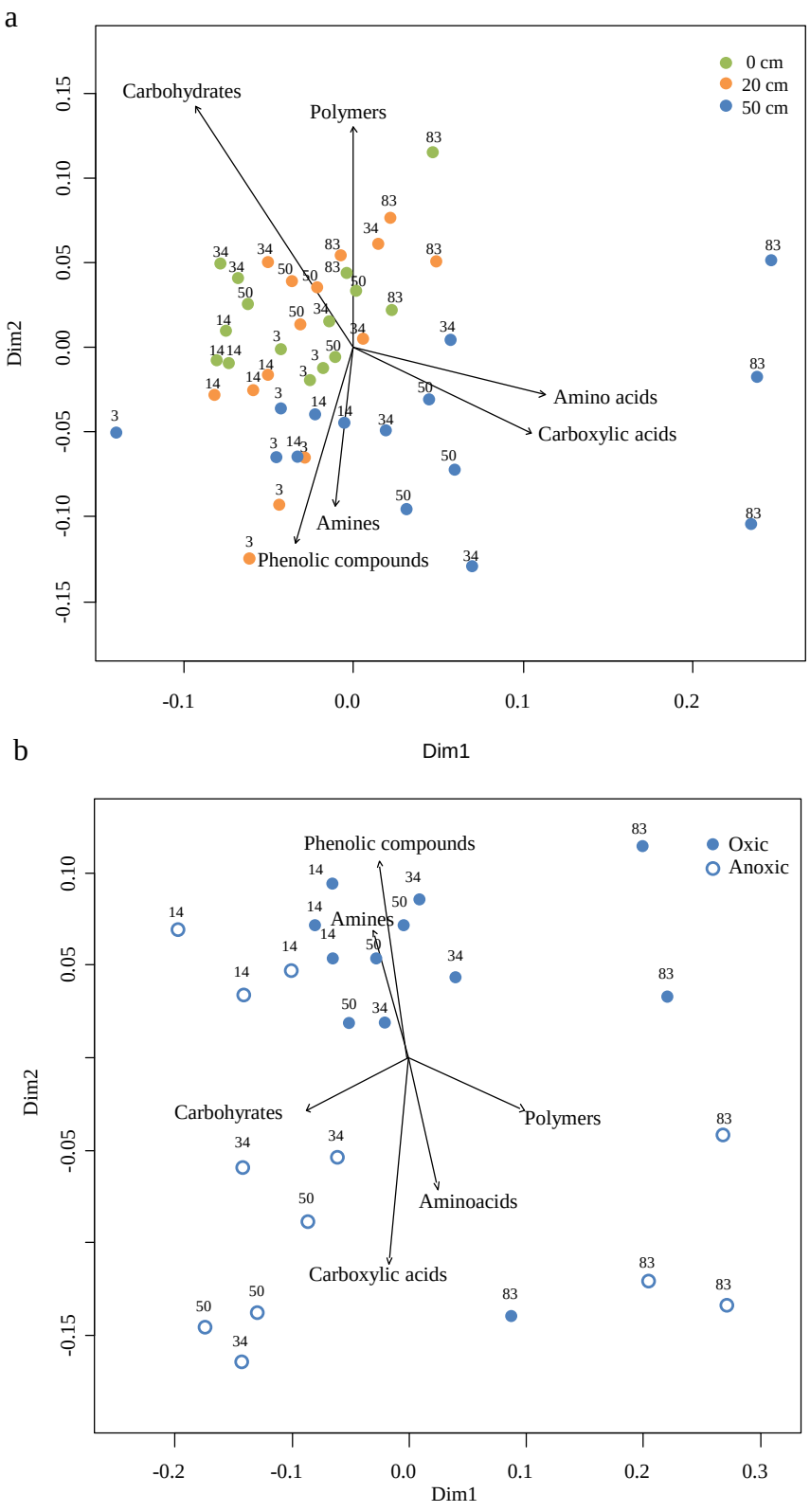
837



838

839

Fig. 7


a
0 cm
20 cm
50 cm
Carbohydrates
Polymers
Amino acids
Carboxylic acids
Amines
Phenolic compounds
Dim1
Dim2
b
Oxic
Anoxic
Phenolic compounds
Amines
Carbohyrates
Polymers
Aminoacids
Carboxylic acids
Dim1
Dim2

867

868

869

870