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Interaction between physical heterogeneity and microbial processes in subsurface sediments: a laboratory-scale column experiment

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Complete List of Authors:	Perujo, N.; Universitat Politècnica de Catalunya (UPC), Department of Civil and Environmental Engineering. Associated Unit: Hydrogeology Group (UPC-CSIC); Universitat de Girona, GRECO-Institut d'Ecologia Aquàtica Sanchez-Vila, X.; Universitat Politècnica de Catalunya (UPC), Department of Civil and Environmental Engineering. Associated Unit: Hydrogeology Group (UPC-CSIC) Proia, L.; Universitat de Girona, GRECO-Institut d'Ecologia Aquàtica Romaní, A.; Universitat de Girona, GRECO-Institut d'Ecologia Aquàtica

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1 Interaction between physical heterogeneity and microbial processes
2 in subsurface sediments: a laboratory-scale column experiment

3 N. Perujo^{a,b,c,*}, X. Sanchez-Vila^{a,b}, L. Proia^c A.M. Romani^c

4 ^a Department of Civil and Environmental Engineering, Universitat Politècnica de Catalunya (UPC), Jordi
5 Girona 1-3, 08034 Barcelona, Spain

6 ^b Associated Unit: Hydrogeology Group (UPC-CSIC)

7 ^c GRECO - Institute of Aquatic Ecology, Universitat de Girona, Girona, Spain

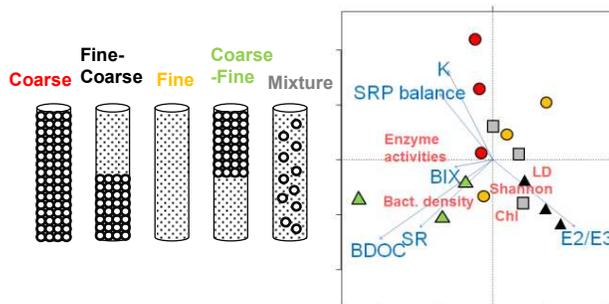
8 *Corresponding author, e-mail address: nuria.perujo@upc.edu

9 **Abstract**

10 Physical heterogeneity determines interstitial fluxes in porous media. Nutrients and organic matter
11 distribution in depth influence physicochemical and microbial processes occurring in subsurface.
12 Columns 50 cm long were filled with sterile silica sand following 5 different setups combining fine
13 and coarse sands or a mixture of both mimicking potential water treatment barriers. Water was
14 supplied continuously to all columns during 33 days. Hydraulic conductivity, nutrients and organic
15 matter, biofilm biomass and activity were analysed in order to study the effect of spatial grain size
16 heterogeneity on physicochemical and microbial processes and their mutual interaction. Coarse
17 sediments showed higher biomass and activity in deeper areas compared to the others; however, they
18 resulted in incomplete denitrification, large proportion of dead bacteria in depth, and low functional
19 diversity. Treatments with fine sediment in the upper 20 cm of the columns showed high
20 phosphorous retention. However, low hydraulic conductivity values reported in these sediments
21 seemed to constraint biofilm activity and biomass. On the other hand, sudden transition from coarse-
22 to-fine grain sizes promoted a hot-spot of organic matter degradation and biomass growth at the

23 interface. Our results reinforce the idea that grain-size disposition in subsurface sandy sediments
24 drives the interstitial fluxes, influencing microbial processes.

25 TOC Art



26
27 **Keywords:** sediment heterogeneity, infiltration columns, hydraulic conductivity, biofilm biomass,
28 hot-spots, microbial processes

29 1. Introduction

30 Bacterial communities inhabiting surface and subsurface sediments catalyse a number of ecosystem
31 processes, including uptake, storage and mineralization of dissolved organic matter, as well as
32 assimilation of inorganic nutrients.^{1,2} Processes occurring in subsurface sediments are not only
33 relevant in natural environments (such as in river hyporheic zones), but also in man-made
34 applications for water quality improvement (such as land based wastewater disposal or managed
35 aquifer recharge facilities). Infiltration systems are water treatment systems that rely on water
36 percolation³ through a porous medium whereby the quality of the effluent improves progressively
37 during the infiltration path as a consequence of the combination of biological, chemical and physical
38 processes^{4,5} driven by microbial activity⁶ at the cost of progressively reducing infiltration rates.⁷ In
39 this sense, infiltration systems may be advantageous in many aspects; they may increase (by
40 recharge) groundwater supplies, provide further treatment to infiltrating water, and reduce
41 degradation of stream-water quality.⁸ Infiltration systems may also enable water reuse thereby

42 preserving valuable freshwater resources.⁹ Some examples of infiltration systems are Rapid
43 Infiltration Basin Systems (RIBS), Slow Sand Filtration Systems (SSFS), Soil-Aquifer treatment
44 (SAT) among others.

45 Biofilms colonizing subsurface sediments offer the potential for biotransformation of organic
46 compounds, thereby providing an in situ method for treating contaminated groundwater supplies,¹⁰
47 also relevant for emerging compounds degradation.¹¹ Processing by extracellular enzymes is the
48 primary mechanism for the microbial degradation of polymeric and macromolecular organic matter
49 into low-molecular-weight molecules which can then cross bacterial cell membranes, becoming
50 available for bacterial growth and nutrient cycles.¹² Extracellular enzyme activities are good proxies
51 to determine nutrient demands and decomposition capabilities of microorganisms, as well as to
52 characterize the quantity and quality of available dissolved organic carbon and nutrients in the
53 environment.¹²

54 Heterotrophic bacteria assimilate dissolved organic carbon (DOC) and concomitantly release
55 substantial amounts of carbon in the form of extracellular polymeric substances (EPS).¹³ The EPS
56 layer traps and stores particulates and nutrients for cell metabolism and is generally thought to
57 comprise the major component of bacterial biofilm.¹⁴ It can also affect the physical characteristics of
58 porous medium through the reduction of available pore spaces for flow and alteration of water
59 retention,¹⁵⁻¹⁶ significantly reducing hydraulic conductivity and enhancing dispersion of solutes.¹⁷
60 Microbial processes and biomass accrual in subsurface sediments are determined by the surrounding
61 physical and chemical conditions. The link between physicochemical and biological parameters is
62 complex^{18,19} but the consideration of the interactions of soil microorganisms with their physical and
63 chemical environments is crucial for substantially advance in our understanding of microbial
64 ecology.¹⁹⁻²¹ Recently, some studies addressed water quality changes resulting from infiltration in
65 porous media^{3,5} and biofilm accumulation in infiltration systems (a term called bioclogging).²² Link

66 between physicochemical and biological processes in porous media has also been studied by other
67 authors.^{14,19,20,23,24,25}

68 Spatial heterogeneity of particle grain sizes distribution determines the specific physical and chemical
69 conditions in subsurface sediments. Sediment grain size and distribution are key parameters
70 determining interstitial fluxes, which also modulate the distribution of electron donors and acceptors
71 and, consequently, the distribution of microbial processes in subsurface sediments.²⁶ Related to this,
72 Higashino²⁷ proposed a model where grain diameter plays an important role in determining both
73 hydraulic conductivity and microbial oxygen uptake rate. Small hydraulic conductivity resulted in
74 small dissolved oxygen transfer but large microbial oxygen uptake rate. In coarse sand they stated
75 that even dissolved oxygen transfer rate can be large owing to a large hydraulic conductivity,
76 microbial oxygen uptake rate is small since available surface area for colonization by biofilms is
77 reduced. On the other hand, Essandoh, Tizaoui and Mohamed²⁸ concluded that the type of soil affects
78 the performance of soil columns; specifically they stated that low hydraulic conductivity results in
79 low microbial growth and low DOC removal. Similarly, Dodds, Randel and Edler²⁹ stated that
80 microbial activity may be greatest with the largest particle size because of increased water exchange
81 through pores, and smallest particle size would promote denitrification.

82 As the influence of substratum type or grain size on biogeochemical processes and biofilm
83 accumulation is not clear and it remains poorly understood, further investigation is needed to focus
84 on the interaction between physicochemical and biological parameters in different spatial grain size
85 distributions. The present work addresses the link between physicochemical and microbial processes
86 in subsurface sediments using laboratory-scale infiltration columns of different sediment grain sizes
87 and distribution. The objectives are to understand the influence of subsurface sediment heterogeneity
88 on (1) physicochemical water parameters; (2) biofilm biomass and activity and (3) the relationship
89 between these parameters and how they influence biogeochemical processes occurring in sediment
90 infiltration systems. For this purpose, we designed a number of column setups (mimicking potential

91 sand filter treatments) with different combinations of fine and coarse sands placed in different
92 columns.

93 We expected that coarse sediment would display higher infiltration rates, which would transfer higher
94 quantity of dissolved oxygen (DO), nutrients and organic matter during the infiltration process. This
95 will promote biofilm activity and biomass in deeper areas in columns having coarse sediment. On the
96 other hand, low hydraulic conductivity in fine sediments would promote anaerobic zones potential to
97 denitrification processes, but biofilm activity and biomass in depth will be limited due to reduced
98 transport of nutrients and organic matter in depth. Also we expect high phosphorous retention in fine
99 sediments compared to coarse ones. Mixture of coarse and fine sand would enable the coexistence of
100 slow and rapid zones which would promote aerobic and anaerobic processes at the same layers, as
101 well as enhancing biogeochemical processes and biomass development which could be responsible of
102 stronger bioclogging. Bilayer columns of coarse sediment in the upper part and fine sediment in the
103 bottom part would take advantage of high transfer of DO, nutrients and organic matter in the coarse
104 layer, and anaerobic conditions and phosphorous retention in the fine layer.

105 **2. Experimental**

106 **2.1 Experimental design and sampling**

107 The laboratory experiment consisted in flow-through columns filled with sediments of different grain
108 sizes. We used two different grain sizes: coarse sand (0.9 – 1.2 mm) and fine sand (0.075 – 0.250
109 mm), placed in columns 50 cm long and 4.6 cm diameter to create 5 treatments (3 replicate per
110 treatment for a total of 15 columns) with different spatial distribution of fine and coarse sand. All the
111 sand had been previously burned (450 °C for 4 hours) and cleaned with distilled water to ensure it
112 was free from organic matter.

113 We designed a column setup (mimicking potential sand filter treatments) with five combinations of
114 fine and coarse sands (see Fig. 1). Each column was filled to a height of 40 cm. A layer of 10 cm of

115 water was left above the sediment surface. Infiltration was performed with synthetic water ($13 \text{ mg}\cdot\text{L}^{-1}$
116 Na_2SO_4 , $16.1 \text{ mg}\cdot\text{L}^{-1}$ Na_2SiO_3 , $29.4 \text{ mg}\cdot\text{L}^{-1}$ $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$, $0.6 \text{ mg}\cdot\text{L}^{-1}$ KCl , $3 \text{ mg}\cdot\text{L}^{-1}$ $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$,
117 $26.5 \text{ mg}\cdot\text{L}^{-1}$ Na_2CO_3 , $0.6 \text{ mg}\cdot\text{L}^{-1}$ $\text{NH}_4\text{H}_2\text{PO}_4$, $7.3 \text{ mg}\cdot\text{L}^{-1}$ $(\text{NH}_4)(\text{NO}_3)$, and $4.27 \text{ mg}\cdot\text{L}^{-1}$ humic acids in
118 MQ water) reproducing the chemical signature of a well characterized pristine river (Fuirosos stream,
119 Spain).³⁰ Nutrient and organic matter concentrations were slightly enhanced to facilitate biofilm
120 colonization of the sediment. An inlet water tank (50 L) was placed on top of each group of 5
121 columns to produce a flow-through system. Water tanks were refilled when necessary to ensure
122 continuous infiltration. The experiment was performed at a constant temperature ($20 \text{ }^\circ\text{C}$) with a 12:12
123 light:dark cycle (incident light was $130\text{-}150 \text{ }\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). The portion of the columns filled
124 with sand was kept in the dark to mimic subsurface conditions by wrapping them with opaque
125 material. Light conditions were allowed in the surface sediment as in real infiltration sand basins. At
126 the start of the experiment, a bacterial inoculum extracted from natural sediment (from Fuirosos
127 stream) was added to all the columns (700 ml , $1.27\cdot 10^7 \text{ cel}\cdot\text{ml}^{-1}$).

128 During the 33 days of experiment, physical and chemical water characteristics (pH, DO, conductivity
129 and temperature) were measured twice per week in the inlet tanks to ensure homogeneous conditions
130 during all the experiment. Water samples from the inlet tanks and the outlet of each of the columns
131 were taken on days 15, 20, 30 and 33 to measure dissolved nutrients and organic matter content
132 (nitrates/nitrites, ammonium, phosphates, dissolved organic carbon –DOC-, and several dissolved
133 organic matter –DOM- quality properties). DO in sediment at three different depths and flow at the
134 outlet of the columns, were measured weekly. All measurements were performed during the light
135 cycle and at the same time (after 6 hours of the start of the light conditions) to reduce variability
136 between measurements due to day/night cycles.

137 At the end of the experiment, columns were dismantled for sediment biofilm biomass and activity
138 measurements at three different depths (0-2 cm, 18-22 cm, 36-38 cm). These depths corresponded to
139 the top (inlet) and the bottom (outlet) of the column, and an intermediate point which in two of the

140 configurations correspond to the interface between coarse and fine grain sizes. Sediment samples
141 were analysed for bacterial density, bacterial viability, chlorophyll-a content –chl-a-, extracellular
142 polymeric substances –EPS- content, extracellular enzyme activity and functional diversity. Each
143 layer of sediment was sampled totally and homogenized. Sub-samples of 1ml of sediment were then
144 collected using an uncapped syringe.

145 **2.2 Physical and chemical water analyses**

146 *2.2.1 Flow and hydraulic conductivity*

147 Flow rate (Q) was measured manually at the outlet of each individual column. Hydraulic
148 conductivity K (in cm/s) was calculated using Darcy's law:

$$149 \quad K = \frac{QL}{\Delta h A} \quad (1)$$

150 where Δh is the piezometric head difference (set at a constant value of 1108 ± 9 cm), L is total
151 length of the sediment (= 40 cm), A is the cross-section area (= 16.619 cm^2), and Q is measured in
152 cm^3/s .

153 Advection time is a measure of the time that takes water to go through the sediment. Advection time
154 (t) was calculated using the formula:

$$155 \quad t = (\emptyset \cdot L \cdot A) / Q \quad (2)$$

156 where \emptyset is the porosity of the sediment (0.4 for the coarse sediment and 0.32 for the fine sediment).

157

158 2.2.2 Chemical water analyses

159 Physicochemical water parameters (pH, DO, conductivity and temperature) were measured with
160 specific probes (HQd Field Case, HACH) in the supply tanks. To measure DO at different depths
161 (surface, 20 cm and 40 cm) without perturbing the sediment biofilm, a non-invasive method was used
162 by fixing oxygen sensor spots inside the wall of the columns and measuring dissolved oxygen
163 concentration using an optical fiber (PreSens).

164 Samples for dissolved nutrients and organic matter determination were filtered in pre-burned (4
165 hours, 450°C) filters (GF/F, 0.7 µm, Whatmann). After filtering, samples for dissolved inorganic
166 nutrients were frozen until analysis. DOM spectroscopic properties were analysed in fresh. Samples
167 for DOC analysis were acidified and kept at 4°C until analysis. Inorganic nutrients were analysed as
168 following: nitrate by ionic chromatography (761 Compact IC 1.1 Metrohm), phosphate by the
169 Murphy-Riley³¹ spectrophotometric method, and ammonium by the spectrophotometric sodium
170 salicylate protocol.³² DOC was analysed with TOC-V CHS/TNM-1 SHIMADZU. Spectroscopic
171 properties were analysed in order to characterize potential changes in DOM quality and included the
172 following parameters: the Slope ratio (SR) described in Helms et al.³³ which is inversely correlated to
173 organic matter molecular weight; the Fluorescence Index (FI) described in Cory and Mcknight³⁴
174 indicative of the origin of the organic matter; the Biological Index (BIX, Huguet et al.)³⁵ as indicator
175 of recent biological activity and the E2/E3 index which is related to photo reactivity (Minero et al.).³⁶
176 Biodegradable dissolved organic carbon (BDOC) was analysed once, following the protocol
177 described by Servais et al.³⁷

178 2.3 Sediment biofilm biomass and activity

179 2.3.1 Bacterial density

180 Bacterial density was determined by flow cytometry (FACSCalibur, Becton Dickinson) following a
181 protocol adapted from Amalfitano et al.³⁸ Filter-sterilized (filtered by 0.2 µm) simplified synthetic
182 water (without nutrients and organic carbon, 10 ml) and formaldehyde (100 µl, 37%) were added to

183 each sediment sample. Samples were kept in the dark at room temperature until analysis. Sediment
184 samples were sonicated for 1 minute, shook for 30 seconds, and sonicated again for 1 minute to
185 extract the biofilm from sediment grains (Ultrasons, Selecta). A sub-sample of the obtained extract (1
186 ml) was pipetted into a glass vial and 9 ml of detaching solution was added. Detaching solution
187 consists of NaCl (130mM), Na₂HPO₄ (7 mM), NaH₂PO₄ (3 mM), formaldehyde (37%), sodium
188 pyrophosphate decahydrate 99% (0.1% final concentration), and tween 20 (0.5% final concentration),
189 and it helps to separate cells avoiding aggregation. Samples were then shaken for 30 minutes (150
190 rpm) at dark and room temperature conditions. Samples were left 10 minutes at 4 °C, and sonicated
191 with ice during two cycles of 1 minute. After shaking for 1 minute, samples were left for 5 minutes
192 for sedimentation of larger particles and 1 ml of supernatant was transferred in an Eppendorf.
193 Nycodenz (1 ml) was added to the bottom of the Eppendorf and samples were centrifuged (14000
194 rpm) for 90 minutes at 4 °C. Purified extract (400 µl) was stained with Syto13 (4µl Fisher, 5µM
195 solution) and incubated in the dark for 30 minutes. Stained samples were counted using flow
196 cytometry (FACSCalibur, Becton Dickinson). To normalize fluorescence data, a bead solution (10µl
197 of 10⁶ beads·ml⁻¹, Fisher 1.0 µm) was added to the samples in a known concentration. Results are
198 reported as bacterial cells·10⁶/g sediment dry weight.

199 **2.3.2 Bacterial viability**

200 A bacterial extract from fresh sediment samples was first prepared to obtain a homogeneous and
201 dispersed cell suspension. Pyrophosphate (5ml, 50mM) was added to fresh sediment samples³⁹ and
202 they were incubated for 15 minutes at room temperature and soft shaking. Samples were then
203 sonicated for one minute with ice to avoid cell disruption.⁴⁰ A sub-sample of the obtained extract (1
204 ml) was diluted with filter-sterilized simplified synthetic water (1:50). A sub-sample of the diluted
205 extract (400 µl) was stained with propidium iodide and Syto 9 (8 µl, BacLight Bacterial Viability
206 Kit).⁴¹ Syto 9 penetrates all bacterial membranes and stains the cells fluorescent green, while
207 propidium iodide only penetrates cells with damaged membranes, and the combination of the two

208 stains produced red fluorescing cells.⁴² Samples were incubated in the dark for 15 minutes. According
209 to Falcioni et al.⁴¹ to normalize fluorescence data, a bead solution (40 μ l of 10⁶ beads·ml⁻¹, Fisher 1.0
210 μ m) was added to the samples in a known concentration. Bacterial viability was measured by flow
211 cytometry (FACSCalibur, Becton Dickinson). Results are reported as the ratio between live cells (L)
212 and dead cells (D) -LD ratio-.

213 ***2.3.3 Chlorophyll-a***

214 Samples for chl-a analysis were placed in glass vials and kept in dark at (-20°C) until analysis. Chl-a
215 concentration was determined as described by Jeffrey and Humphrey⁴³. Acetone 90% (10 ml) was
216 added to each sediment sample in order to extract the chl-a and kept in dark for 8-12 hours at 4°C.
217 Sediment samples were sonicated and filtered (GF/C, 1.4 μ m, 47 mm). Absorbance was measured at
218 430, 665, and 750 nm. Results are given as μ g of chlorophyll-a/g sediment dry weight.

219 ***2.3.4 Content of polysaccharides in extracellular polymeric substances***

220 EPS were extracted by a cation exchange resin (CER) and the content of polysaccharides measured
221 spectrophotometrically following the protocol described by Dubois et al.⁴⁴ Sediment samples for EPS
222 analysis were placed in plastic flasks and frozen until analysis. Previous to analyses, CER (Dowex
223 Marathon C sodium form, Sigma-Aldrich) was conditioned with HCl (4M) and NaOH (1M)
224 following manufacturer instructions, and the samples were left to reach room temperature. Then,
225 samples were placed in an Eppendorf with 1ml of simplified synthetic water plus 0.3 g of CER. After
226 shaking them carefully, samples were incubated with ice for one hour in a shaker (250 rpm). Samples
227 were then centrifuged (11000 rpm) for 15 minutes at 4 °C. The supernatant (500 μ l) from each sample
228 was pipetted into glass tubes. A phenol solution (12.5 μ l, 80% w/w) was added to the glass tubes.
229 After carefully shaken, 1.25 ml of H₂SO₄ (95.5%) was added to the samples. Glass tubes were
230 capped. After 10 minutes, samples were carefully shaken and incubated for 20 minutes in a water
231 bath (30 °C). Absorbance (485 nm) was measured in a spectrophotometer. To determine EPS

232 concentration, a glucose standard was prepared. Further transformation of results to sediment dry
233 weight was performed. Results are given in μg glucose-equivalents/g dry weight.

234 ***2.3.5 Extracellular enzyme activities***

235 Extracellular enzyme activities β -glucosidase (EC 3.2.1.21), β -xylosidase (EC 3.2.1.37), phosphatase
236 (EC 3.1.3.1 -2) and leucine-aminopeptidase (EC 3.4.11.1) were measured with spectrofluorometry
237 using fluorescent-linked artificial substrates (Methylumbelliferyl (MUF)- β -D-glucopyranoside,
238 MUF- β -D-xyloside, MUF-phosphate and L-leucine-7-amido-4-methylcoumarin hydrochloride (Leu-
239 AMC), Sigma-Aldrich). All enzyme activities were measured under saturating conditions (0.3 mM).⁴⁵
240 Fresh sediment samples were placed in a 15 ml tube with synthetic water (4 ml) and 120 μl of
241 artificial substrate. A blank for each artificial substrate was prepared with synthetic water in order to
242 determine the abiotic hydrolysis of the substrate itself. Samples and blanks were incubated for 1 hour
243 in the dark with agitation. After 1-hour incubation, glycine buffer (4 ml, pH 10.4) was added in order
244 to stop the reaction and maximize MUF and AMC fluorescence. Samples were centrifuged (2000 g)
245 for 2 minutes, and the supernatant (350 μl) of each sample was placed into a 96 wells black plate
246 (Greiner bio-one). Fluorescence was measured at excitation/emission wavelengths of 365/455 (MUF
247 fluorescence) and 364/445 (AMC fluorescence) in a fluorimeter plate reader (Tecan, infinite M200
248 Pro). To determine extracellular enzyme activities, MUF and AMC standards were prepared and
249 measured for their fluorescence. Results are given in nmol MUF/g dry weight \cdot h or nmol AMC/g dry
250 weight \cdot h.

251 ***2.3.6 Functional diversity***

252 Biolog Ecoplates microplates (AEX Chemunex) were used to determine functional diversity of
253 sediment communities. Each microplate contains three replicate wells of 31 carbon sources and a
254 blank (no substrate). To obtain an extract of the microbial community from the sediment samples a
255 similar procedure to that used for bacteria viability was used. Pyrophosphate (5 ml, 50 mM) was
256 added to the sediment samples which were then incubated for 15 minutes at room temperature and

257 soft shaking. Samples were sonicated for one minute with ice. A sub-sample of the obtained extract
258 (1 ml) was diluted with filter-sterilized simplified synthetic water (1:50). Microplates were inoculated
259 under sterile conditions with 130 μ l of the diluted extract to each well and incubated in dark
260 conditions at 20 °C for 14 days. Absorbance was measured every 24 hours at 590 nm (Tecan, infinite
261 M200 Pro). The color measured in each well, a measure of the capability of the inoculated
262 community to metabolize the specific substrate, was corrected by the color measured in the blank
263 well from each microplate. During the incubation, absorbance measurements increased following a
264 sigmoidal pattern, and after 14 days of incubation the absorbance was saturated. Absorbance data of
265 each substrate, when the average well color (AWCD) was 0.5, was used to calculate functional
266 diversity by means of the Shannon diversity index.⁴⁶

267 **2.4 Data treatment**

268 Normalized hydraulic conductivity with respect to the original value (K/K_0 , K being actual hydraulic
269 conductivity and K_0 the initial one at each column) was calculated as a function of time and analyzed
270 with ANCOVA analysis. Oxygen balance was calculated from the differences between column
271 outlets and inlets. To study the relationship between oxygen balance and normalized hydraulic
272 conductivity Pearson's correlation was performed. Nutrient and DOC balances were calculated from
273 the differences between column outlets and inlet tanks and process rates were calculated dividing
274 nutrient balances by advection time. For these parameters, differences between treatments were
275 analyzed with two factors ANOVA (factor: day and treatment). Differences in DOM properties
276 between treatments were also analyzed with ANOVA (factor: day and treatment). For better
277 understanding the relationship between physic-chemical parameters and biological processes
278 occurring in the columns, values of hydraulic conductivity measured the last day of the experiment
279 were analyzed through ANOVA to detect differences between treatments. Biological data from
280 sediment samples and DO from the last sampling day were analyzed by a two-way ANOVA test for
281 differences between treatments and depths and their interaction. Whenever significant differences

282 were detected, further Tukey's post hoc tests were performed. Differences between treatments at each
283 depth were further analyzed.

284 To integrate physic-chemical and biological data along the column, a redundancy analysis (RDA)
285 was performed using one matrix with biofilm biomass and activity values, fitted with another matrix
286 containing physic-chemical parameters (nutrient and DO balances, DOM properties and absolute K
287 values) measured the last day of the experiment in each treatment. Since biofilm biomass and activity
288 was measured at three different depths, data was integrated by depth layers to obtain one number per
289 parameter and treatment. Complementarily, ANOSIM analysis was performed to detect differences
290 between treatments. Further, Pearson's correlation was performed. All statistical analyses were
291 carried out with R statistics (vegan package) excepting ANOSIM analysis which was performed
292 using PRIMER v.6 Software. For multivariate analysis, variables were previously scaled using the
293 scale command in R. For ANOVA analysis all variables were logarithmically transformed to bring
294 the variables close to the normal distribution (Shapiro-Wilk normality test). In all the parameters
295 three replicates were used.

296 **3. Results**

297 **3.1 Physicochemical parameters**

298 Flow measured at the start of the experiment was 1.07 ± 0.42 ml/s in Coarse treatment; 0.17 ± 0.03
299 ml/s and 0.13 ± 0.02 ml/s in Fine-Coarse and in Fine treatments, respectively; 0.39 ± 0.18 ml/s in
300 Coarse-Fine treatment and 0.31 ± 0.1 ml/s in Mixture treatment. Hydraulic conductivity displayed a
301 clear decreasing trend with time (Fig. S1). ANCOVA analysis did not show significant differences in
302 normalized K values between treatments although results showed that in the Coarse and Coarse-fine
303 columns K reduction started later as compared to the other treatments, which showed a sharp
304 reduction in the first days. All columns showed a negative oxygen balance indicating consumption of
305 oxygen from the column inlet to the outlet. Oxygen consumption increased along the experiment
306 reaching values of -6 mg O₂/L at the end of the experiment. Oxygen consumption was correlated with

307 reduction of hydraulic conductivity (Fig. S2) however at the start of the experiment slightly positive
308 oxygen balance values were reported possibly due to still high instability of the system. On the last
309 day of the experiment, the highest K values were measured in treatments displaying coarse sand at
310 the upper layers (Coarse and Coarse-fine treatments, Table 1). Absolute DO values showed a
311 significant decrease in depth ($p < 0.01$, Table 1). The minimum value reported for DO was 2 mg/L.
312 No significant differences in DO were detected between treatments at any given depth but slight
313 oxygen production occurred at the surface of the sediment especially in Fine-Coarse, Fine and
314 Mixture treatment (Table 1). Coarse and Coarse-Fine treatment resulted in high DO consumption rate
315 (Table 2). Coarse treatment showed also the shortest advection time meaning that water passed faster
316 through the sediment. On the other hand, Fine-Coarse and Fine treatments showed the longest
317 advection times indicating more time for water to pass through the sediment (Table 2).

318 After 30 days from the start of the experiment all the ammonium supplied at the inlet (1.26 mg N-
319 NH_4/L) was eventually fully transformed in all treatments (Fig. S3). However, Coarse treatment was
320 showing the highest ammonium consumption rate (Table 2). N- NO_x balance showed mainly positive
321 values indicating nitrate/nitrite production. No significant differences were detected between
322 treatments in N- NO_x balances, but when analyzing N- NO_x production rates Coarse treatment
323 resulted in the highest values (Table 2). Phosphorus was mainly retained through all sediment
324 columns and the highest retention was measured for Fine-Coarse and Fine treatments (Fig. S4). Mean
325 DOC at the inlet tanks was 1.39 ± 0.38 mg/L, at the outlet was 1.44 ± 0.28 mg/L, this results in a very
326 small balance and no differences between treatments were detected.

327 Even though no differences were detected in DOC concentrations differences in DOM properties
328 were reported (Table S1): the Coarse treatment showed the lowest SR and BIX values, while the
329 Coarse-Fine one reported the highest BIX value. E2/E3 values were highest for the Fine-Coarse
330 treatment. The highest BDOC value was observed in the Coarse-fine treatment and the lowest one
331 corresponded to the Fine.

332 **3.2 Sediment biofilm biomass and activity**

333 Bacterial density, chlorophyll and EPS content in sediments showed a strong vertical gradient in
334 depth with highest values at the surface declining sharply in the top 20 cm (Fig. 2). This depth pattern
335 was different depending on the treatment. Bacterial density at the surface was not significantly
336 different between treatments, but at 20 cm depth, the highest values were measured at the Coarse-fine
337 treatment and at 40 cm the highest values were measured at the Coarse treatment. The highest
338 chlorophyll-a concentration at the surface was measured at the Fine-coarse and fine treatment, and at
339 20 cm depth highest values were found in the Coarse-fine treatment. Mixture treatment showed the
340 lowest EPS concentration at 20 and 40 cm depth.

341 The LD ratio (live to dead bacteria) was below 1 for all treatments, and increased in depth except in
342 the Coarse treatment (Fig. 3). Functional diversity decreased with depth (Fig. 3); the lowest value
343 was detected in the Coarse column at 20 and 40 cm depths, and the highest was reported for the Fine-
344 coarse column at 20 cm. Analyzing the functional fingerprint, no significant differences were
345 detected between treatments, but that at the surface was different from the ones observed at 20 and 40
346 cm depth (ANOSIM, $r = 0.567$, $p = 0.0001$).

347 Extracellular enzyme activities showed a gradient in depth (Table 3). The Coarse-fine treatment
348 showed higher β -glucosidase and β -xylosidase activities in the surface compared to the other
349 sediments. This treatment also showed higher β -xylosidase and phosphatase activities at 20 cm depth.
350 The Coarse treatment showed higher β -glucosidase and leucine-aminopeptidase activities at 40 cm
351 depth.

352 **3.3 Integrating physicochemical and biological responses**

353 Integrating values for each individual column and performing an RDA analysis, data corresponding
354 to biofilm activity, biomass and functional diversity was fitted with the environmental variables
355 (nutrient balances, hydraulic conductivity and DOC properties measured the last day of the
356 experiment) to study the conjunction between biofilm and physical properties (Fig. 4). Treatments

357 displaying coarse sand in the first 20 cm (Coarse and Coarse-fine), are placed on the left of the graph;
358 showing the lowest E2/E3 values and the highest β -glucosidase, β -xylosidase and leucine-
359 aminopeptidase activities. However, differences between the Coarse-fine and the Coarse treatments
360 do exist. The former resulted in higher phosphatase activity, bacterial density, BDOC, BIX, FI, and
361 SR. On the other hand, the Coarse treatment was characterized by highest hydraulic conductivity,
362 lowest phosphorous retention, highest NO_x production, and low LD ratio as well as low functional
363 diversity.

364 On the right part of the same graph (Fig.4) we can find the treatments with low hydraulic
365 conductivity (Fine, Fine-coarse and Mixture), all involving fine sand in the upper 20 cm and sharing
366 low values of β -glucosidase, β -xylosidase and leucine-aminopeptidase activities, and high E2/E3
367 values and oxygen consumption. However, interpretation of E2/E3 index should be done cautiously
368 since its values and the tendencies between treatments vary among time. Significant differences were
369 detected between all treatments (ANOSIM, $r = 0.6$, $p = 0.001$), except for Fine and Mixture treatment
370 which could not be discriminated.

371 Pearson's correlations were performed for the last day of the experiment with biological and
372 physicochemical parameters. Significant correlations ($r > 0.5$, $p < 0.05$) are described as follows:
373 hydraulic conductivity was positively correlated with positive balances of N-NO_x and phosphorous
374 indicating production of N-NO_x and no retention of phosphorous. BDOC was positively correlated
375 with bacterial density. Extracellular enzyme activities were positively correlated between them and
376 bacterial density was positive correlated with all of them. Shannon Index was positively correlated
377 with chl-a content, LD ratio and E2/E3 index. Negative balance of DO was positively correlated with
378 transformation of N-NH₄.

379

380 **4. Discussion**

381 **4.1 Effects of sediment heterogeneity on physicochemical parameters**

382 Saturated hydraulic conductivity (K) is the most relevant parameter driving flow and transport in
383 porous media. As expected, hydraulic conductivity was highest in the Coarse treatment, while the
384 presence of fine sediments in the other treatments resulted in lower conductivity values. This
385 coincides with Baveye et al.⁴⁷ and Pavelic et al.⁹ who found higher saturated hydraulic conductivity in
386 coarse-textured materials as compared to fine-textured materials. As expected, high hydraulic
387 conductivity results on high transfer of nutrients, organic matter and DO in depth, which allow for
388 high nitrification rates. Reduction in K as a function of time was mostly associated to biological
389 clogging. However, sharp K reduction at the beginning of the experiment in treatments with fine
390 sediment in the upper layer could be related to sediment compaction⁴⁸. In the columns, reduction of
391 hydraulic conductivity was correlated to oxygen consumption. DO is energetically the most
392 favourable electron acceptor and strongly influences the succession of biogeochemical processes
393 within the subsurface.⁴⁹ Specifically, DO is consumed during the mineralization of organic matter
394 and nitrification of ammonium in the oxic zone. However, decrease of oxygen in subsurface
395 sediments could be also related to slow DO supply resulting from the reduction of K and
396 corresponding water fluxes with time.⁵⁰ Contrarily to what expecting, no denitrification was achieved
397 in any treatment due to DO concentrations were not low enough. As phosphorous reduction is
398 enhanced by the presence of fine sediment, we expect adsorption to be the main process affecting
399 phosphorous reduction. However, it also could be related to high P uptake by autotrophs,⁵¹ as
400 treatments with fine sediment in the upper part showed high Chl-a concentration at the surface and
401 high phosphorus reduction.

402 Low SR values reported in the Coarse treatment are indicative of low organic matter degradation (SR
403 values are inversely correlated to organic matter molecular weight).³³ Oppositely, transition from

404 coarse-to-fine sediment could promote biological activity as indicated by high BIX values³⁵ and high
405 SR values.

406 **4.2 Linking physicochemical parameters to biofilm biomass and activity**

407 In general, biomass and biofilm activity decrease with depth (e.g., Freixa et al.)⁵². This is related to
408 oxygen and nutrients being the limiting factor controlling bacterial growth and metabolic activity¹⁸
409 and these resources decreasing in depth.^{53,54} The experiment show significant interaction between
410 treatment and depth for most biological parameters, indicating that the sediment grain size
411 distribution was affecting differently the activity and biomass patterns in depth.

412 Sediments displaying high hydraulic conductivity values are expected to lead to fast transport of
413 organic matter into deeper sediments⁵⁵ due to high infiltration rates. This could explain high bacterial
414 biomass concentrations at large depths in coarse sediments. However, the low proportion of live
415 bacteria in depth and the high reduction on functional diversity in these sediments coincide with less
416 degraded organic matter. Also high leucine-aminopeptidase activity achieved in coarse sediments
417 could be an indicator of organic material released because of cell lysis.⁵⁶

418 The coarse-to-fine transition promotes the accumulation and transformation of organic matter at the
419 interface. This was suggested by the highest capacity to degrade polysaccharides as demonstrated by
420 high C-acquiring enzyme activities, β -glucosidase and β -xylosidase activities. The former is related
421 to cellulose degradation, while the latter is promoted by the presence of hemicellulose.⁵⁷ High
422 phosphatase activity in the transition compared to the other treatments could be related to high chl-a
423 content, since algae are also responsible for this activity but may be also linked to low availability of
424 inorganic phosphorus due to its low retention capacity which may enhance bacterial phosphatase
425 activity. High enzyme activities in Coarse-fine treatment coincide with biogeochemical aspects
426 explained above (high BIX and SR values) implying that the coarse-to-fine transition promotes the
427 transformation of organic matter into biodegradable, low-molecular-weight molecules.

428 In the treatments displaying low hydraulic conductivity, nutrients and organic matter transport to
429 deeper areas are limited, resulting in low microbial activity and biomass in depth. High E2/E3 values
430 measured in such treatments on the last day of the experiment could be indicative of the photo-
431 degradability and photo-reactivity of DOC;^{58,59} however this statement should be interpreted with
432 caution since results in E2/E3 index are not consistent throughout the sampling days. High Chl-a
433 concentration measured in these treatments could be favored by high advection time which resulted
434 in slow flow and increased the contact time between water, sediment and light in the upper part of the
435 columns. This in turn could be responsible of slight higher values of DO in the upper part of these
436 columns due to release of oxygen from photosynthetic activity. However, as advection times were
437 much shorter (between 12 minutes and 1 hour) than day/night cycles we expect that the pulses in DO
438 due to algal metabolism will be rapidly dislocated through the columns and then having limited effect
439 on biogeochemical processes. Further work will be necessary to clearly understand specific effects of
440 daily primary production pulses and consequent daily variability on the physicochemical parameters
441 in infiltration systems.

442 The non-homogeneity of sediment grain size, despite the spatial homogeneity (Mixture treatment)
443 contrarily to what expected, did not favor microbial colonization or extracellular enzyme activity.
444 Furthermore, it resulted in the lowest values of EPS concentration in depth. Since not many
445 differences were accountable between the Fine and the Mixture treatments, we could state that the
446 presence of fine grain size sediments would determine the majority of the biogeochemical processes
447 that take place in the subsurface.

448 To sum up, sediments composed even partially by coarse sands which display high infiltration rates,
449 transfer high quantity of nutrients and organic matter in depth which promote high bacterial density
450 in deeper areas compared to fine sand sediments. Although not seeing differences in oxygen
451 concentration between treatments; nitrification rates and oxygen consumption rates are greater for
452 coarse sediment. Related to this, higher rates of infiltration may be associated with higher potential

453 process rates. However, low water residence times in coarse sediments result in low functional
454 diversity and a decrease in the proportion of live bacteria in depth. On the other hand, the presence of
455 fine sands limits biofilm activity and biomass in depth due to low infiltration which at the same time
456 reduce nutrient load in depth. According to this, biofilm activity, biomass and process rates could be
457 limited by low nutrient load. On the other hand, phosphorous retention is enhanced by fine sediment.
458 Transition of coarse to fine grain size sediments promote the accumulation of organic matter in the
459 interface, favoring its decomposition to smaller and more biodegradable compounds and creating hot-
460 spots of bacterial activity and biomass.

461 The present work concludes that biological and physicochemical parameters are influenced by the
462 grain size and the grain size distribution of the sediment. In relation to our hypothesis, coarse
463 sediment allows for high biomass in depth and high process rates due to high input load, while fine
464 sediment promotes accumulation of algae in the upper part of the columns and ameliorates
465 phosphorous retention but biomass in subsurface is constrained by low input loads. However, in
466 contrast to our hypothesis mixture of coarse and fine sediment behaves similarly than only fine
467 sediment. Interestingly, bilayer of coarse sediment in the upper part and fine sediment in the bottom
468 promotes high biomass in the interface between the two layers resulting in high microbial organic
469 matter degradation and nutrient recycling and also allows for phosphorous retention mainly thanks to
470 the fine layer.

471 In short, it is important to account for the implications of grain size and spatial transitions between
472 layers in subsurface sediments in order to understand and improve biological and physical knowledge
473 about processes occurring either in natural or in artificial infiltration systems. It is important to take
474 into account that implications of sediment heterogeneity on microbial biomass and activity are not
475 fully characterized by the topsoil few cm, but rather influenced by the grain size spatial distribution
476 of at least the top 40 cm.

477 **Supporting Information**

478 The supporting information is available free of charge via the Internet at <http://pubs.acs.org>.
479 DOM properties measured in each treatment during the experiment (Table S1), temporal
480 variation of normalized hydraulic conductivity for each treatment (Figure S1), relationship
481 between oxygen balance and normalized hydraulic conductivity (Figure S2), temporal
482 variation of ammonium, nitrate, and nitrite balances (Figure S3), temporal variation of
483 phosphorous balance (Figure S4).

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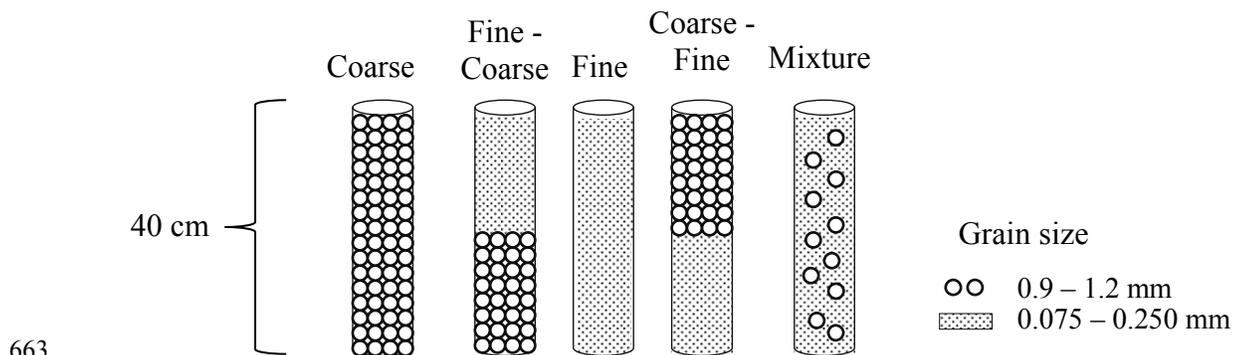
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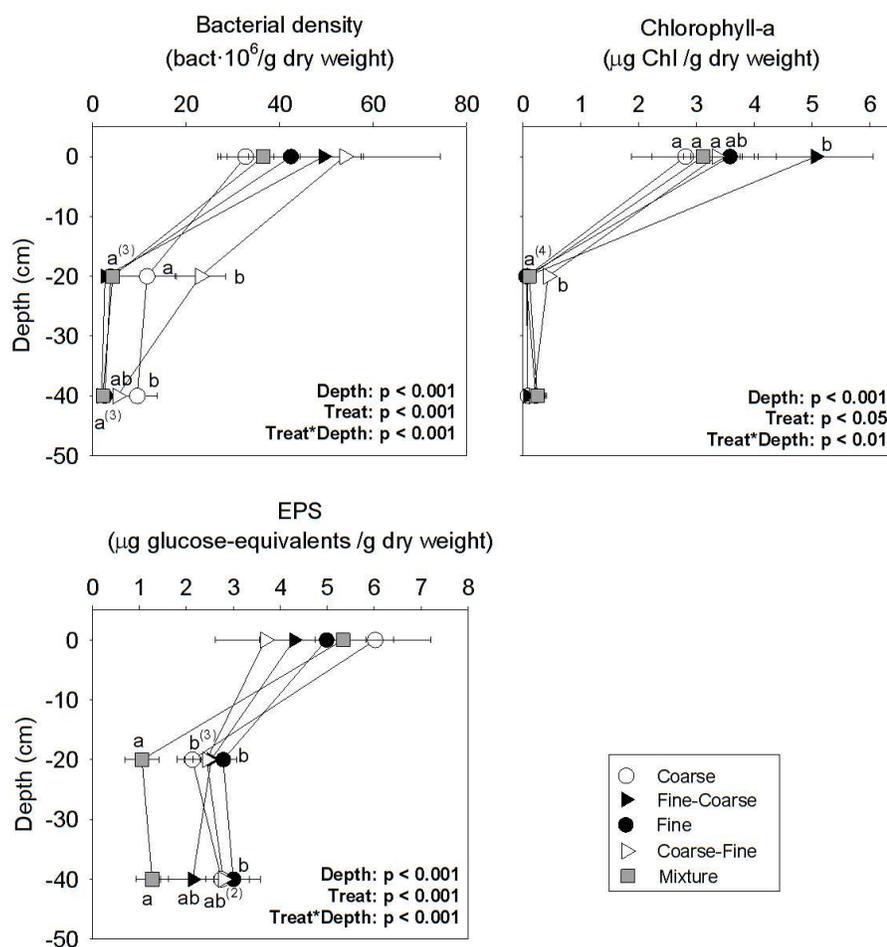
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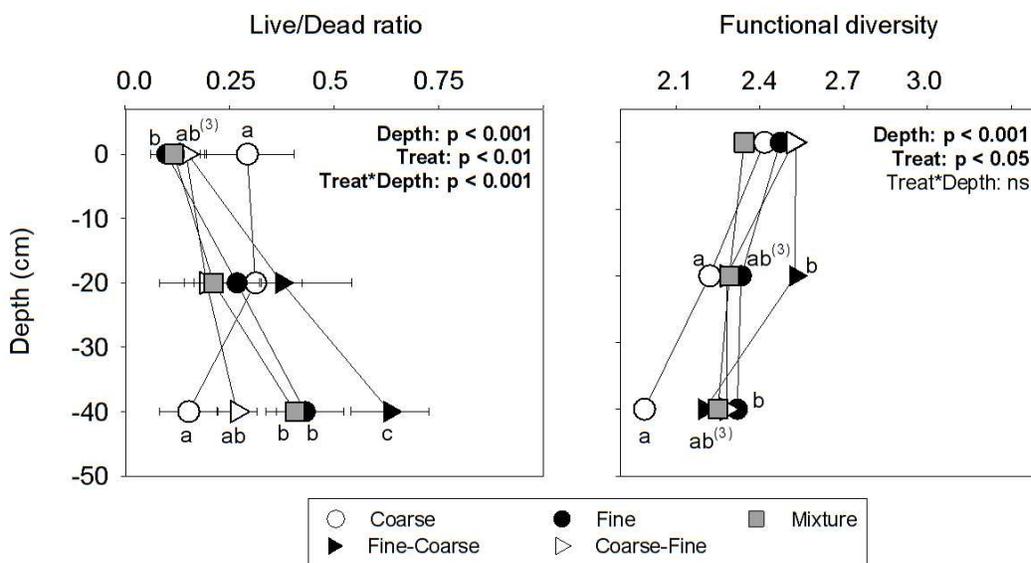
662 **Figures & Tables**

664 **Figure 1** Scheme of the column configurations regarding grain size distributions used in this experiment. Three
665 replicate columns were used for each treatment.



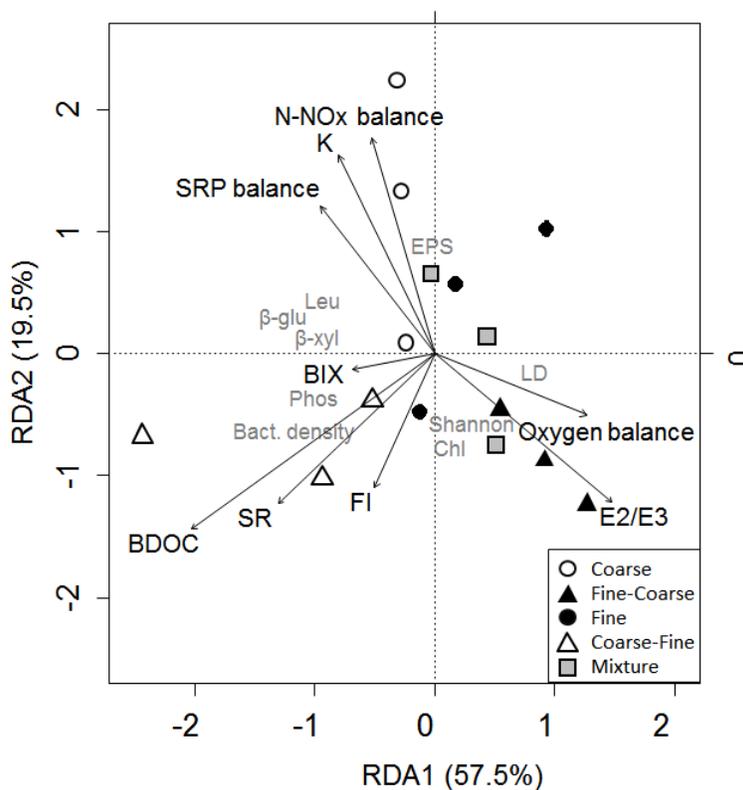
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667 **Figure 2** Absolute values of biofilm biomass (bacterial density; chlorophyll-a content and EPS content)
668 measured in sediment at different depths at the end of the experiment. Letters indicate significant differences
669 between treatments (Treat) on each depth after Tukey's post-hoc analysis. Superscripts indicate the number of
670 treatments in the same group.



671

672 **Figure 3** LD ratio values and functional diversity measured as Shannon diversity. Letters indicate significant
 673 differences between treatments (Treat) on each depth after Tukey’s post-hoc analysis. Superscripts indicate the
 674 number of treatments in the same group.



675

676 **Figure 4** RDA analysis with data from sediment biofilm fitted with physicochemical data from the last day of
 677 the experiment. ANOSIM analysis detect differences between treatments (ANOSIM R = 0.604, p = 0.001).

678 **Table 1** Hydraulic conductivity and dissolved oxygen measured the last day of the experiment in each
679 treatment

	K (m/day)	O₂ surface (mg/L)	O₂ – 20 cm (mg/L)	O₂ – 40 cm (mg/L)
Coarse	0.3078^b ± 0.1086	5.46 ± 0.78	3.86 ± 0.13	2.92 ± 0.44
Fine – coarse	0.1019 ^a ± 0.0602	7.75 ± 2.90	5.43 ± 4.10	3.44 ± 2.27
Fine	0.1319 ^a ± 0.0505	8.22 ± 3.68	3.47 ± 0.71	1.91 ± 0.73
Coarse – fine	0.1867 ^{ab} ± 0.1133	5.84 ± 0.85	2.98 ± 0.35	2.15 ± 0.74
Mixture	0.1120 ^a ± 0.0334	8.66 ± 0.19	4.86 ± 1.29	3.33 ± 0.85

680 Values are the mean of the replicates (n=3) ± sd. Letters next to the means indicate significant different groups
681 after Tukey's post-hoc analysis (p < 0.05).

682 **Table 2** Advection time and process rates for ammonium, nitrates and nitrites (NO_x), phosphorous (SRP) and
683 dissolved oxygen (DO) along the infiltration columns

	Advection time (seconds)	N-NH₄ (µg N/L·s)	N-NO_x (µg N/L·s)	SRP (µg P/L·s)	DO (µg O₂/L·s)
Day	p < 0.001	p < 0.001	p < 0.001	p < 0.001	p < 0.001
Treat	p < 0.001	p < 0.001	p < 0.001	p < 0.001	p < 0.001
Treat*day	ns	p < 0.001	p < 0.001	p < 0.001	p < 0.01
Coarse	736 ^a ± 700	-1.70 ^b ± 1.17	2.14 ^b ± 1.61	0.0007 ^a ± 0.0380	-5.05 ^c ± 1.61
Fine - Coarse	3890 ^c ± 2313	-0.36 ^a ± 0.10	0.22 ^a ± 0.29	-0.0181 ^b ± 0.0222	-0.82 ^a ± 0.83
Fine	2500 ^{bc} ± 1482	-0.52 ^a ± 0.23	0.38 ^a ± 0.29	-0.0231 ^b ± 0.0299	-1.53 ^{ab} ± 1.50
Coarse – Fine	1577 ^{ab} ± 1160	-0.75 ^a ± 0.43	0.63 ^a ± 0.73	-0.0142 ^b ± 0.0170	-2.03 ^b ± 2.00
Mixture	2029 ^{ab} ± 1231	-0.49 ^a ± 0.15	0.49 ^a ± 0.19	-0.0124 ^b ± 0.0177	-1.01 ^{ab} ± 1.20

684 Values are the mean of the four sampling days (n = 12) ± sd. Positive process rates indicate production while
685 negative process rates means removal/consumption. Letters next to the means indicate significant differences
686 between treatments (Treat) after Tukey's post-hoc analysis.

687

688

Table 3 Enzyme activities measured at different depths in each treatment

	Depth (cm)	Coarse	Fine - coarse	Fine	Coarse - fine	Mixture
β-glu	0	3.90 ^{ab} ± 0.28	2.30 ^a ± 0.92	2.39 ^a ± 0.13	7.39^b ± 1.51	3.93 ^{ab} ± 0.04
Depth: p < 0.001	20	1.32 ± 0.56	0.32 ± 0.38	0.71 ± 0.75	2.30 ± 1.73	0.84 ± 0.79
Treat: p < 0.001	40	1.08^b ± 0.58	0.15 ^a ± 0.12	0.48 ^a ± 0.35	0.42 ^a ± 0.04	0.38 ^a ± 0.12
Treat*Depth: ns						
β-xyl	0	1.14 ^{ab} ± 0.39	0.61 ^a ± 0.51	1.06 ^{ab} ± 0.37	1.22^b ± 0.45	0.66 ^a ± 0.24
Depth: p < 0.001	20	0.17 ^{ab} ± 0.14	0.01 ^a ± 0.02	0.06 ^a ± 0.11	0.90^b ± 0.74	0.07 ^a ± 0.13
Treat: p < 0.001	40	0.10 ± 0.09	0.00 ± 0.00	0.02 ± 0.03	0.18 ± 0.31	0.00 ± 0.00
Treat*Depth: ns						
Phos	0	6.20 ± 0.29	12.31 ± 3.68	8.69 ± 2.91	9.56 ± 1.01	9.88 ± 2.95
Depth: p < 0.001	20	3.36 ^a ± 0.57	2.24 ^a ± 0.30	3.38 ^a ± 2.43	6.06^b ± 1.63	2.81 ^a ± 0.57
Treat: p < 0.05	40	2.31 ^{ab} ± 0.69	1.14 ^a ± 0.28	2.41 ^{ab} ± 0.76	3.86^b ± 0.89	1.98 ^{ab} ± 0.56
Treat*Depth: ns						
Leu	0	4.10 ± 0.58	5.84 ± 2.94	6.40 ± 3.09	7.43 ± 0.83	2.58 ± 0.66
Depth: p < 0.001	20	4.86^b ± 0.84	1.96 ^a ± 1.12	2.52 ^{ab} ± 1.14	3.98 ^{ab} ± 0.95	3.28 ^{ab} ± 0.78
Treat: p < 0.05	40	4.42^b ± 0.59	1.18 ^a ± 0.41	1.73 ^a ± 1.01	3.37 ^{ab} ± 1.78	1.59 ^a ± 0.98
Treat*Depth: p < 0.1						

689 Values are the mean of the replicates (n=3) ± sd, expressed as nmolMUF/g dry weight·h for β-glucosidase (β-
690 glu), β-xylosidase (β-xyl) and Phosphatase (Phos); and nmolAMC/g dry weight·h for Leucine-aminopeptidase
691 (Leu). Letters next to the means indicate significant differences between treatments (Treat) after Tukey's post-
692 hoc analysis comparing treatments at each depth. Values in bold indicate the highest activity measured at each
693 depth.