

**Modeling long term Enhanced in situ Bionitrification and induced heterogeneity in column experiments under different feeding strategies**

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19 **Abstract**

20 Enhanced *In situ* Biotenitrification (EIB) is a capable technology for nitrate removal in  
21 subsurface water resources. Optimizing the performance of EIB implies devising an  
22 appropriate feeding strategy involving two design parameters: carbon injection frequency  
23 and C:N ratio of the organic substrate nitrate mixture. Here we model data on the spatial  
24 and temporal evolution of nitrate (up to 1.2 mM), organic carbon (ethanol), and biomass  
25 measured during a 342 day-long laboratory column experiment (published in Vidal-Gavilan  
26 et al., 2014). Effective porosity was 3% lower and dispersivity had a seven-fold increase at  
27 the end of the experiment as compared to those at the beginning. These changes in transport  
28 parameters were attributed to the development of a biofilm. A reactive transport model  
29 explored the EIB performance in response to daily and weekly feeding strategies. The latter  
30 resulted in significant temporal variation in nitrate and ethanol concentrations at the outlet  
31 of the column. On the contrary, a daily feeding strategy resulted in quite stable and low  
32 concentrations at the outlet and complete denitrification. At intermediate times (six months  
33 of experiment), it was possible to reduce the carbon load and consequently the C:N ratio  
34 (from 2.5 to 1), partly because biomass decay acted as endogenous carbon to respiration,  
35 keeping the denitrification rates, and partly due to the induced dispersivity caused by the  
36 well-developed biofilm, resulting in enhancement of mixing between the ethanol and nitrate  
37 and the corresponding improvement of denitrification rates. The inclusion of a dual-domain  
38 model improved the fit at the last days of the experiment as well as in the tracer test  
39 performed at day 342, demonstrating a potential transition to anomalous transport that may  
40 be caused by the development of biofilm. This modeling work is a step forward to devising

41 optimal injection conditions and substrate rates to enhance EIB performance by minimizing  
42 the overall supply of electron donor, and thus the cost of the remediation strategy.

43

## 44 **1 Introduction**

45 Nitrate is a priority environmental pollutant in many countries due to the combination of  
46 high toxicity and widespread presence (European Environment Agency, 2007; Organisation  
47 for Economic Co-operation and Development, 2008). Agricultural leaching has been  
48 identified as the primary source of groundwater nitrate contamination (Böhlke, 2002;  
49 Jahangir et al., 2012). Additional sources of nitrate pollution include landfill leachate,  
50 leaking septic tanks, and municipal storm water runoff (Hiscock et al., 1991; Panno et al.,  
51 2008).

52 Different options to reduce the high nitrate concentration levels in groundwater are  
53 available, including improved farming practices, delineation of aquifer protection zones, or  
54 dilution with low-nitrate water sources. However, these options are seldom available due to  
55 legal, logistic, or economical constraints. Thus, groundwater remediation technologies,  
56 such as ion exchange, reverse osmosis, electro dialysis, and Enhanced *in situ*  
57 Bionitrification (EIB) (Haugen et al., 2002), are often the only practical options left to  
58 deal with nitrate-contaminated aquifers.

59 EIB holds environmental and economic advantages over the other remediation  
60 methods mentioned, because it is simple, selective, and cost efficient (Smith et al., 2001).  
61 The technology is based on the reduction of nitrate to dinitrogen gas by anaerobic  
62 heterotrophic facultative bacteria that use nitrate as electron acceptor. Such bacteria are  
63 ubiquitous in soil and groundwater (Beauchamp et al., 1989). EIB is feasible anywhere  
64 bacteria may thrive, organic electron donors can be supplied, and oxygen levels are below  
65 1-2 mg/L (Korom, 1992). In natural aquifer conditions, a major limiting factor for

66 biodenitrification is organic matter. Therefore, the main idea behind EIB is the addition of  
67 an organic carbon source (acting as electron donor for nitrate reduction and as a carbon  
68 source for biomass growth), while controlling a suite of environmental parameters such as  
69 the concentrations of other oxidants (e.g. O<sub>2</sub>), pH, and nutrient levels (e.g. phosphorous or  
70 oligo-elements). Optimal configuration of EIB, involving the presence of one or more  
71 injection and extraction wells, is site specific, depending on pumping rate, groundwater  
72 flow velocity, and residence time of nitrate in the system (Khan and Spalding, 2004).

73         The injection of organic carbon during EIB creates a bioactive zone, characterized  
74 by the growth of denitrifier biomass, heterogeneously distributed throughout the porous  
75 media depending on nutrient availability. Biomass can be found either as suspended matter  
76 or as biofilms attached to the solid matrix. Biofilms occur as micro-colonies or aggregates  
77 composed by denitrifier microorganisms, extracellular polymeric or proteinic substances  
78 (EPS), and potentially trapped dinitrogen gas formed during denitrification (Dupin and  
79 McCarty, 2000; Hand et al., 2008; Rittmann, 1993; Vandevivere and Baveye, 1992).

80         As biofilm develops and the pore space is occupied, partial bioclogging might take  
81 place, affecting a number of hydraulic properties. In addition to bioclogging, a reduction of  
82 hydraulic conductivity can be associated with the presence of trapped N<sub>2</sub> gas (Amos and  
83 Mayer, 2006; Jarsjö and Destouni, 2000). While the word clogging is traditionally defined  
84 in terms of the overall reduction in hydraulic conductivity (Vandevivere and Baveye,  
85 1992), the decrease in effective pore volume caused by biofilm growth also changes  
86 porosity. Due to the variation of these two hydraulic parameters, changes in groundwater  
87 velocity might be recorded (Pavelic et al., 2007; Taylor and Jaffé, 1990; Taylor et al.,  
88 1990), changing residence time between injection and extraction wells, thus influencing the

89 overall capacity for biodenitrification. Furthermore, the spatial heterogeneity of hydraulic  
90 properties caused by the inhomogeneous distribution of biofilm throughout the porous  
91 media also promotes changes in dispersivity (Seifert and Engesgaard, 2007). Dispersivity is  
92 an important parameter as it affects the mixing of nitrate with injected organic substrate,  
93 and it is sometimes the limiting factor for the reaction processes (Dentz et al., 2011).

94 Thus, the amount of biomass and the way it grows significantly affect the  
95 performance of EIB facilities. Biomass growth is driven among other things by the feeding  
96 strategy, i.e., the frequency of injection, the total carbon supplied, and the resulting carbon-  
97 nitrogen ratio (C:N). With the objective of limiting the biomass growth, some authors  
98 suggested injecting the electron donor in discrete pulses rather than as a continuous supply  
99 (Franzen et al., 1997; Gierczak et al., 2007; Peyton, 1996; Semprini et al., 1991; Semprini  
100 et al., 1990; Shouche et al.). Nevertheless, little is known about how the frequency of  
101 injection pulses affects biomass growth and nitrate degradation. Regarding the C:N ratio,  
102 Vidal-Gavilan et al. (2014) observed that even working with low C:N ratios (C:N=1; below  
103 the stoichiometric one: C:N = 2.5), high denitrification rates were achieved after biofilm  
104 development. The authors attributed this to the occurrence of endogenous bacterial decay.

105 Proper understanding of processes occurring during EIB involves the need for  
106 multispecies reactive transport modeling (RTM) (Chen and MacQuarrie, 2004; Lee et al.,  
107 2006; Rodríguez-Escales et al., 2016). Such models can facilitate exploring a variety of  
108 remediation strategies such as injection duration and rate, and concentration of reactants.  
109 Nevertheless, there is a need to develop specific models to evaluate how different feeding  
110 strategies interact with transport processes.

111 The present work is aimed at developing a model capable of reproducing different  
112 feeding injection frequencies (from weekly to daily) with different C:N ratios in a long term  
113 column experiment of Enhanced *in situ* Biotenitrification, lasting 342 days (Vidal-Gavilan  
114 et al., 2014). This modeling study focusses on the EIB performance in response to the  
115 frequency of organic substrate addition as well as the changes in hydraulic and transport  
116 properties promoted by the growth of biofilm. Proper understanding of the processes taking  
117 place allow defining the optimal injection strategy (frequency and rate) capable of  
118 enhancing EIB performance (high performance at low cost) by minimizing the overall  
119 supply of labile organic carbon substrate.

## 120 **2 Materials and Methods**

### 121 **2.1 Description of the experiment and data set**

122 A full description of the experiment is provided in Vidal-Gavilan et al. (2014), and  
123 sketched here in Figure 1 for completeness. It consisted of a glass cylindrical column (70  
124 cm length, 8 cm inner diameter) filled with unconsolidated sediment from a sandy alluvial  
125 aquifer (located in Argenton, NE Spain). The sediment was composed by medium and  
126 coarse-grained sand mainly made up of quartz and feldspar and with a small silt content,  
127 the organic matter content in the sediment was negligible (Vidal-Gavilan et al., 2014).  
128 Water was forced to flow from the bottom to the top of the column with a pump-controlled  
129 average flow-rate of 180 mL/d resulting in a residence time in the column of about 6.4  
130 days. A total of eight sampling ports were installed: one at the inflow reservoir, six along  
131 the column (at 6, 16, 26, 36, 46 and 56 cm from inlet), and one at the outflow, allowing the  
132 delineation of aqueous compounds and suspended biomass profiles at different predefined



133 times. The data set provided in Vidal-Gavilan et al. (2014) and used in the modeling effort  
134 includes aqueous concentrations of ethanol, nitrate, and biomass at selected times at the  
135 sampling ports placed within the column. A control experiment without carbon substrate  
136 addition ran for 2 months, and natural denitrification was not observed, as changes in  
137 nitrate along the column were lower than 1% (Vidal-Gavilan et al., 2014).

138         The water used in the experiment was obtained from an existing large-diameter well  
139 located at the site. Three 25-L containers were used to store the input water for the  
140 experiment, filled up at different days (August 2011, December 2011, and April 2012). The  
141 well was always purged prior to sampling. No forced deoxygenation took place, so that the  
142 input water (see Table 1) was oxic and saturated with oxygen. The experiment ran for 342  
143 days at aquifer temperature (15°C). Ethanol was added as an external organic carbon source  
144 by means of four injectors located 16 cm from the inlet (see Figure 1). It was added by  
145 mixing it with the input water previous to injection (Table 1). Different feeding strategies  
146 were tested during the experiments (Table 2), characterized by different injection  
147 frequencies (weekly *versus* daily) and carbon to nitrogen molar ratios (from 2.5 to 1). In  
148 this ratio the amount of C is computed from the concentration of ethanol multiplied with  
149 the duration of injection (0.5 min). Feeding was twice discontinued, first between days 150  
150 and 175 due to pump failure (no water was supplied), and then between days 286 and 311,  
151 this time to evaluate the resilience of the system to the absence of feeding (water with no  
152 ethanol was supplied during that second period).

153         Two tracer tests were performed, one previous to the start of the experiment, before  
154 any feeding took place, and a second one at day 342. The tests were conducted under  
155 continuous flow with constant concentration of bromide (1.45 and 2.23 mM, respectively).

156 During the two tracer tests the flow rate was 835 mL/d. The bromide breakthrough curves  
157 were monitored at the outflow point.

## 158 **2.2 Model construction**

159 Here we describe first the biogeochemical equations used in the biodenitrification model;  
160 second, the hydrogeological parameters derived from the two tracer tests; third, the codes  
161 used in the modeling effort; and fourth, the calibration process.

### 162 **2.2.1 EIB biogeochemical model**

163 Biodenitrification was modelled considering both nitrate respiration and biomass growth  
164 (see e.g., Rodríguez-Escales et al., 2014). The reactions considered are:

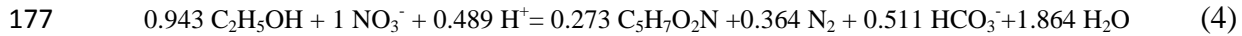
$$165 \quad r_{ED} = -k_{max} \frac{[ED]}{[ED] + K_{S,ED}} \frac{[EA]}{[EA] + K_{S,EA}} [X] \quad (1)$$

$$166 \quad r_{EA} = Qr_{ED} - Sb[X] \quad (2)$$

$$167 \quad r_X = -Y_h r_{ED} - b[X] \quad (3)$$

168 where [ED] is the concentration of the electron donor (ethanol, C<sub>2</sub>H<sub>5</sub>OH); [EA] that of the  
169 electron acceptor (nitrate), and [X] the denitrifier biomass concentration, all expressed in  
170 [ML<sup>-3</sup>]; k<sub>max</sub> [T<sup>-1</sup>] is the consumption rate of electron donor per unit value of biomass; K<sub>S,ED</sub>  
171 [ML<sup>-3</sup>] and K<sub>S,EA</sub> [ML<sup>-3</sup>] the half saturation constants of electron donor and acceptor,  
172 respectively; b [T<sup>-1</sup>] a biomass decay constant; Y<sub>h</sub> the microbial yield [C biomass / C  
173 ethanol], and Q [N nitrate / C ethanol] and S [N nitrate / C endogenous]. Both K<sub>max</sub>  
174 (μ<sub>max</sub>/Y<sub>h</sub>) and K<sub>s</sub> were fitting parameters, whereas S and Q were stoichiometric factors

175 determined by the driving denitrification reaction (4). Biomass was conceptualized as  
176 having an average chemical composition of C<sub>5</sub>H<sub>7</sub>O<sub>2</sub>N (Porges et al., 1956).



178 Equation (4) was determined following the instructions of Rittmann and McCarty (2001)  
179 and it applies to the following determined parameter values: (i) the portion of substrate  
180 (ethanol) used for cell synthesis during denitrification (Y<sub>h</sub>) was 0.724 C-biomass/C-ethanol  
181 (in agreement with Rodríguez-Escales et al. 2014); and (ii) the portion of nitrate consumed  
182 by substrate oxidation (Q) was 0.53 mol nitrate-mol C-ethanol. The stoichiometric  
183 relationship between nitrate and endogenous carbon (S) was 0.92 mol nitrate-mol C  
184 endogenous, following (Rodríguez-Escales et al., 2014).

185 Although the injected solution was partly to almost fully oxic (oxygen concentrations  
186 measured varied between 0.06 and 0.2 mM), ethanol oxidation by oxygen was and could be  
187 neglected. This assumption was based on ethanol consumption by oxygen being between  
188 0.1 and 4% of ethanol injected (depending on initial concentrations). Moreover, preliminary  
189 models considering instantaneous reduction of oxygen showed that oxygen was consumed  
190 within the first 5 cm of the column (results not shown). Considering all of this and in order  
191 to simplify the model, ethanol oxidation by oxygen was not contemplated.

192 Nitrite accumulation was not relevant in the experiment (only present during the first  
193 20 d, in concentrations below 0.1 mM; whereas nitrate decreased then with 1.2-1.6 mM.).  
194 Therefore, the model contemplates only one step reduction from nitrate to dinitrogen gas.  
195 The potential accumulation of NO and N<sub>2</sub>O was discarded because the system was  
196 maintained at low oxygen concentrations, with enough labile organic carbon, and with pH

197 values between 7 and 8; under these conditions complete denitrification is expected (Rivett  
198 et al., 2008; Tallec et al., 2008).

199 Most often, the amount of bacteria suspended in the aqueous phase is quite small as  
200 compared to that attached to the aquifer matrix (Barry et al., 2002; Rittmann, 1993). As a  
201 way to implement a practical model, minimizing the number of fitting parameters, we  
202 assumed that all biomass was attached to the solid matrix, and thus immobile, without  
203 considering attachment and detachment processes, described for example in Clement et al.  
204 (1997). The initial biomass concentration was estimated in  $6.5 \times 10^{-8}$  mmol/kg, considering  
205 a most probable number for denitrifying cells equal to 37.5 cel/ml (Vidal-Gavilan et al.  
206 2014) and converted to moles using a denitrifier cell weight of  $10^{-9}$  mg (Alvarez et al.,  
207 1994). The initial value used in PHT3D was normalized by liter of water.

208 Finally, the column was considered as an open system in equilibrium with the  
209 atmosphere because it was open at its upper part. Thus, degassing was allowed if the sum of  
210 partial pressures of gases (mainly dinitrogen gas and carbon dioxide) exceeded the  
211 atmospheric pressure. Prior to the simulation process, and in order to evaluate the potential  
212 hydraulic conductivity variations due to bubble formation, we evaluated the potential  
213 building up of denitrification gases. Thus, we ran the model under closed system  
214 conditions. The results showed that the hydrostatic pressure was exceeded in most feeding  
215 strategies illustrating that degassing could occur. To limit the chance for gas entrapment,  
216 which would be the main responsible of changes in hydraulic conductivity (Amos and  
217 Mayer, 2006), we purposely ran the column experiment in vertical mode with water  
218 flowing upwards. In this way, gas entrapment should have been limited as any gas formed  
219 could escape at the top outlet of the column and the flow of gas bubbles and water in the

220 column were aligned. Furthermore, we expect that the coarse sand (grain size between 1  
221 and 2 mm) used in the column further limited any gas entrapment.

## 222 **2.2.2 Transport model parameters evaluated from the tracer tests**

223 Two tracer tests with a conservative tracer (Br<sup>-</sup>) were performed at days 0 and 342 in order  
224 to build a conceptual model for conservative transport and to estimate the corresponding  
225 hydraulic parameters. Invoking the parsimony principle, we first tried to fit the  
226 breakthrough curves with the simplest model, that of the one-dimensional advection-  
227 dispersion equation (ADE).

228 The ADE model could properly reproduce the test performed at time 0, but failed to  
229 fit the tail of the experimental BTC obtained during the second test at day 342. As an  
230 alternative model we selected a dual porosity model (Delay et al., 2013; Haggerty and  
231 Gorelick, 1995; Lawrence et al., 2002; Seifert and Engesgaard, 2007), representing the  
232 porous medium as composed of a mobile and of an immobile region that coexist at any  
233 given point in the domain. The first one was an aqueous phase where advection and  
234 dispersion were the main transport processes, whereas the second one was a (diffusion zone  
235 governed by biofilm dynamics). Both regions exchange mass proportionally to the  
236 difference in their concentrations at any given time. The equation describing the  
237 concentration of species *i* in the mobile zone,  $c_{m,i}$ , is:

$$238 \quad \phi_m \frac{\partial C_{m,i}}{\partial t} = -q \frac{\partial C_{m,i}}{\partial x} + \phi_m \frac{D \partial^2 C_{m,i}}{\partial x^2} - \Gamma_i \quad (5)$$

239 where *D* is the dispersion coefficient, *q* is Darcy's velocity,  $\phi_m$  the porosity corresponding  
240 to the mobile zone (aqueous phase with aqueous solution), and  $\Gamma_i$  the source-sink term

241 controlling the mass transfer of species  $i$  between the mobile (m) and the immobile regions  
242 (im) (biofilm phase with microorganisms attached to the sediment), given by:

$$243 \quad \Gamma_i = \alpha \phi_{im} (C_{m,i} - C_{im,i}) \quad (6)$$

244 with  $\alpha$  the mass transfer rate [ $T^{-1}$ ],  $\phi_{im}$  [-] the porosity associated with the immobile region  
245 (volume fraction occupied by the biofilm), and  $C_{im,i}$  the concentration of species  $i$  in the  
246 immobile region. The actual total porosity is  $\phi_t = \phi_m + \phi_{im}$ , and remains constant during  
247 biofilm formation. The rationale behind it is that the biofilm colonizes pores that were  
248 initially occupied by water in sediments not affected by consolidation or swelling, so that  
249 the sediment occupied the same volume at the beginning and end of the experiment. A key  
250 parameter characterizing the shape of the BTC in the dual porosity model is the ratio of  
251 porosities (Fernàndez-Garcia and Sanchez-Vila, 2015) given by:

$$252 \quad \beta = \frac{\phi_{im}}{\phi_m} = \frac{\phi_t}{\phi_m} - 1 \quad (7)$$

### 253 **2.2.3 Used codes and calibration process**

254 The PHT3D model code (v. 2.17) (Prommer and Post, 2010) was used to simulate the  
255 evolution of groundwater hydrochemistry during enhanced biodegradation in the column.  
256 This model couples the transport simulator MT3DMS (Zheng and Wang, 1999) and the  
257 geochemical model PHREEQC-2 (Parkhurst and Appelo, 1999), by means of a sequential  
258 split-operator technique. Regarding solute transport, PHT3D incorporates either the  
259 traditional ADE, or else the dual domain model through MT3DMS. Since the PHT3D  
260 reaction module uses the original PHREEQC-2 database syntax, equilibrium and non-  
261 equilibrium reaction chains can be defined. For reactions in equilibrium, the constants were

262 taken directly from the database. Kinetic reactions such as ethanol degradation and  
263 bacterial growth/decay (1-3), not being part of the standard database, were incorporated  
264 into the module in the form of BASIC routines, as explained in Rodríguez-Escales et al.  
265 (2014) and Carrey et al. (2014).

266       Regarding the tracer tests, the interpretation using the traditional ADE and the dual  
267 domain model was carried out with the CXTFIT code (Toride et al., 1999). We developed  
268 the inverse modelling of transport processes using the experimental information of the  
269 BTCs from the tracer tests and we determined the following parameters: dispersivity  
270 coefficient, total, mobile and immobile porosities, and, dual domain transfer coefficient.  
271 Furthermore, CXTFIT provides the confidence interval (95%) of each parameter as well as  
272 their corresponding standard deviations. In order to avoid the correlation between immobile  
273 porosity and dispersivity coefficient in the transport equation (e.g. Wehrer et al. 2012), the  
274 calibration process was divided in two steps. First of all, we calibrated the dispersivity  
275 coefficient and the mobile porosity without considering the tail. Then, we incorporated the  
276 dual domain model to improve the fittings of the tail, allowing an independent estimation of  
277 the immobile porosity. Following this methodology, we only related the dispersivity to the  
278 change into the geometry and not also to the diffusion processes avoiding its correlation  
279 with immobile porosity.

280       To assist the biodegradation model calibration process, the model independent  
281 parameter estimation program PEST (Doherty, 2005) was coupled to PHT3D and used to  
282 estimate the reaction rate parameters ( $k_{max}$ ,  $K_{S,ED}$ ,  $K_{S,EA}$ , and  $b$ ). PEST computed the  
283 sensitivities, correlations, and linear uncertainties (confidence intervals) of the optimized  
284 model parameters. For the calibration process, the error associated with the measurement

285 was treated as 95% confidence interval, and weights were applied using the inverse of the  
286 standard deviation of this confidence interval (Karlsen et al., 2012). Using this method,  
287 values with a higher accuracy get assigned a higher weight and the resulting objective  
288 function became dimensionless. Standard ranges for measurement error of chemical  
289 sampling were given with an accuracy of 5%. Weights ( $w$ ) for each chemical species  
290 observation  $i$  were thus calculated:

$$291 \quad w_i = \frac{1.96}{\varepsilon_i C_i} \quad (8)$$

292 where  $\varepsilon$  is the measurement error described above and  $C$  is the observed concentration. For  
293 the calibration process, we used the experimental data of nitrate during the first 100 days of  
294 the experiment (35 points). The calibration process of the reactive transport was performed  
295 by fixing the conservative transport parameters. Finally, we also evaluated the likelihood of  
296 the models comparing the Akaike information criterion values (AIC) calculated by PEST.

### 297 **3 Results and discussion**

#### 298 **3.1 Tracer tests interpretation: derivation of transport processes and parameters**

299 The first step is the interpretation of the 1-D conservative tracer tests. The traditional ADE  
300 equation was capable of properly fitting the curve corresponding to the first test, but it  
301 failed to provide a good fit of the tail of the BTC corresponding to the second test, with a  
302 maximum error in estimated concentrations of 3%. On the other hand, the dual domain  
303 model was capable to reproduce the tail of the BTC corresponding to the second test  
304 indicating a transition from a Fickian description of transport at the start to an anomalous  
305 description of transport at the end of the EIB experiment. The reported BTCs are presented



306 in Figure 2, together with the best fits obtained either with code CTXFIT at day 0 (single  
307 porosity) and at day 342 (dual porosity); the fitted parameters are listed in Table 3.  
308 Groundwater velocity was very similar in the two tests (see Table 3). The hydraulic  
309 gradient could not be measured in the applied experimental setup. Therefore, any reduction  
310 in hydraulic conductivity due to biofilm growth could not be assessed. Total (single-phase)  
311 porosity and dispersivity were estimated from the first test; total porosity, the proportion of  
312 immobile and mobile porosity, dispersivity, and the mass transfer rate were estimated from  
313 the second one. Total porosity values estimated from both tests were statistically not  
314 different, with best estimates of  $0.33\pm 0.03$  to  $0.34\pm 0.05$ , and estimation intervals largely  
315 overlapping (Table 3). However, the dual porosity model estimated an immobile porosity of  
316  $0.015\pm 0.009$  at day 342.

317         There was a remarkable seven-fold increase in the dispersivity coefficient estimated  
318 from the two tests, with the mean value changing from  $0.48\pm 0.01$  to  $3.44\pm 0.25$  cm (see  
319 Table 3). This result is consistent with the observations by Taylor and Jaffé (1990) who  
320 also described an increase in immobile porosity linked to an increase in dispersivity in a  
321 column experiment colonized by biomass. Several studies also report significant changes in  
322 dispersivity, ranging from two- to eight-fold increases, in bioremediation experiments  
323 lasting 2-7 weeks (Arnon et al., 2005; Bielefeldt et al., 2002; Hill and Sleep, 2002; Seifert  
324 and Engesgaard, 2007; Sharp et al., 1999; Taylor and Jaffé, 1990; Taylor et al., 1990), and  
325 as high as a 10-100 fold variation for long duration experiments (Taylor and Jaffé, 1990;  
326 Bielefeldt et al., 2002). This increase in dispersivity is generally associated to denitrifier  
327 biomass colonizing the sand grains forming the soil skeleton. Thus, while total porosity  
328 remained basically constant, a small fraction was colonized by biomass aggregates and

329 micro-colonies, changing its behavior from water accessible by flow (mobile) to  
330 inaccessible (immobile). Such aggregates have been reported to induce irregular surfaces of  
331 the solid particles (Rittmann, 1993), and consequently, to increase the heterogeneity in the  
332 pore size distribution (Seifert and Engesgaard, 2007), thus enhancing dispersivity.

333         The change in the conceptual model of transport was associated with the growth of  
334 biofilm during the duration of the experiment. Thus, the fitted parameters of the dual  
335 domain model have a clear physical explanation; for example, the calibrated  $\alpha$  parameter ( $\alpha$   
336 =  $0.019 \pm 0.018 \text{ d}^{-1}$ ) can be interpreted as the inverse of the characteristic diffusive time of  
337 bromide transport through the immobile phase (thus being equal to 45 days). Moreover, the  
338  $\beta$  value ( $\beta = 0.046 \pm 0.030$ ) represented the proportion of the void volume occupied by the  
339 biofilm ( $4.65 \pm 2.96 \%$ ).

340         Regarding the calibration process of the transport parameters, the automatic  
341 calibration showed that during the two steps of calibration the parameters were not  
342 correlated because the correlation coefficients were lower than 0.95 (Hill et al., 1998).  
343 During the first step (calibration using ADE of velocity and dispersion), the correlation  
344 among parameters was lower than 0.025 for the two tracer tests. During the second step, the  
345 correlation between immobile porosity and the mass transfer coefficient was 0.21. The  
346 coefficients of variation (CV) of the parameters of ADE were well estimated, as their  
347 values were generally low (less than 0.15). Regarding the parameters of the dual domain  
348 model, they were estimated as highly uncertain.

### 349 **3.2 Long-term modeling of EIB. Impact of organic carbon injection strategies**

350 Based on tracer tests results the column experiment was first interpreted using a Fickian  
351 representation of transport, i.e., based on the ADE. Emphasis was placed on the  
352 performance of the daily and weekly feeding strategies upon the observed temporal  
353 evolution of the concentrations of nitrate, ethanol, and biomass. Since Table 3 displays two  
354 dispersivity values corresponding to days 0 and 342, but no intermediate values were  
355 obtained, the 342-day column experiment was modeled using both dispersivity values, by  
356 assuming that they lasted the full duration of the experiment, thus providing two limiting  
357 cases. The column was discretized into 70 elements of 1 cm length. The time discretization  
358 was selected to satisfy Peclet and Courant criteria. Dispersive transport was computed by  
359 the third-order Total Variation Diminishing solution, a feature available in PHT3D.

360 The actual data and the fittings with the two dispersivity values are shown in Figure  
361 3. Neither porosity (obtained from the tracer test, 0.33), nor the geochemical parameters of  
362 reactions in equilibria (selected from the PHREEQC2 database) were calibrated. The only  
363 calibrated parameters were the microbiological ones (Table 4) and, all were in range  
364 compared to values reported in the literature. Note that we compared the  $\mu_{\max}$  parameter  
365 instead the  $k_{\max}$  with literature values, because it only depends on velocity reaction and it is  
366 easier to compare. The automatic calibration procedure used for the estimation of kinetic  
367 parameters in the denitrification model showed that the evaluated parameters were not  
368 cross-correlated, as indicated by their values in the coefficient correlation matrix being  
369 below 0.747 (data not shown). That is, given the available observations for model  
370 calibration, each model parameter affected the simulated equivalents to the observations  
371 sufficiently differently. The values of the coefficients of variation, CVs, were relatively

372 high, ranging from 0.26 to 0.61. As pointed out by Greskowiak et al. (2005), large CVs do  
373 not necessarily imply an incorrect model concept. Instead, it may indicate that the available  
374 observation data are insufficient to uniquely constrain (estimate) the parameter, or that  
375 there is an underlying physical basis for relatively high CVs.

376         The lowest dispersivity value (0.48 cm) resulted in a good fitting of the experimental  
377 data during the weekly feeding strategy (Figure 3), lasting the first 98 days, indicating that  
378 during this period dispersivity did not change significantly. This result is in contrast with  
379 other works based on column experiments using somewhat different experimental  
380 conditions like organic substrate but were all fed continuously (Bielefeldt et al., 2002;  
381 Seifert and Engesgaard, 2007; Taylor and Jaffé, 1990) (Table 5). For example, Seifert and  
382 Engesgaard (2007), using acetate and oxygen as electron acceptor, reported an increase in  
383 dispersivity from 0.33 cm to 1.1 cm in 64 days. On the other hand, Bielefeldt et al. (2002),  
384 in an experiment on propylene glycol degradation using nitrate as electron acceptor,  
385 observed a 20-60 fold increase in 15 days in clean sand. Finally, Delay et al. (2013)  
386 reported a noticeable change in dispersivity in a 1.4 day column denitrification experiment.  
387 In short, from the data in Table 5, it seems that a weekly feeding strategy limits dispersivity  
388 increases with time.

389         We note that Figure 4 reports the modeling results assuming a constant representative  
390 dispersivity value all throughout the column. We expect though that most of the biomass  
391 colonization took place around the injection point (Kildsgaard and Engesgaard, 2001),  
392 associated with the highest EA and ED concentrations and, consequently, the modification  
393 of the transport parameters too. Although the general trends were well captured, the

394 limitation of considering only one set of transport parameters could explain the  
395 discrepancies between the experimental data and the simulated results.

396         During the daily feeding strategy, starting after day 99, the best overall fit of nitrate  
397 concentration was obtained with the final dispersivity value of 3.43 cm. This is visible both  
398 for time-series (Figure 3) and for spatial profiles (Figure 4). Consequently, the increase in  
399 dispersivity seems triggered by the changes in feeding strategy, from weekly to daily  
400 pulses. During weekly injection, biomass was not fed homogenously, and probably biomass  
401 growth was through colonies or aggregates that did not colonizing the whole sandy media.  
402 On the other hand, daily injection drove a more continuous growth of biomass (probably in  
403 biofilm form) and favoring the colonization of the whole column (Rittmann, 1993). We  
404 thus contend that induced heterogeneity was larger in the daily scenario as compared to the  
405 weekly one, and consequently, a seven-fold increase of dispersivity in the former feeding  
406 strategy was observed. This increase was smaller than others reported in the literature for  
407 continuous feeding (see Table 5 for values and references). This can be explained because  
408 the injection was performed in the form of a daily pulse, rather than fully continuous.  
409 Besides this change in feeding strategy, the two stop periods in daily feeding strategies  
410 could also facilitate the increasing of heterogeneity due to the detachment of biomass and  
411 its redistribution through the column (Wehrer et al., 2012). This suggests that both the  
412 feeding frequency and the stop periods are key operational parameters that may affect  
413 hydraulic parameters and thereby control the transport of chemical species during EIB.

414         We want to emphasize that the increase of dispersivity was evaluated in a column  
415 experiment (1D), thus only considering longitudinal dispersivity. Although it is still  
416 unknown how biofilm growth will disturb the dispersivity in 3-D (e.g. field applications),

417 we would expect an increase in the three directions of dispersivity, longitudinal, and  
418 transversal both horizontally and vertically. The last two of those having a most significant  
419 impact upon the enhancement of spreading and mixing of nutrients (Chiogna et al., 2012;  
420 Rolle et al., 2009).

421 The biomass concentration decreased corresponding to the low C:N ratios (Figure 3).  
422 Note that the biomass concentration did not differ between feeding strategies I and II,  
423 indicating that the injection frequency played a lower role than the C:N ratio. Nevertheless,  
424 we hypothesize that the biomass growth was different for each strategy. Whereas during  
425 weekly feeding strategy, the biomass distribution should not be continuous, in the daily one  
426 we should expect that a connected biofilm was formed. This idea follows the observations  
427 of Rittmann (1993), who determined that a continuous feeding causes a biofilm whereas a  
428 discontinued one resulted in disconnected biomass aggregates. Although the  
429 characterization of the attached biomass could be done at the end of the experiment (e.g.  
430 Clement et al. (1997)), we recommend for future research the characterization of the  
431 biofilm structure through SEM (Scanning Electron Microscope) images.

### 432 **3.3 The implication of introducing non-Fickianity in the conceptual model**

433 The incorporation of a dual domain transport model resulted in a slight improvement of the  
434 model fit from day 183 onwards (Figure 3 and 4, blue dashed-dotted line). The parameters  
435 used in the model are reported in Table 3 for transport processes (last row) and Table 4 for  
436 the biogeochemical ones. Note that the mass transfer coefficient had a high standard  
437 deviation (0.019) in relation to the parameter value (0.018). Considering that, we run the

438 model with different mass transfer values. The results showed that the model was not very  
439 sensitive to this change (results not shown).

440 As the fit obtained during the weekly feeding strategy by ADE was quite good, much  
441 better than the obtained with the non-Fickian model (Figure 3), we contend that during this  
442 period the diffusive transport through the biofilm was negligible. Thus, the  
443 conceptualization of the porous medium as a dual domain was not considered until the daily  
444 feeding strategy started, that supposed to enhance the biofilm developing (conceptualized  
445 as immobile porosity). This improvement in fitting is attributed to modeling the partial  
446 transformation of initial pores to non-flowing volume (immobile porosity or diffusive  
447 layer) that act as electron donor sink. Yet, it is still unknown at which point of the  
448 experiment this process was relevant. This could only be assessed by the incorporation of  
449 non-invasive techniques to monitor biofilm evolution in future studies. We emphasize that  
450 water velocity conditioned the significance of involving a dual domain into the conceptual  
451 transport model. Thus, the impact of a dual domain model in the tracer test interpretation  
452 (Figure 2) was more significant than that on the biodenitrification experiment (Figure 3-4)  
453 because the water velocity was higher (0.5 m/d instead of 0.1 m/d) and thus the time  
454 available for mass transfer between the domain was less. Although this difference in  
455 velocity, we want to remark that the dual domain model was more likely than the ADE  
456 model because its AIC value was the lowest one (203.22 compared to 212.55).

#### 457 **3.4 Significance of the C:N ratio and implications for EIB design**

458 In the scenarios with the lowest C:N ratios (strategies III, days 206-252 and IV, days 253-  
459 342), the model correctly reproduces the experimental data of nitrate and ethanol being

460 completely consumed inside the column (not detected at the outlet). This means that the  
461 source of organic carbon was used optimally, fully consumed, as opposed to that observed  
462 in strategy I. Note that the increase in dispersivity resulted in enhanced spreading and then  
463 mixing of the injected ethanol with nitrate, enabling a more efficient substrate use.

464 Another parameter that helped defining the success of the different injection  
465 strategies is the stress produced upon the biomass population. When the carbon load was  
466 reduced (strategies III and IV), the modeled biomass diminished (see Figure 3). However,  
467 nitrate remained undetected, indicating that denitrification was partially linked to biomass  
468 decay (endogenous respiration) meaning that there was not enough external carbon to  
469 maintain the large biomass population (see Figure 5). The use of endogenic carbon as  
470 electron donor in bioremediation facilities has already been reported in other works  
471 (Béranger et al., 2006; Rodríguez-Escales et al., 2016). The decrease in biomass  
472 concentration indicated that the low C:N strategies were not sustainable in time.  
473 Nevertheless, working with low C:N could be a good tool to reduce the risk of clogging.

474 Besides this, the amount of ethanol used in these strategies was lower than in  
475 strategies I and II (Figure 6), which would imply important savings (the main cost in an  
476 EIB operation is electron donor injection). A proper design of the amount of carbon  
477 supplied could represent significant savings in an EIB technology. For these reasons, we  
478 recommend applying low C:N strategies when the system has reached maturity (complete  
479 denitrification achieved, mature biofilm, no nitrite accumulation) and/or when an important  
480 risk of clogging exists (monitored with continuous or semi continuous measurement of  
481 hydraulic conductivity and mobile porosity).



#### 482 **4 Summary and conclusions**

483 An Enhanced *In situ* Biotenitrification experiment, performed in a 70 cm long column  
484 under virtually constant flow rate and different feeding strategies was modeled. Injection  
485 strategies were defined in terms of periodicity of injection of organic carbon (ethanol), and  
486 thus resulting C:N ratio. A long term reactive transport (342 d) model based on the  
487 Advection Dispersion Equation (ADE) fitted properly most of the experimental data.

488 Throughout the experiment, estimated dispersivity varied from the beginning to the  
489 end of the experiment. During the weekly supply strategy I (first 98 days), the best fit was  
490 obtained using a low dispersivity value (0.48 cm), whereas during the daily strategy, it was  
491 best fitted with a larger dispersivity value (3.43 cm). We attributed this increase to the  
492 change in injection periodicity, from weekly to daily, after day 98, resulting in biofilm  
493 growth. Furthermore, after day 252, with a very mature system, data fitted better using a  
494 dual-domain model (i.e., non-Fickian) as compared to one based on the ADE. This change  
495 was associated with the presence of a diffusive layer (biofilm) increasing its relevance with  
496 time. Although the dynamic conditions of the system, the presented model has been capable  
497 of reproducing satisfactorily the experimental observations in all feeding strategies.

498 On the other hand, reducing the C:N ratio below the stoichiometric requirements  
499 allowed the optimization of ethanol injection into the system avoiding its presence at the  
500 column outlet. At this point, biomass decay increased and the endogenous carbon acted as  
501 partial source of electron donor during the denitrification process. Nevertheless, the  
502 decrease of modelled biomass concentration in time showed that this strategy is not

503 sustainable at long term and that it only can be used when a mature biofilm exists in the  
504 subsurface.

505 Our work has shown that besides other parameters (nutrient loading, flow rate, or grain  
506 size), injection frequency is a significant operational parameter that can affect a number of  
507 hydraulic parameters, notably dispersivity. This finding could be extended to promote field  
508 Enhanced *In Situ* Bionitrification (EIB) applications. A larger dispersivity value offers  
509 the possibility of enhancing spreading of injected solutes, increasing the area treated per  
510 injection point and limiting the organic carbon loss in this particular *in situ* technique. Thus,  
511 this will promote the growth of biofilm and, when a mature system is eventually reached,  
512 reducing the C:N ratio can minimize the risk of clogging. So, in order to improve efficiency  
513 and saving costs in real field scale applications, feeding strategy in terms of frequency and  
514 C:N relationship should be evaluated before the design and construction of EIB  
515 installations, as well as during its operation.

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707

708 **Figure captions**

709 **Figure 1.** Experimental flow-through system and location of the sampling ports.

710 **Figure 2.** Model fits using the ADE (black lines) and a dual domain model (red dashed  
711 lines). Square symbols ( $\square$ ) correspond to measurements corresponding to the tracer test  
712 performed at day 0 (red and black lines run in top of each other), whereas circles ( $\circ$ )  
713 correspond to the BTC from the test at day 342. The error bars are related to the bromide  
714 analyses.

715 **Figure 3.** Results of the EIB models considering different injection strategies at the outflow  
716 of the column (70 cm). The black and the red solid lines were obtained with dispersivity  
717 values of 0.48 and 3.43 cm, respectively, considering an ADE equation. The dashed-dotted  
718 blue line corresponds to a model using a dispersivity value of 3.43 cm and a dual model  
719 mass transfer. The dashed black line corresponds to ethanol concentration in the injection  
720 solution. Grey areas represents the two periods without feeding. The bottom plot shows the  
721 simulated biomass concentrations (represented in mM) at the last cell of the model domain  
722 (70 cm).

723 **Figure 4.** Nitrate distance profiles (simulated vs. measured) at 12 different times. In each  
724 plot, the first number represents the sampling day, and that in brackets reflects the elapsed  
725 time since the last injection period. The black and the red dashed lines were obtained with  
726 dispersivity values of 0.48 and 3.43 cm, respectively, considering an ADE equation. The  
727 blue dashed dotted line in the last two plots corresponds to a model using a dispersivity  
728 value of 3.43 cm and a dual model mass transfer. The grey zone corresponds to the  
729 injection point.

730 **Figure 5.** Detail of feeding strategies with low C:N ratio without considering decay of  
731 biomass (top line) and biomass decay (bottom line). Filling zone indicates the importance  
732 of biomass decay on nitrate consumption rates under low C:N.

733 **Figure 6.** Comparison of the amount of ethanol used in injections and percentage of  
734 denitrification achieved by the different feeding strategies. The percentage of denitrification  
735 was calculated as the difference in nitrate mass between the inlet and the outlet of the  
736 column divided by the nitrate mass at the inlet.

### 737 **Table captions**

738 **Table 1.** Average concentration of different species in the input water. Nitrate  
739 concentration (\*) varied during the experiment due to seasonal nitrate oscillations within  
740 the aquifer.

741 **Table 2.** Summary of the different feeding strategies (I to IV) tested during the experiment,  
742 in terms of feeding frequency, ethanol concentration supplied, ratio of C (external organic  
743 carbon source concentration) to N (nitrate concentration), and duration.

744 **Table 3.** Hydraulic parameters estimated for the two bromide tracer tests. The standard  
745 deviation was calculated by using the inverse problem with CTXFIT. The interpretation  
746 models were different for the two tests: the initial one (day 0) with an ADE model; the  
747 second one (day 342) with the dual domain model, thus involving two additional  
748 parameters. The  $R^2$  of two fitted curves were 0.999 and 0.998, respectively.

749 **Table 4.** Biogeochemical constants used in the denitrification model, compared with values  
750 compiled from the literature. Both the median and the standard deviation were determined  
751 by automatic calibration using PEST.

752 **Table 5.** Comparison between the values obtained in this work and in similar experiments  
753 compiled from the literature, including feeding strategies, organic carbon inflow, flow rate  
754 and estimated increase in the dispersivity after some period of time.

Figure1  
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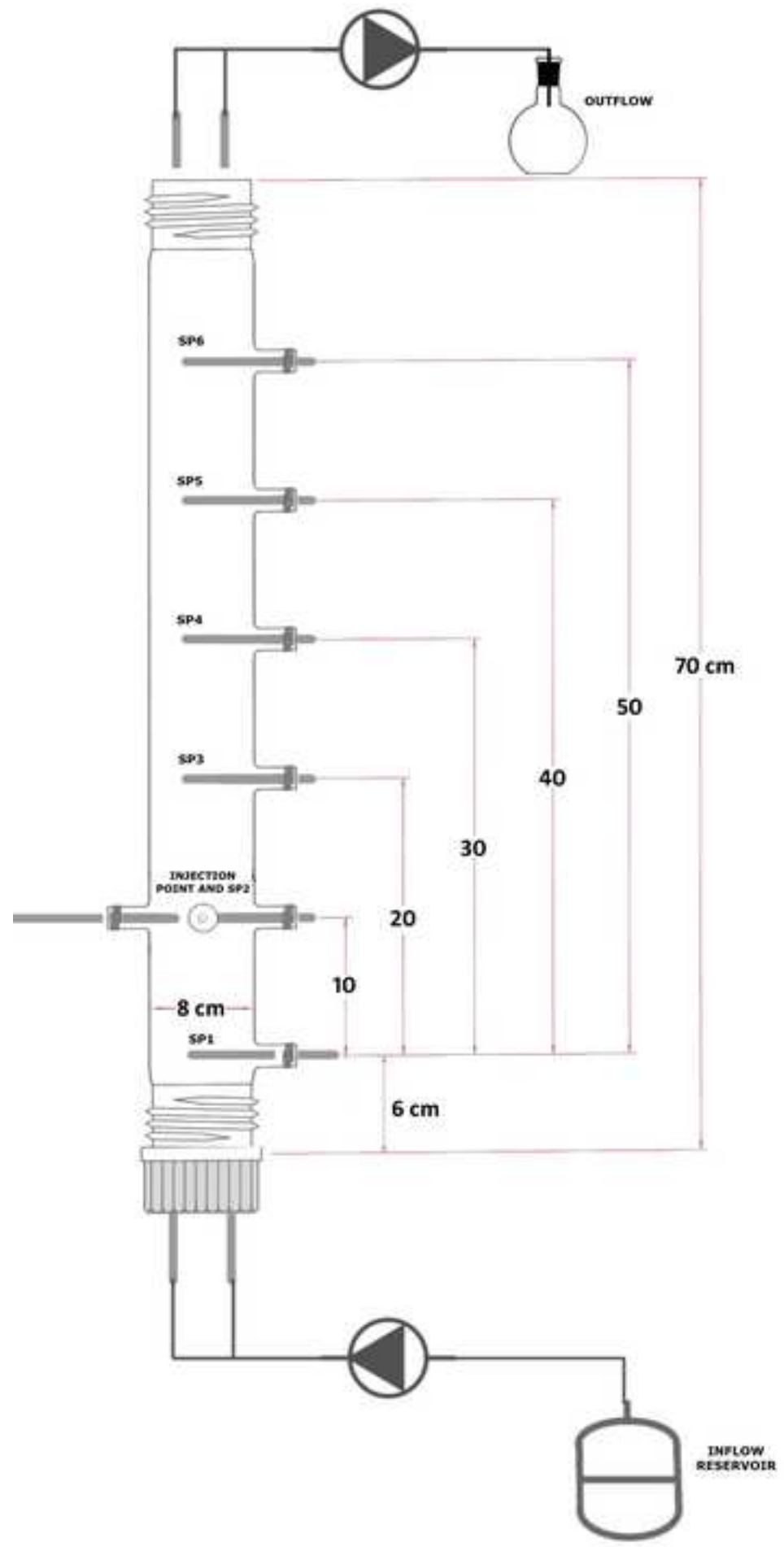


Figure2

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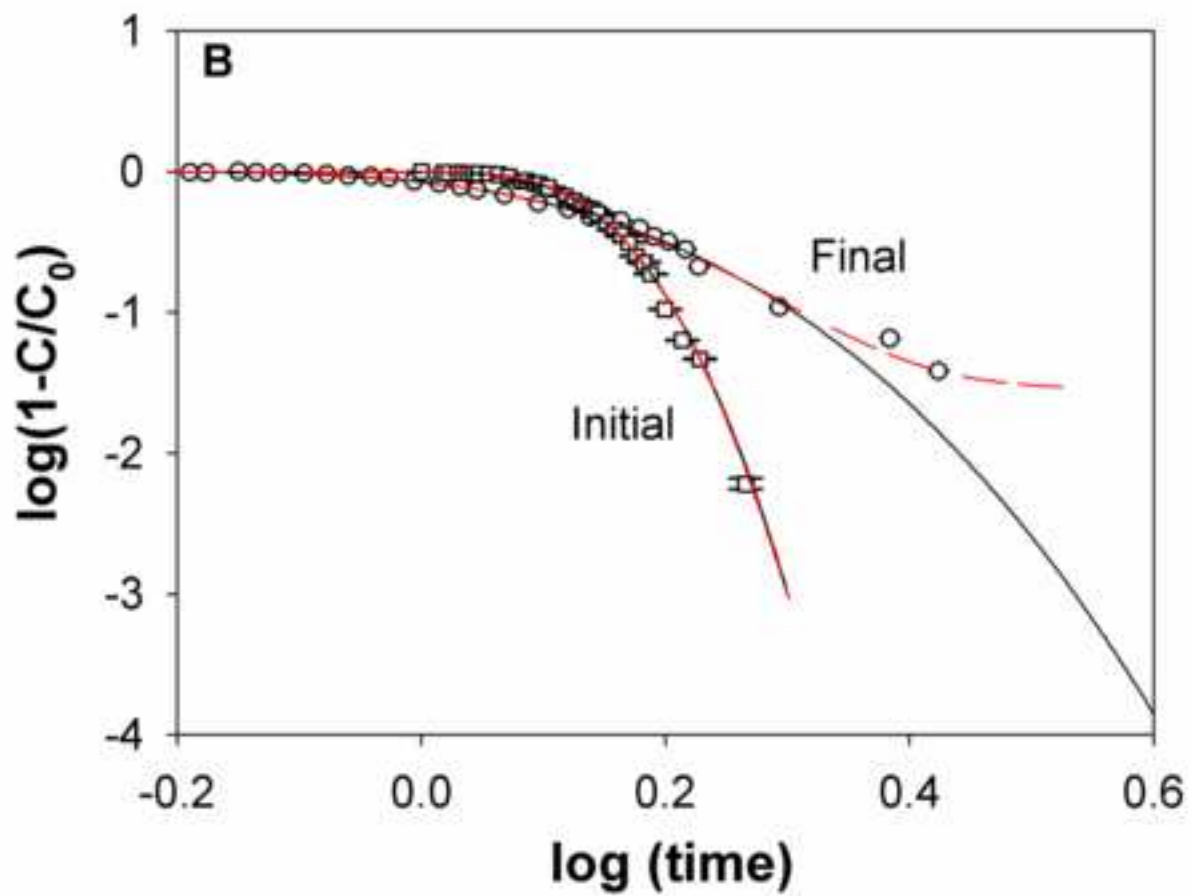
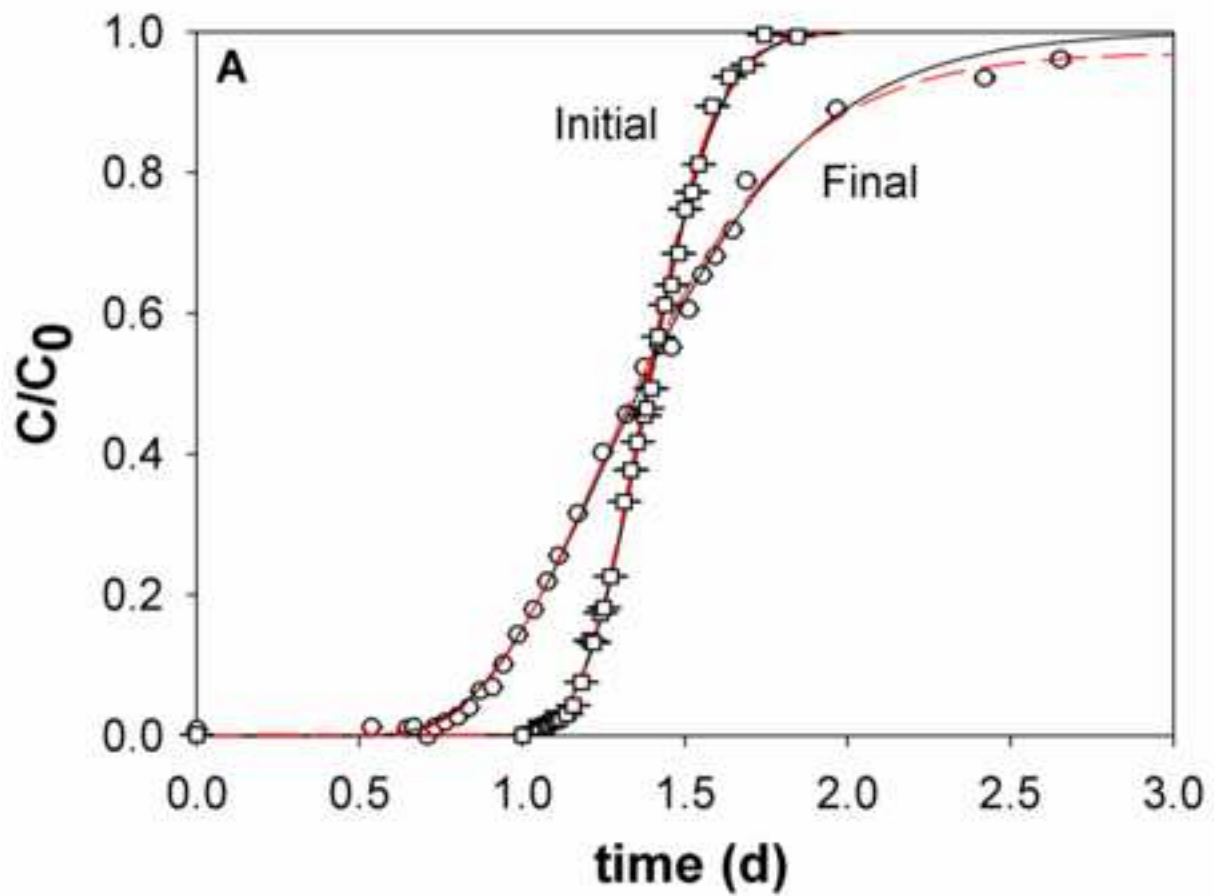


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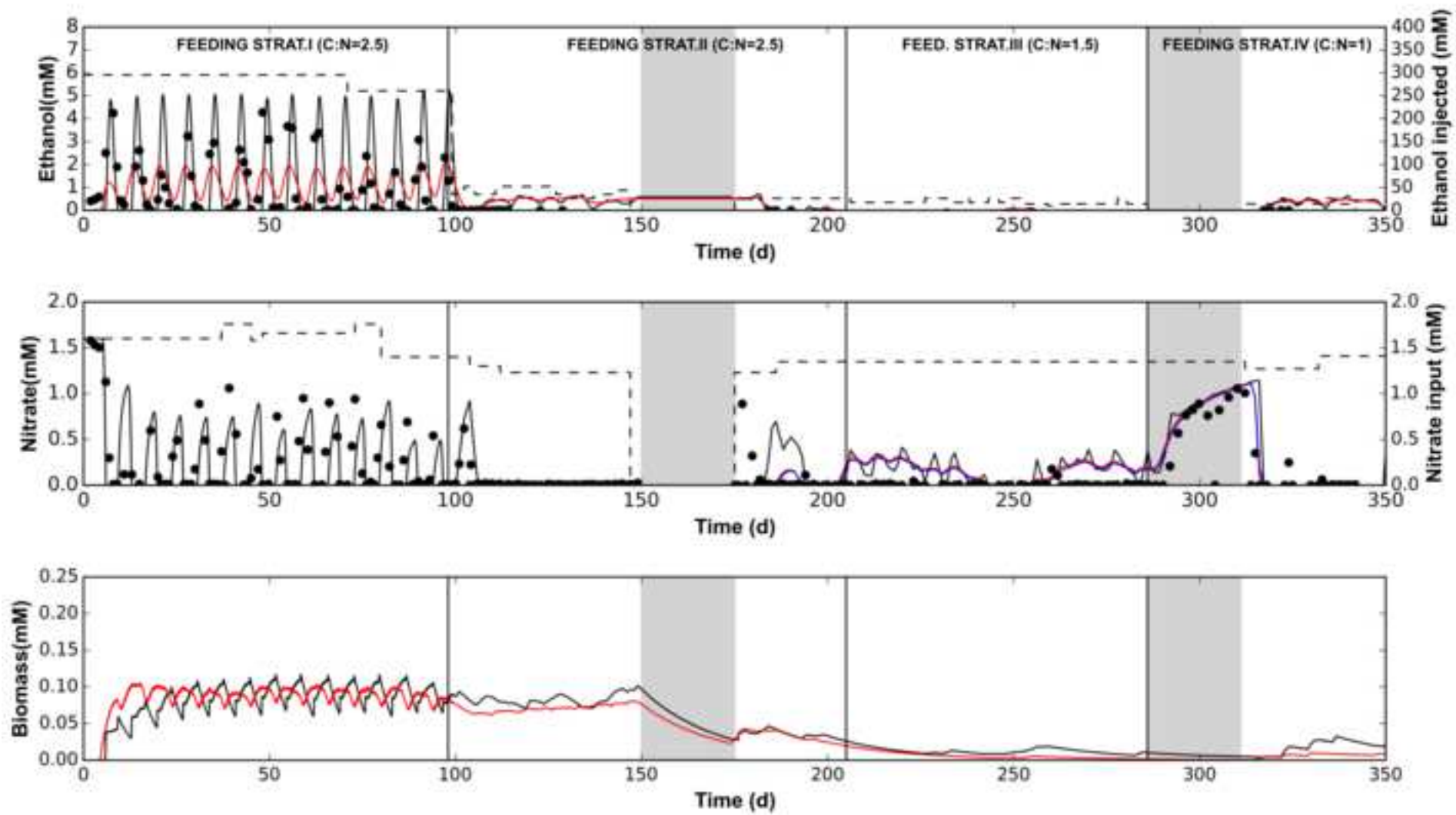




Figure4

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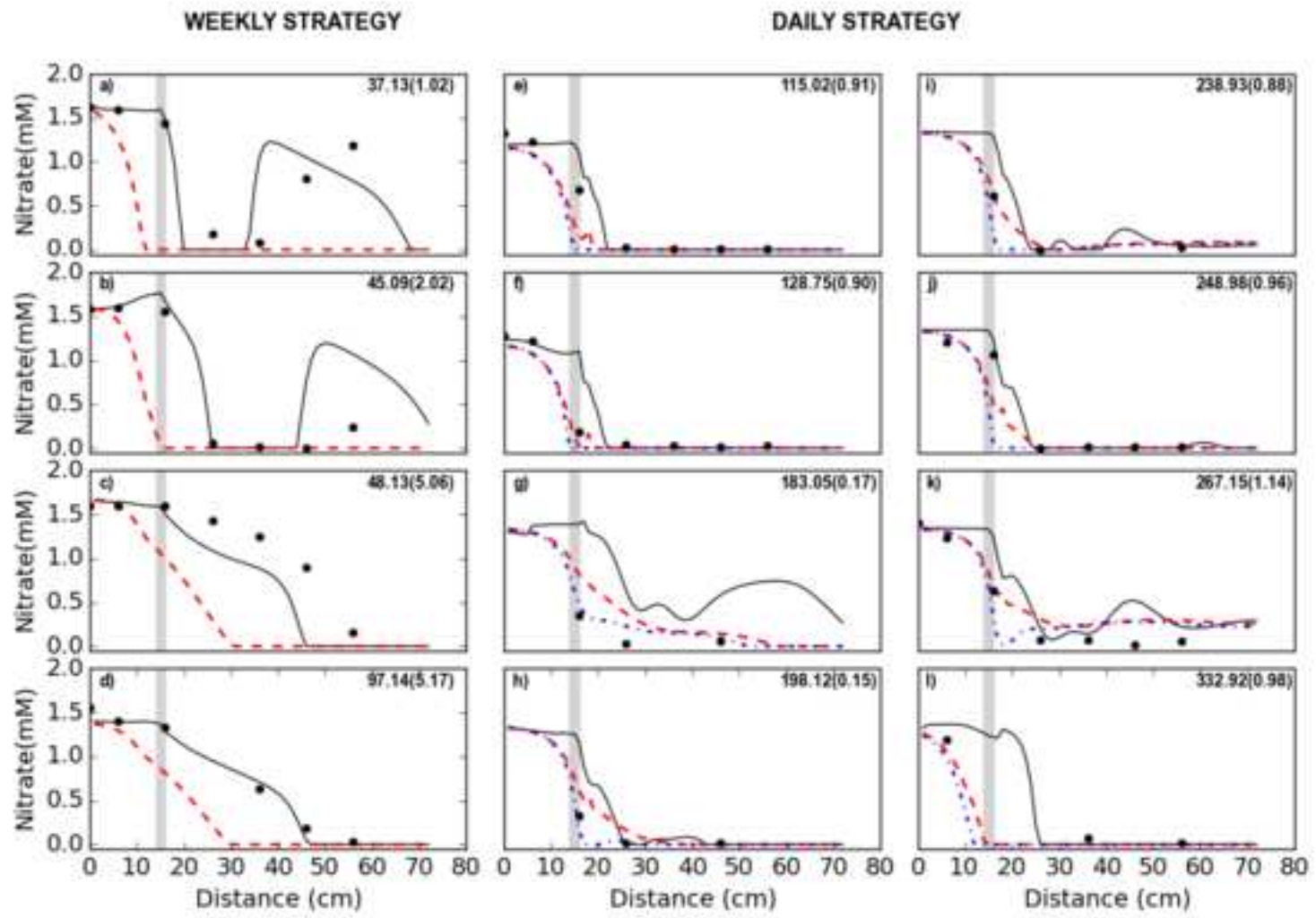


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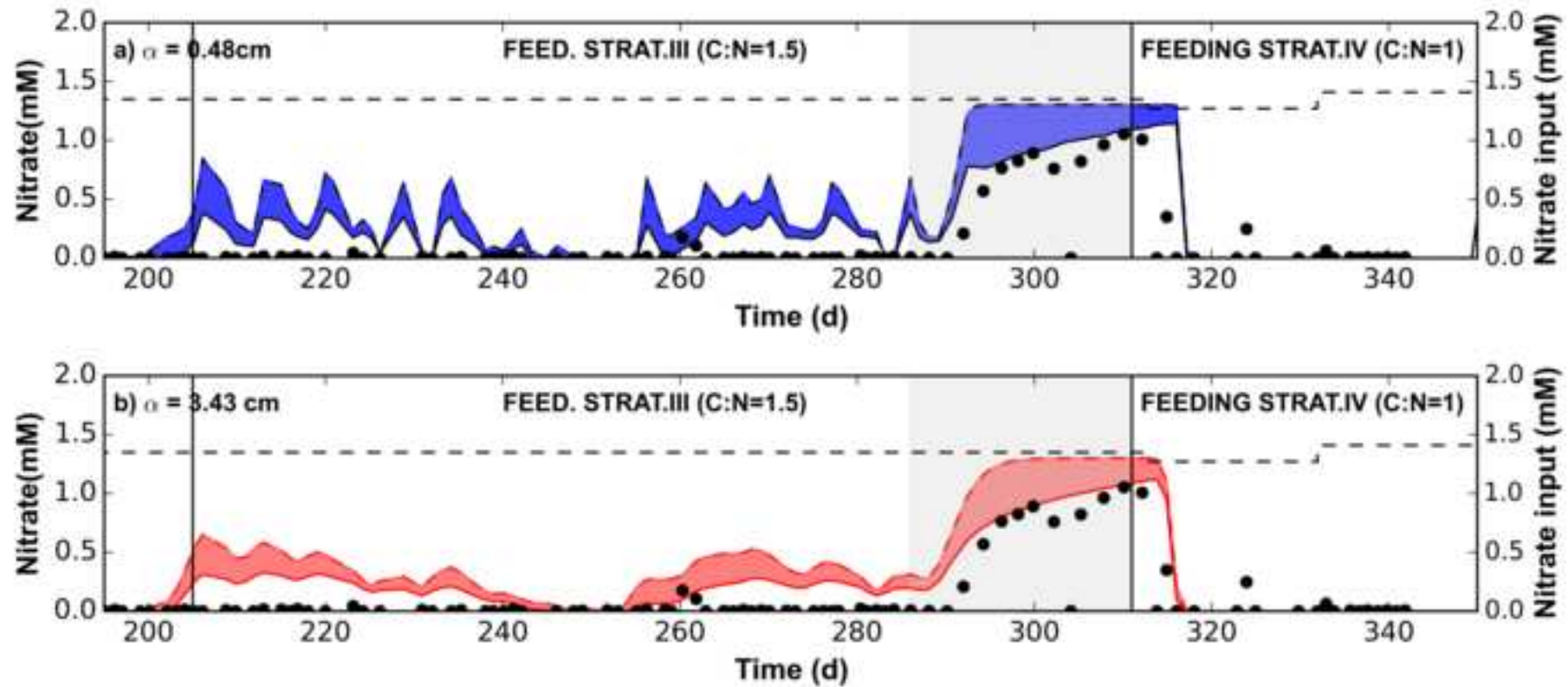


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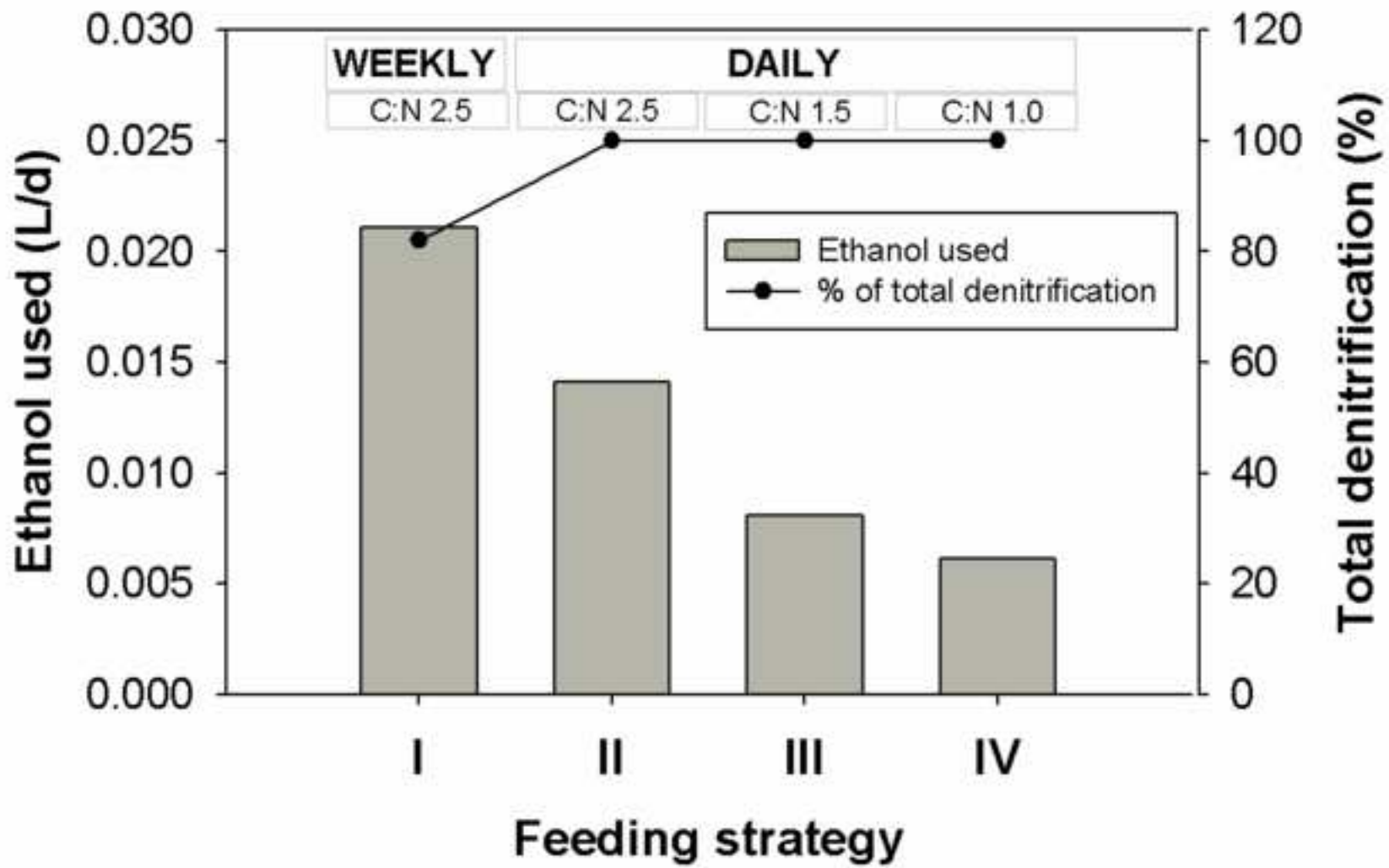


Table1

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Parameter	Unit	Column solution	Injected solution
pH		7.2 ± 0.1	7.2 ± 0.1
Temperature	°C	15	15
Nitrate	mM	1.2-1.6 (*)	1.2-1.6 (*)
DIC	mM	7.2 ± 1.0	7.2 ± 1.0
Chloride	mM	0.10 ± 0.04	0.10 ± 0.04
Sulfate	mM	1.2 ± 0.1	1.2 ± 0.1
Calcium	mM	3.40 ± 0.07	3.40 ± 0.07
Sodium	mM	2.20 ± 0.04	2.20 ± 0.04
Magnesium	mM	1.60 ± 0.03	1.60 ± 0.03
Potassium	mM	0.100 ± 0.001	0.100 ± 0.001
Ethanol	mM	-	14-292
Biomass	mM	2.3 x 10 <sup>-7</sup>	

**Table2**[Click here to download Table: Table2\\_v2.docx](#)

Feeding strategy	Feeding frequency	Average C:N	Ethanol injected (mM ethanol)	Days of experiment
I	Weekly	2.5	261-292	1-98
II	Daily	2.5	26-35	99-205*
III	Daily	1.5	17	206-287
IV	Daily	1	14	287-342**

\*Supply of water was discontinued between days 150 and 175

\*\*Supply of organic carbon was discontinued between days 286 and 311

**Table3**[Click here to download Table: Table3\\_v2.docx](#)

	Groundwater velocity (m d <sup>-1</sup> )	MODEL TYPE	Mobile Porosity	Dispersivity (cm)	Mass transfer parameter ( $\alpha$ , d <sup>-1</sup> )	Immobile porosity
Initial	0.5036 ± 0.0004	ADE	0.331 ± 0.033	0.485 ± 0.006	N/A	N/A
End	0.5107 ± 0.0018	Dual domain	0.326 ± 0.044	3.440 ± 0.246	0.019 ± 0.018	0.015 ± 0.009

**Table4**[Click here to download Table: Table4\\_v2.docx](#)

Parameter	Unit	This work	Literature values	Reference <sup>a</sup>
$\mu_{\max}$	[d <sup>-1</sup> ]	$3.01 \times 10^1 \pm 1.82 \times 10^1$	$1 \times 10^1$ ; $1.1 \times 10^1$ ; $2 \times 10^1$ ; $1.08 \times 10^2$	1,2,3,4
$K_{S,EA}$ (nitrate)	[M]	$8.18 \times 10^{-6} \pm 4.84 \times 10^{-6}$	$1.6 \times 10^{-6}$ ; $3.2 \times 10^{-6}$ ; $1.2 \times 10^{-5}$ ; $1.8 \times 10^{-4}$	1,3,2,4
$K_{S,ED}$ (ethanol)	[M]	$1.18 \times 10^{-4} \pm 4.55 \times 10^{-5}$	$8.3 \times 10^{-6}$ ; $1.7 \times 10^{-4}$ ; $6.6 \times 10^{-4}$ ; $7.3 \times 10^{-2}$	1,2,3,4
b	[d <sup>-1</sup> ]	$1.73 \times 10^{-1} \pm 4.66 \times 10^{-2}$	$6 \times 10^{-2}$ ; $1.5 \times 10^{-1}$ ; $2 \times 10^{-1}$	2,4,3

<sup>a</sup> References are 1, Chen and MacQuarrie (2004); 2, Lee et al., (2009); 3, Kinzelbach et al., (1991); 4, Rodríguez-Escales et al., (2014).

Table5

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Authors	Feeding strategy	Sediment	Time of feeding strategy (d)	Biological process	Organic carbon inflow (mM C)	Velocity (m/d)	$\alpha / \alpha_0$ (observed time)
This work	Weekly	Sand (0.5-0.8 mm)	100	Denitrification using ethanol	260-235	0.5	1 (100 d)
	Daily		200		14-35		7 (200 d)
Taylor and Jaffé (1990) and Taylor et al. (1990)	Continuous	Sand	284	Methanol oxidation (aerobic conditions)	0.22	27.2	100-1000
			356		0.17		100-1000
Bielefeldt et al. (2002)	Continuous	Sand (0.32 mm)	50	Naphthalene degradation (aerobic conditions)	1.6	3.3	1.8 (25-46)
						7.5	3-4.8 (37-44)
						11.2	2.2 (32-50)
Seifert and Engesgaard (2007)	Continuous	Sand (0.4-0.8 mm)	150	Acetate oxidation (aerobic conditions)	0.15	5.1	2 (13 d)
							7 (45 d)
Delay et al. (2013)	Continuous	Coarse crushed limestone (2 cm)	1.4	Denitrification using ethanol	1.52	34.6	1 (1.4 d)
					2.23	138.2	1 (1.4 d)