Macroinvertebrate community traits and nitrate removal in stream sediments

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1   # Macroinvertebrate community traits and nitrate

2   # removal in stream sediments

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29 # SUMMARY

30 1. In-stream nitrate removal capacity may be used as a proxy for the ecosystem

31 service of water quality regulation. It is well known that this natural function is driven

32 by abiotic and biotic factors in running water environments. With regard to biotic

33 drivers, most of the literature focuses on the microbial community influences, but

34 there has been very little emphasis on the relationship with the benthic

35 macroinvertebrate community. Since this community feeds on microbial assemblages

36 (autotrophic and/or heterotrophic biofilms) that live on the streambed and in the

37 hyporheic zone of the river, macroinvertebrates also have the potential to influence

38 nitrate removal via its influences on microbiological processes.

39 2. The objective of this study was to examine the potential relationship between the

40 macroinvertebrate communities and nitrate removal. A dataset of in-stream nitrate

41 removal rates measured in nine third-order streams was analysed. The simultaneous

42 influences of abiotic (hydromorphological, physical and chemical characteristics) and

43 biotic (biofilm and macroinvertebrate) drivers were examined and together explained

44 56 % of the in-stream nitrate removal variance. An analysis of the independent

45 contributions of each driver showed that abiotic drivers (e.g. ammonium, dissolved

46 organic carbon, temperature and transient zone) contributed 40 % of this nitrate

47 removal variance, while the macroinvertebrate community contributed 39 %.

48 3. The potential relationship between macroinvertebrates and nitrate removal was

49 subsequently explored using trait-based approaches of the macroinvertebrate

50 community. This method allows for the selection of trait modalities assuming a

51 top-down control of microbial communities by macroinvertebrates, with in-stream

52 abiotic conditions correlated to nitrate removal (assuming that environmental

53 conditions affect macroinvertebrate community composition).

54 4. The main trait modalities positively correlated with nitrate removal were scraper

55 (feeding habit), flagstones/boulders/cobbles/pebbles (substrate preference), crawler

56 and interstitial (locomotion) and detritus (food). The main modalities negatively

57   correlated with nitrate removal were silt and mud with microphytes (as substrate

58   preference), and with fine sediment with microorganisms, and dead animals (as food

59   sources). These results agreed with the hypothesis of top-down control and enhanced

60   understanding of the influence of hydromorphological factors on nitrate removal.

61   5. This study highlights the involvement of the macroinvertebrate community in

62   in-stream nitrate processing, and demonstrates the usefulness of applying a functional

63   approach to explaining relationships between biodiversity and ecosystem function.

64

Introduction

66   Anthropogenic loading of nitrogen into freshwater ecosystems has increased by

67   more than one order of magnitude over the past two decades (Vitousek 1997;

68   Galloway *et al.*, 2004; Ruehl *et al.*, 2007). Streams and rivers are important sinks for

69   nitrogen (Grizzetti *et al.*, 2015), and about half of the nitrogen input is ultimately

70   removed by streams and rivers before flowing into coastal waters (Galloway *et al.*,

71   2004). In-stream nitrogen retention is the set of processes by which nitrogen is stored,

72   transformed and removed from the water column of streams and rivers or stored in

73   biota (Alexander *et al.*, 2000). This retention contributes to the regulation of

74   downstream nitrogen exports. Nitrate is one of the major forms of dissolved inorganic

75   nitrogen in rivers. Nitrate retention may be used as an ecosystem service indicator of

76   water quality regulation (Millennium Ecosystem Assessment 2005; Cardinale, 2011).

77   A number of processes are involved in in-stream nitrate retention, including

78   abiotic processes such as hydrologic storage (Triska *et al.*, 1989 a, b) and biotic

79   retention (Gücker *et al.*, 2006). Biotic nitrate removal includes assimilatory processes

80   *via* uptake by aquatic plants, algae and fungi, and dissimilatory processes such as

81   denitrification and dissimilatory nitrate reduction to ammonium (DNRA) (Ranalli and

82   Macalady, 2010). These biotic processes are driven by microbial assemblages

83   (bacteria, fungi and algae) as well as by macrophytes in rivers (Pusch *et al.*, 1998;

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84   Sabater *et al.*, 2002; Battin *et al.*, 2003; Simon *et al.*, 2005; Ensign & Doyle, 2005;

85   Teissier *et al.*, 2007; Von Schiller *et al.*, 2008). In running-water ecosystems, these

86   processes mainly occur on the streambed and in the hyporheic zone (Triska *et al.*,

87   1989a; b; Fellows *et al.*, 2001; Marti *et al.*, 2004; Fischer, 2005; Argerich *et al.*,

88   2011). Nitrate removal can be estimated by conducting a pulse addition experiment

89   based on the nutrient spiralling concept (Newbold *et al.*, 1981; Stream Solute

90   Workshop, 1990).

91   In-stream biotic nitrate removal is controlled by abiotic factors such as nutrient

92   concentrations (Bernot & Dodds, 2005; Mulholland *et al.*, 2008) and the

93   hydromorphological features of the stream channel and the hyporheic zone (Gücker &

94   Boëchat, 2004; Ensign & Doyle, 2006). In-stream nitrate removal by biota is known to

95   be regulated by the bottom-up control of resources (such as carbon and nutrient

96   availability) and environmental conditions (Dodds *et al.*, 2002; Roberts & Mulholland,

97   2007). However, biotic nitrate removal could also be regulated by top-down controls

98   from consumers, such as macroinvertebrate communities feeding on biofilms

99   (Wallace & Webster, 1996; Mermillod-Blondin *et al.*, 2003; Covich *et al.*, 2004;

100   Karlson *et al.*, 2007; Stief, 2013). This top-down control has rarely been explored as a

101   biotic driver of in-stream nitrate removal (Lawrence *et al.*, 2002; Sabater *et al.*, 2002;

102   Law, 2011). The top-down control due to macroinvertebrate grazing could allow the

103   continuous growth of the microbial community in the hyporheic sediment and thus

104   help prevent clogging of the porous media of sediments where nitrogen

105   transformations take place. This influence should favour the transfer of surface water

106   and nutrients into the hyporheic zone and hence promote the interaction between

107   nitrate and microbial assemblages responsible for nitrate reduction

108   (Mermillod-Blondin *et al.*, 2000, 2003; Mermillod-Blondin & Rosenberg, 2006;

109   Mermillod-Blondin, 2011; Stief, 2013). Additionally, macroinvertebrate microhabitat

110   occupancy, burrowing and bioirrigation (the flushing of burrows with overlying water

111   by the peristaltic movements of the invertebrates) (Gerino *et al.*, 2003;

112   Mermillod-Blondin *et al.*, 2003; Schaller *et al.*, 2014) in streambed sediments can

113   change the physical structure of the abiotic microenvironment and modify the spatial

114   distribution of the redox conditions in sediments (Aller. 2004; Meysman *et al.,* 2006).

115   The research referred to above has mainly been based on laboratory experiments,

116   and the relationship between macroinvertebrate communities and nitrate retention in

117   the field requires further exploration. There are two reasons why examination of the

118   macroinvertebrate community as a potential driver of in-stream nitrate removal is

119   warranted:

120   (i)   There is a strong association of many macroinvertebrate taxa with the streambed

121   and hyporheic substrata, which are recognised as major sites of biogeochemical

122   reactions in streams (Giere, 2009)

123   (ii) the sensitivity of macroinvertebrates to in-stream environmental conditions

124   (Rosenberg & Resh, 1993; Statzner & Bêche, 2010; Menezes *et al.*, 2010) is likely to

125   affect in-stream nitrate retention *via* the community compositions.

126   Although taxonomy-based metrics are widely recognised as being useful as

127   bio-indicators, they are unlikely to adequately mirror ecosystem functioning (Sandin

128   & Solimini, 2009). Instead, a trait-based approach using multiple biological and

129   ecological traits of organisms (e.g. mobility, feeding type, size, lifespan of aquatic

130   macroinvertebrates) is more likely to reflect ecological processes and ecosystem

131   functioning (Hooper *et al.*, 2005; Bremner *et al.*, 2006; Colas *et al.*, 2013). This

132   approach allows (i) a description of macroinvertebrate community responses to many

133   abiotic and biotic stressors, (ii) a reduction in uncertainties related to seasonal effects

134   by integrating the variations in environmental conditions over time, such as seasonal

135   variability, and (iii) a more direct and easier detection of the mechanism by which the

136   community composition influences the ecosystem functioning. Furthermore, the trait

137   composition of macroinvertebrate communities is comparable across large spatial

138   scales, and even across ecoregions harbouring communities of a different taxonomic

139   composition (Dolédec *et al.*, 2006; Feio *et al.*, 2010). Consequently, there is

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140   increasing interest in trait-based approaches to improve assessments of stream health

141   and link diversity to ecosystem functions (e.g. McKie *et al.*, 2008; Colas *et al.*, 2013;

142   2014; Frainer *et al.*, 2014).

143   The objective of this study was to explore the influence of both abiotic (i.e.

144   physico-chemical and hydromorphological) and biotic (i.e. biofilm and

145   macroinvertebrate) drivers on in-stream nitrate removal using a dataset from nine

146   third-order streams. A variance partitioning approach was used to assess the

147   independent contributions of biotic and abiotic drivers to nitrate removal, before

148   focusing on the potential relationship between macroinvertebrate and biotic nitrate

149   removal using trait-based approaches. It was hypothesised that the biological traits of

150   macroinvertebrate communities reflect a combination of environmental conditions

151   (examples of mobility and substrate preferences) and some functional relationships

152   between macroinvertebrates and biofilm microbes (examples of feeding habits and

153   food resources) that together influence the local nitrate retention rate. This

154   information should depict the processes that are controlled by macroinvertebrate

155   activity and related to nitrate removal according to the top-down control assumption.

156   **Methods**

157   Data collection

158   The data were collected *in situ* as part of the EU-funded STREAMES project

159   (STream REAch Management, an Expert System,

160   http://cordis.europa.eu/project/rcn/54747_en.html). The objective of this project was

161   to identify the relationships between in-stream nutrient retention capacity and

162   potential biotic and abiotic factors in a set of streams in different ecoregions. The

163   STREAMES project originally involved 11 third-order streams across seven European

164   countries plus Israel. In each stream, several *in situ* experimental measurements were

165   performed to cover contrasting hydrological conditions during 2002.

166   For the specific objective of the present study, only the dates on which there

167 were simultaneous *in situ* records in the stream reaches of nitrate uptake and physical,

168 chemical, hydrological, biofilm and macroinvertebrate characteristics were selected.

169 Only reaches located upstream from wastewater treatment plants (WWTP) were

170 chosen to avoid disturbance from local outflows into the river water. Finally, after this

171 data selection, 27 measurements from nine streams (Fig. 1) were included in this

172 study (see Table 1).

173   *In situ* and laboratory measurements followed common procedure guidelines,

174 ensuring comparability of all data (Gorden *et al.*, 1992; Clesceri, 1998; Gordon *et al.*,

175 2004; Vellido *et al.*, 2007; Morais *et al.*, 2009). During each *in situ* study, hydrology,

176 substrate and macroinvertebrate communities were measured in six equidistant

177 transects (with an average transect length of 3.5 m). Three water samples were

178 collected in each transect, immediately filtered through pre-combusted glass

179 microfibre filters (Whatman GF/F, 0.7μm) and stored in coolers before chemical

180 analysis. Three surface sediment samples in each transect were also collected (18

181 samples per reach) for quantification of biofilm chlorophyll a concentration after

182 pigment extraction from the sediment.

183 Nitrate removal measurements

184   Nitrate addition experiments, using the slug addition technique, were conducted

185 to estimate the retention capacity for nitrate (NO_3-N) along each stream reach over a

186 short period of time, according to the procedure of Gorden *et al.* (1992) and applied

187 by Ruggiero *et al.* (2006) and Sánchez-Pérez *et al.* (2009). The estimation of the

188 stream nutrient retention rate for NO_3-N were made on each sampling date with a

189 solute addition of a known quantity of nutrient (NO_3-N) into the stream.

190 Simultaneously, a conservative element (Cl^-) was added as a tracer to allow

191 downstream corrections for any dilution that may occur along the reach. Nitrate (as

192 $NaNO_3$ or KNO_3) and the conservative tracer (as NaCl) were added at the same time

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193   as a pulse input from a carboy in the mid-channel at the top end of the reaches. The

194   experimental distances were calculated so as to be dependent on stream discharge (Q)

195   (Table 1). Water samples were then collected at the downstream end of the reach,

196   with an increase in sampling frequency during the NaCl solution passage.

197   Concentration-time curves (mg. $L^{-1} .s^{-1}$) of nitrate and NaCl were then used to

198   calculate the nutrient mass retained (mg). The nutrient uptake rate at experimental

199   level (U_{exp}, mg.m^{-2}.min^{-1}) was equal to the nutrient mass retained during the addition

200   experiment divided by the stream bottom area A (m^2) and by time duration (min).

201   Uptake length Sw (m) was the average distance travelled by a nutrient ion before

202   uptake, therefore estimated as:

203        $Sw = ([Nut]_b *Q)/(U_{exp}*w)$

204   where $[Nut]_b$ is the nutrient background concentration (mg.L^{-1}), Q is the discharge

205   (m^3.s^{-1}) and w (m) is the average stream width of the reach. The first-order uptake rate

206   coefficient (Kc, m^{-1}) was calculated by:

207      $Kc = v/Sw$

208   where v is the stream water average velocity (m, s^{-1}). Uptake velocity (Vf, m.s^{-1}; the

209   vertical velocity at which nutrients move to the stream bottom) was estimated from:

210      $Vf = Kc*d$

211   where d is the mean stream depth. The gross nutrient uptake rate at ambient level (U,

212   mg.m^{-2}.min^{-1}) was calculated from:

213      $U = Vf*[Nut]_b$

214   (see details in Ruggiero *et al.*, 2006).

215   Uptake rate (U) was selected as the preferred metric for quantifying the benthic

216   nutrient removal because it is relatively independent of nutrient concentrations and of

217   the stream's hydrologic characteristics. "$U_{NO_3^- -N}$" denotes the assimilation (uptake by

218   plant and algae) and dissimilation (mainly denitrification) processes for biotic nitrate

219   removal. It is a good indicator of variations in biotic nitrate removal and enables

220   intra-site and inter-site comparisons (Simon *et al.*, 2005; Ensign & Doyle, 2006).

Abiotic factors

Catchment factors

221   The catchments of the experimental reaches were characterised for total area,
222   mean slope and percentage of land uses by means of geographic information system
223   (GIS) data layers. These data were then combined using ArcGIS (Environmental
224   Systems Research Institute, Redlands, CA, USA). Land uses (%) were grouped into
225   natural areas (including forest and open land), agricultural land use (including arable
226   and grassland) and urban areas (including towns, residential areas and industrial and
227   commercial zones).

Hydromorphological factors

231   River depths, widths and current velocities were measured to estimate discharges
232   according to the velocity-area method, before calculating the Froude number and
233   Reynolds number according to Gorden *et al.* (1992).
234   The Froude number (Fr) represents the relationship between inertial forces (due
235   to downstream water movement) and gravitational forces, indicating the strength of
236   the water current. The Reynolds number (Re) represents the relationship between
237   inertial forces and viscosity forces, indicating the degree of turbulence in the water.
238   The size of the transient storage zone within each reach was estimated by performing
239   independent additions of conservative tracers (NaCl) (Stream Solute Workshop, 1990).
240   The following two parameters were included to describe the normalised storage zone
241   area (As/A), with the transient storage zone cross-section (A_s, m^2) that accounts for
242   regions of the stream ecosystem where water moves at a slower velocity than the
243   average surface velocity. In this estimation, A is the surface of the cross-sectional area
244   of the stream main channel. The variable was used to estimate the relative importance,
245   among physical factors that might influence solute transport and retention in stream
246   reaches, of zones with slow water velocities, such as the hyporheic zone and pools. The

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247 hydraulic retention factor (HRF, $s.m^{-1}$) is the transient storage zone (A_s, m^2) divided by

248 the uptake length of water (Sw) and this variable reflects the potential transient storage

249 zone effect (Morrice *et al.*, 1997).

Substrate factors

251 Substrate size composition was assessed by eye and categorised into percentages

252 of boulder (>40 cm), rock (20 to 40 cm), cobble (6 to 20 cm), pebble (2 to 6 cm),

253 gravel (0.2 to 2 cm), sand (0.006 to 20 mm) and silt and mud (< 0.006 mm) (Gorden

254 *et al.*, 1992).

Physicochemical factors

256 Water samples were collected and filtered through pre-ashed Whatman GF/F

257 glass fibre filters. Nutrient concentrations, including ammonium (NH_4^+-N), nitrate

258 (NO_3^--N), phosphate (PO_4^{3-}-P) and dissolved organic carbon (DOC) concentrations,

259 were analysed using high-performance ionic chromatography with a DIONEX system.

260 Temperature, dissolved oxygen, conductivity and pH were measured *in situ* using

261 multi-parameter probes (YSI 6920).

Biotic factors

Biofilm biomass

264 Autotrophic biofilm samples from a known area of substrate were collected. The

265 type of device used to collect the biofilm samples depended on the size and type of

266 the dominant stream substrata covered by biofilm. Samples were frozen and stored in

267 the dark before estimating chlorophyll a concentration following standard protocols

268 (Steinman *et al.*, 1996). In the laboratory, samples were extracted in 90 % acetone

269 over 24 h at 4 °C, sonicated or homogenised for 5 min and then centrifuged for 10

270 min. Chlorophyll a concentrations were then determined by spectrophotometry.

271 Macroinvertebrate community

272   Benthic macroinvertebrates were sampled using a Surber net with a 200 μm

273 mesh size. Six equidistant transects per reach were investigated in sampling locations

274 following standard requirements in terms of substrate type selection (Verneaux *et al.*,

275 1982; Compin & Céréghino, 2003). At each location two replicates were taken with

276 respect to microhabitat distributions. Samples were preserved in 96 % ethanol before

277 taxonomic identification. Macroinvertebrate individuals were identified and counted

278 using stereo dissecting microscopes. The family taxonomic level was used for most

279 organisms, except for some groups identified at a different taxonomic level (such as

280 Chironomidae and Oligochaeta at super family level). Taxa densities (individuals.m^{-2})

281 were calculated by taking into account the sampled area for each sample. Rare taxa (n

282 < 3 individuals in all records) were excluded from the analysis (Colas *et al.*, 2013).

283 Densities were then log (x+1) transformed to stabilise variances and normalise the

284 dataset, producing a 'taxa by measurement' matrix (27 measurements x 71 taxa).

285 Macroinvertebrate functional trait profile

286   The biological and ecological traits of benthic macroinvertebrates were used to

287 determine the functional structure of the assemblage (Tachet 2000; Statzner *et al.*,

288 2001). Each trait was described by a set of modalities. Four biological traits including

289 morphology ('maximum size'), feeding behaviour (including 'food' sources and

290 'feeding habits') and locomotion ('locomotion and substrate association') and one

291 ecological trait ('substrate preference') were chosen (following Usseglio-Polatera *et*

292 *al.*, 2000; Castella et al. 2012; Gallardo *et al.*, 2014; Szöcs *et al.*, 2014; see Table 2) in

293 order to reflect the possible influence of macroinvertebrates on nitrate removal by

294 biofilm. The biological trait 'feeding habit' includes eight modalities describing the

295 regular functional feeding groups and was intended to indicate the top-down effects of

296 macroinvertebrates on the biofilm with the modalities of scrapers for surface biofilm

297   and deposit feeders for interstitial biofilm (Statzner & Bêche, 2010). The 'food'

298   modalities supply additional information about the feeding habits, with the modalities

299   'fine sediment and micro-organisms' being associated with deposit feeders that ingest

300   sediment. The modalities of microphytes are related to microscopic algae, such as

301   diatoms, that can live in surface biofilm covering large or fine particles and may be

302   ingested by scrapers or deposit feeders respectively. The 'locomotion and substrate

303   association' trait was selected to reveal the type of movement of macroinvertebrates

304   above the streambed or inside the hyporheic sediment. The 'body size' trait was also

305   included since large animals can have a more significant effect on ecosystem function

306   than smaller ones (Basset *et al.*, 2004; Badosa *et al.*, 2006; Brucet *et al.*, 2006; Gascón

307   *et al.*, 2009). The ecological trait 'substrate preference' was included to encompass

308   potential microhabitat occupancy by macroinvertebrates. All these macroinvertebrate

309   traits have already been reported as responding to chemical and hydromorphological

310   conditions (Kearney *et al.,* 2010, Colas *et al.*, 2014).

311   Statistics

312   Several centred-normed principal component analyses (PCA) were performed for

313   each group of abiotic factors (i.e. catchment properties, physicochemical

314   characteristics of water, substrate size and hydrological characteristics). The

315   coordinates of each measurement from the main axis (i.e. axes 1 and 2) were extracted

316   and used as synthetic variables for each group of abiotic factors. Correspondence

317   analysis (CA) was used for log-transformed densities of macroinvertebrate taxa.

318   Similarly, the coordinates of each measurement from the main axis of CA were

319   extracted and used as synthetic variables of macroinvertebrate assemblages.

320   Generalised linear models (GLMs) with the 'Gaussian family' followed by a

321   stepwise procedure based on the Akaike information criterion (AIC) were carried out

322   to assess the abiotic and biotic drivers that were significantly correlated with $U_{NO_3^--N}$.

323   The coordinates of all measurements from axes 1 and 2 of each PCA or CA were

324   combined as predictors. A total of 11 predictors were used (i.e. Catch 1+ Catch 2 +

325   Phy-che 1+ Phy-che 2+ Sub 1 + Sub 2 + Hydro 1 +Hydro 2+ Biofilm Chl-*a* +

326   M.Inv1+ M.Inv2; see Table 3 and Fig. 2 for details). $U_{NO_3^--N}$ as a dependent variable

327   was previously log-transformed to fulfil normality.

328   The D^2 of each model was calculated to account for the amount of deviance

329   according to the formula proposed by Guisan and Zimmermann (2000) (Eq. (1)):

330   D^2 = (model\$null · deviance − model\$deviance)/model\$null · deviance (1)

331   The D^2 of GLMs is the equivalent of the R-squared value of linear models that

332   measures the proportion of variation accounted for by the model. Model checking

333   included homogeneity of variance and normal distribution of model residuals.

334   The relative importance of each predictor in the best-fitted model was then

335   examined using hierarchical partitioning (HP). A randomisation test, which was based

336   on the upper 0.95 confidence limit, was then run on the hierarchical partitioning

337   results to provide statistical significance (Nally, 2002). HP determined the

338   independent contribution of each predictor to the response variable and separated it

339   from the joint contribution resulting from the correlation with other variables. This

340   enabled a ranking of the importance of the covariates in explaining the response

341   variable independently of the other covariates.

342   For trait-based analyses, the mean functional trait profiles of the communities

343   were calculated from taxonomic data for each measurement using fuzzy-coded

344   biological and ecological traits (Chevenet *et al.,* 1994) described for each taxon from

345   the literature. Fuzzy coding uses positive scores (between 0 and 3 or 5) to describe the

346   affinity of a species for different modalities of a given trait, accounting for phenotypic

347   and ecological preference variability among taxa. The fuzzy coding procedure helps

348   to extract different types and levels of information available for different taxa

349   (Chevenet *et al.*, 1994), addressing spatial or temporal differences in the traits of a

350   given taxon (Statzner & Bêche, 2010). The mean weighted (by log-transformed

351   densities) trait profiles of community assemblages were then calculated for each

13

352   measurement and expressed as relative density distributions of trait categories within

353   the assemblages (Thioulouse *et al.*, 1997). Partial least squares (PLS) regressions

354   (Abdi, 2003) were then carried out to identify macroinvertebrate trait modalities that

355   significantly predicted $U_{NO_3^- -N}$. PLS was particularly suitable for this case because (i)

356   there were few replicates (n=27) and several predictors (n=21) and (ii) many

357   predictors showed high collinearity (Carrascal *et al.*, 2009). PLS reduced a set of

358   predictors to a few components that had maximum covariance with the response

359   variable. These components were defined as a linear combination of original variables,

360   so the original multi-dimensionality was reduced to a smaller number of components.

361   PLS analysis generated variable importance in projection (VIP) values, as well as the

362   variance (R^2) explained by each of the two components. VIP values reflect the

363   importance of each predicted variable of the model, with VIP > 0.7 indicating

364   important predictors (Eriksson, 1999).

365   For all steps in the statistical analysis, 'ade' (Chessel *et al.*, 2012), 'MASS',

366   'hier.part' (Walsh *et al.*, 2013) and PLS (Mevik & Wehrens, 2007) packages in R

367   software (R development Core Team, 2011) were used.

368   **Results**

369   Nitrate uptake rate ($U_{NO_3^- -N}$) and environmental characteristics

370   Taking all the measurements into consideration, $U_{NO_3^- -N}$ ranged from 0.04 to 10.75

371   mg.m^{-2}.min^{-1}, with a mean value equal to 1.64 $\pm$ 2.39 mg.m^{-2}.min^{-1} (standard

372   deviation) (Table 1). The catchment size of the investigated streams ranged from 11.2

373   to 480.0 km^2 (Table 1). The average catchment slope was 10 %. The studied

374   catchments included wide ranges of natural (20-87 %) and agricultural (11-79 %) land

375   uses. Urban land use accounted for less than 20 % in all the catchments (Table 1). The

376   first axis of the PCA performed on catchment variables accounted for 44 % of the

377   variance and indicated a gradient from natural to agriculture-dominated catchments.

378   The second axis (34 % of the variance) indicated a gradient from urban to

379   agriculture-dominated catchments (Table 3).

380   Concentrations (min - max) of phosphate (0.003-8.2 mg PO_4^{3-}-P. L^{-1}), nitrate

381   (0.05-8.98 mg NO_3^--N. L^{-1}), dissolved organic carbon (0.55-21.9 mg C. L^{-1}) and

382   ammonium (0.003-0.18 mg NH_4^+-N. L^{-1}) spanned wide ranges. Water temperature

383   and conductivity ranged from 5.2 to 22.0 °C and 164 to 1258 uS.cm^{-1} respectively

384   (Table 2). The first axis (Phy-che 1) of the PCA, performed on the water

385   physicochemical variables and explaining 37 % of the variance, was negatively

386   related to NO_3^--N, PO_4^{3-}-P and conductivity. The second axis (Phy-che 2 explaining

387   24 % of the variance) was positively related to NH_4^+-N (0.7), DOC (0.7) and

388   temperature (0.5) (Table 3).

389   Depth (0.02-0.32 m), velocity (0.02-0.50 m^3. s^{-1}) and Froude (0.03-0.43) varied

390   by over an order of magnitude between the studied streams. Discharges (0.001-0.267

391   m^3. s^{-1}), As$/$A ratios (0.04-63.70), HRF (0.4-975.0) and Reynolds (358-73077)

392   spanned wide ranges (Table 1). The first axis of the hydrological PCA (Hydro 1) was

393   representative (49 % of the variability) of a gradient of hydromorphological features

394   (Reynolds, velocity, Q and Froude). The second axis (Hydro 2), accounting for 24 %

395   of the variability, was negatively related with the transient storage zone variables (i.e.

396   HRF and As/A) (Table 3).

397   The substrata in these streams had different compositions (Table 1), as depicted

398   by the substrate size PCA. The first axis, which accounted for 40 % of the total

399   substrate variability, indicated the gradient of sediment granulometry (Table 3).

400   The third components in each PCA accounted for less than 20 % of the variance

401   and are not shown here.

402   Large variations in the concentration of biofilm chlorophyll a were observed

403   between the streams (1 - 484 mg. m^{-2}) (Table 1).

404 Taxonomic structure of macroinvertebrate assemblages

405 Macroinvertebrate densities, richness and diversity (Shannon index) exhibited

406 wide variations between the different streams and within the same stream (Table 1).

407 The first (M. Inv 1) and second (M. Inv 2) axes of the correspondence analysis

408 performed on macroinvertebrate densities explained 15 % and 12 % respectively of

409 the variation in the taxonomic composition of the streams (Fig. 2).

410 Relationship between $U_{NO_3^--N}$ and abiotic and biotic drivers

411 The components listed in Table 4 were selected as the best predictors of $U_{NO_3^--N}$

412 by the step-wise GLM analysis. These results indicate that biotic and abiotic factors

413 together explained 56 % of the total deviance of $U_{NO_3^--N}$ distribution. Hierarchical

414 partitioning (HP) allowed the identification of the independent influences of these

415 seven selected components on $U_{NO_3^--N}$ and simultaneously ranked these influences.

416 The best predictors were the abiotic factors (Hydro 2 and Phy-che 2) and the biotic

417 factors (M.Inv 2 and M.Inv 1), with independent contributions varying from 23 % to

418 16 % of $U_{NO_3^--N}$ variance.

419 For the two abiotic factors, Phy-che 2 was positively related with $U_{NO_3^--N}$ so that

420 higher values of $U_{NO_3^--N}$ were found in sites with a high temperature and high

421 concentrations of NH_4^+-N and DOC (with the positive loading on Phy-che 2, Table 3).

422 Hydro 2 was negatively related with $U_{NO_3^--N}$ and PCA results (Table 3), which

423 indicated that Hydro 2 was negatively loaded by As/A and HRT. As a result, $U_{NO_3^--N}$

424 was higher with higher values of As/A and HRT.

425 For the biotic factors, M.Inv 2 had a significant positive correlation with $U_{NO_3^--N}$.

426 The following taxa had positive loadings of M.Inv 2: e.g. Helophoridae,

427 Odontoceridae, Crambidae Aeshnidae, Stratiomyidae and Atyidae (Fig. 2). The GLM

428 result therefore indicated a positive relationship between $U_{NO_3^--N}$ and the occurrence of

429 these taxa. M.Inv 1 had a significant negative correlation with $U_{NO_3^--N}$. Since M.Inv 1

430 also had a negative loading in the CA results with some other taxa, it was concluded

431 that the combination of these two negative correlations led to positive influences.

432 Thus the higher $U_{NO_3^--N}$ was positively related with the occurrence of taxa such as

433 Culicidae, Mesoveliidae, Nemouridae, Limnephilidae and Planorbidae. These results

434 suggested the occurrence of a particular influence of some macroinvertebrate taxa on

435 $U_{NO_3^--N}$ intensity. At the same time, it also showed the relative contribution of the

436 macroinvertebrate community in the nitrate retention capacities estimated by $U_{NO_3^--N}$,

437 with an influence comparable to that of the abiotic drivers (39 % and 40 %

438 respectively).

Relationship between $U_{NO_3^--N}$ and macroinvertebrate trait modalities

440   In the outcome of the PLS regression model, the first extracted component

441 accounted for 42 % of the variance of the macroinvertebrate functional profile and

442 contributed to 28 % of the variation in $U_{NO_3^--N}$ (Table 5).

443   The modalities identified as having a significant positive association with $U_{NO_3^--N}$

444 were coarse sediment (i.e. flagstones/boulders/cobbles/pebbles) for the substrate

445 preference trait, crawler and 'interstitial' for the locomotion trait, detritus and

446 microphytes for the food trait, and scraper for the feeding habit trait. The association of

447 these modalities is typical of a riverbed with large particle size sediment that forms the

448 hyporheic zone and allows interstitial invertebrates to colonise this macroporous zone.

449 Along with crawlers and scrapers preferentially live on the surface of this coarse

450 sediment where microphytes make up part of the autotrophic biofilm. The modalities

451 exhibiting a negative association with $U_{NO_3^--N}$ were silt or mud with microphytes

452 (substrate preference trait) and fine sediment and detritus (food trait). The association

453 of these modalities describes the habitat of riverbeds or stream beds with low

454 hydrodynamism, where fine sediment accumulates with microphytobenthos in the

455 upper layers and detritus make up part of the deposits.

456   The second component accounted for 36 % of the macroinvertebrate functional

457 profile variance. This is not shown here since it selected similar important modalities as

458 predictors of $U_{NO_3^--N}$ as the first component. This second component explained 20 % of

459 the $U_{NO_3^--N}$ variation.

460 Modalities of the 'maximum potential body size' trait were not selected as

461 important predictors of $U_{NO_3^--N}$ (VIP < 0.7) and were consequently excluded from the

462 final PLS results.

463 These PLS results identified the macroinvertebrate trait modalities, related

464 positively and negatively to $U_{NO_3^--N}$, that allow identification of the community

465 functional profile and substrate composition that have significant influences on nitrate

466 removal and thus on the nitrate removal efficiency of the water quality service.

Discussion

468 The values of nitrate removal rates in the present stream study fall within ranges

469 that have previously been reported (Mulholland *et al.*, 2008). This includes streams

470 with mainly natural land uses and agriculture and few urban areas, which are the same

471 three land-use types that occur in the watersheds of the reaches in the present study.

472 The focus here was on predictors of gross nitrate uptake rate over a short period, and

473 thus the present study did not consider the specific effect of macroinvertebrate N

474 excretion on it nor the detailed influence of varying macroinvertebrate traits on N

475 release and net uptake rates.

Relative contribution of biotic and abiotic drivers to nitrate removal

477 The slug addition method was used to quantify the *in situ* short-term nitrate

478 removal. It includes all the possible in-stream microbial processes that occurred

479 during the experimental period, such as short-term assimilative uptake and permanent

480 removal by denitrification and anammox. $U_{NO_3^--N}$ was found to be strongly regulated

481 by the combination of physicochemical and hydrological factors and by some

482 macroinvertebrate groups. Previous studies of $U_{NO_3^--N}$ drivers have demonstrated the

483   influence of nitrate concentration, temperature and discharge, as well as the biotic

484   influence of microbial community composition and biomass (Simon *et al.*, 2005;

485   Mulholland *et al.*, 2008; Von Schiller *et al.*, 2008). However, none of these studies have

486   combined both types of biotic and abiotic influences together in the same analyses.

487   Furthermore, the biotic drivers generally considered the biofilm and macrophyte

488   composition of the biotic assemblages, without including macroinvertebrate

489   community composition. This study showed an additional independent and significant

490   contribution by the macroinvertebrate community to the microbial processes at the

491   source of the nitrate removal, as quantified by $U_{NO_3^--N}$. This offers a complementary

492   insight into the organisms possibly involved in relationships with the microbial

493   community responsible for nitrogen processing.

494   Abiotic drivers of nitrate removal

495   DOC and NH_4^+ concentrations and temperature were positively correlated with

496    $U_{NO_3^--N}$. DOC is an important source of carbon for stream heterotrophs and occasionally

497   for autotrophs (Bernhardt & Likens, 2011). Previous research has demonstrated how

498   DOC concentrations significantly control nitrate removal, in particular through the

499   in-stream denitrification process (Meyer *et al.*, 2005; Gücker & Pusch, 2006; Peyrard *et*

500   *al.*, 2011). High water temperature may accelerate metabolic processes and

501   consequently nitrate removal (Ortiz *et al.*, 2005).

502   Nitrate concentration was not selected as a predictor of $U_{NO_3^--N}$ by the final model,

503   probably due to the high NO_3^- concentrations in this study (2.2 mg.L^{-1} on average).

504   Under high nitrate concentrations, the microbial pool may become saturated with N,

505   resulting in decreased N-absorbing capacity (Garcia-Ruiz *et al.*, 1998; Kemp & Dodds,

506   2002; Arango *et al.*, 2008; Mulholland *et al.*, 2008). The nitrate concentrations in some

507   reaches in this study might therefore have been too high to allow the microbial

508   communities to sequester additional nutrients.

509   A wide range (0.04-63.7) and relatively high values of As/A (mean= 17.5) were

19

510   observed in this study across different streams. As/A and HRF were found to positively

511   influence $U_{NO_3^--N}$. The transient storage zone has been widely regarded as an important

512   geomorphological feature that has a positive influence on $U_{NO_3^--N}$ of streams (Valett *et*

513   *al.*, 1996; Gücker & Boëchat, 2004; Hall *et al.*, 2009). The spiralling process, which

514   characterises the water flow in the transient zone, facilitates the contact of reactive

515   solutes with a high biotic capacity for biogeochemical processing (Mulholland &

516   DeAngelis, 2000; Runkel, 2007). The significant contribution of transient storage to N

517   removal has also been demonstrated by modelling efforts at reach and watershed scales

518   (Stewart *et al.*, 2011). In contrast, several studies reported no relationship between

519   solute removal and transient storage parameters (e.g. Webster *et al.*, 2003; Niyogi *et al.*,

520   2004; Meyer *et al.*, 2005), probably due to the relatively low values and small range of

521   transient storage in these studies.

522   ## Biotic drivers of nitrate removal

523   No significant independent influence of Chl-*a* on $U_{NO_3^--N}$ was found, although

524   autotrophic biofilm organisms (macro- and microphytes) were likely to contribute to

525   this process. In the same studied reaches, heterotrophic biofilm biomass supported by

526   large transient storage zones may also suggest considerable heterotrophic

527   contributions to the nitrate retention process (Marmonier *et al.* 2012). Thus, it is

528   difficult to detect a probable relationship between surface and autotrophic biofilm

529   biomass alone and nitrate removal.

530   Hierarchical partitioning helped to identify the significant independent influences

531   of the macroinvertebrate community on $U_{NO_3^--N}$ in these streams. Previous laboratory

532   experiments (Mermillod-Blondin *et al.*, 2000; 2003; 2002; Mermillod-Blondin &

533   Rosenberg, 2006; Navel *et al.*, 2011) have already demonstrated a large decrease in

534   nitrate concentrations with sediment depth in down-welling flow-through columns

535   colonised with Oligochaetes or other interstitial macroinvertebrates. The fact that this

536   depletion was more accentuated in the columns with macroinvertebrates than in the

537   columns without fauna suggests the possible enhancement of microbial denitrification

538   under the influence of macroinvertebrate activities. These laboratory experiments

539   using intact macroinvertebrate and microbial assemblages from natural streams

540   sediments may reflect the set of processes that occur in the stream and involve

541   interactions between the two assemblages (Marshall & Hall, 2004). These previous

542   demonstrations allow the examination of the role of macroinvertebrates as possible

543   ecological engineers exerting top-down control on nitrate removal. Stief (2013)

544   explains the effect of benthic macrofauna on nitrate removal by animal-microbe

545   interactions due to sediment burrowing, grazing or symbiosis.

546   In the present study, which was undertaken in natural conditions, the direction of

547   the relationship between macroinvertebrate communities and nitrate removal is hard

548   to demonstrate and may occur simultaneously in several directions. The selected trait

549   profile of the macroinvertebrate community could provide a better understanding of

550   the links that relate the macroinvertebrate community to nitrate removal. These links

551   are summarised in a conceptual model shown in Figure 3 and imply the following:

552   (1) a direct influence of macroinvertebrates on nitrate removal *via* macroinvertebrate

553   feeding on autotrophic and heterotrophic biofilms, promoting top-down control on the

554   microbial community

555   (2) an indirect physical effect by the activities of macroinvertebrates dwelling on or in

556   the sediments, limiting sediment clogging and changing the abiotic interstitial

557   microenvironment

558   (3) an indirect link due to some abiotic conditions (including water quality) that may

559   influence both macroinvertebrate composition and nitrate removal.

560   This last indirect linkage could be the result of the initial improvement in water

561   quality due to natural water quality regulation. Furthermore, macroinvertebrate

562   diversity and nitrate removal may have been controlled by the same abiotic drivers,

563   such as discharge and pollutants, that lead to confounding effects on retention

564   processes and the related biodiversity.

565 Relationships between macroinvertebrate trait profile and nitrate

566 removal

567 The macroinvertebrate community positively associated with $U_{NO_3^--N}$ was

568 characterised by relatively high densities of organisms that live in coarse sediment

569 and exhibit locomotion as crawlers or through interstitial movements in their habitats.

570 In these communities, the organisms positively related to nitrate removal were mainly

571 scrapers using autotrophic biofilm on the surface of the sediment, but also consuming

572 the heterotrophic biofilm and detritus of the interstitial matrix in the sediment column

573 as their main foods (Merritt & Cummins, 2007). These traits are illustrated by the

574 gastropod *Physella* with a positive loading on M.Inv 2 and nemourid stoneflies with a

575 negative loading on M.Inv 1. These results revealed a potential top-down control of

576 scraper macroinvertebrates on biofilm microbes. Indeed, scrapers primarily shear

577 attached algae from autotrophic biofilms, the main food source of macroinvertebrate

578 communities identified as microphytes. Furthermore the potential association of fine

579 and coarse detritus with all types of biofilm agreed with the occurrence of such food

580 sources in the diet of these macroinvertebrates. Both types of autotrophic and

581 heterotrophic biofilm consumption by macroinvertebrates were likely to influence

582 $U_{NO_3^--N}$, although it was difficult to draw conclusions about the main direction of the

583 cross-community relationships between microbial and macro-invertebrate

584 communities that probably exist in both directions: a top-down control of the

585 macroinvertebrates on the biomass and composition of the microbial community and,

586 inversely, a bottom-up effect of available microbial food for the macroinvertebrates.

587 Many arguments can be found to support the indirect contribution of scrapers on

588 nitrate removal by biofilm through the top-down aspect. Indeed, scraping can build

589 and maintain galleries in the biofilms matrix itself (Stief, 2013), as well as in the

590 interstitial sediments, and maintain the microbial diversity mosaic

591 (Mermillod-Blondin *et al.*, 2000; 2003; Law, 2011). Feeding on the biofilm

592   components also prevents the biofilm from clogging the sediments' interstitial pores

593   and fuels the productivity and activity of microbes by favouring nutrient penetration

594   in the whole sediment column (Covich *et al.*, 2004; Stief, 2013). It has been reported

595   that, to some degree, scraping effects may stimulate biofilm regrowth with a high

596   productivity and metabolism (Gasol *et al.*, 2002; Cheever *et al.*, 2011). This differs

597   from some studies in which intense scraping has been shown to substantially decrease

598   biofilm biomass and its nitrate removal capacity (Sabater *et al.*, 2002; Law, 2011).

599   The macroinvertebrate assemblages positively related with nitrate removal prefer

600   to live in coarse sediment instead of silt and muddy substrates. In contrast with coarse

601   sediment, fine sandy sediment and mud was negatively correlated with nitrate

602   removal. This can be explained by the fact that low hydraulic conductivity limits

603   opportunities for water exchange and is a supplementary source of interstitial clogging

604   (Morrice *et al.*, 1997). Moreover, coarse sediment suggests strong hydrological

605   connections between running surface water and interstitial water (advection-dominate

606   system). This allows macroinvertebrate colonisation at depth in the sediment column

607   and enhances the zone of biological influences (Gerino *et al.*, 2003;

608   Mermillod-Blondin & Rosenberg, 2006; Piscart *et al.*, 2011).

609   The locomotion trait modality associated with interstitial macroinvertebrates

610   living in the hyporheic zone was also selected as an important predictor of $U_{NO_3^--N}$.

611   This agreed with previous studies, showing that hyporheic macroinvertebrate

612   assemblages have small but significant effects on nutrient and organic matter

613   processing by changing flow patterns in the interstitial water and associated solute

614   concentrations (Mermillod-Blondin *et al.*, 2000; 2001; Mermillod-Blondin & Gérino,

615   2002; Marshall & Hall, 2004).

616   In summary, the results of this functional trait approach suggest a possible

617   relationship between nitrate removal and macroinvertebrates *via* their top-down

618   feeding control effect on biofilms as well as their dwelling activities in the hyporheic

619   zone that influence microbial capacities. The substrate preferences of

23

620   macroinvertebrate communities are also confirmed to be macro-porous sediment,

621   where interstitial invertebrate communities and the heterotrophic biofilms may

622   contribute to the $U_{NO_3^--N}$ processes, such as in the hyporheic zone. Moreover, these

623   results provide additional information about the influence of hydromorphological

624   factors on $U_{NO_3^--N}$. For example, a higher $U_{NO_3^--N}$ was observed in sites with higher

625   densities of macroinvertebrates that preferred coarse substrates, potentially suggesting

626   that a higher $U_{NO_3^--N}$ was indirectly associated with coarse sediments in these streams.

627   ## Conclusions and perspectives

628   Both abiotic and biotic factors were examined in this study as the main drivers

629   simultaneously of in-stream biotic nitrate removal quantified as $U_{NO_3^--N}$ in *in situ*

630   conditions. Physicochemical factors (e.g. NH_4^+, DOC and temperature) and

631   hydromorphological factors (transient zone) as abiotic drivers and macroinvertebrate

632   assemblages as biotic drivers had both significant and independent influences on

633   $U_{NO_3^--N}$. These results suggest that further studies of the macroinvertebrate

634   communities, in addition to other compartments of the riverine biota, may be

635   necessary to explain the variability of *in situ* nitrate retention. The development of

636   experimental studies in laboratory conditions has previously been required to explore

637   the different hypotheses and links behind the functional relationship between

638   invertebrate diversity and nutrient retention. The present trait-based approach

639   highlighted the potential contribution of macroinvertebrates, such as biofilm grazers,

640   to *in situ* microbial nitrate removal. In particular, invertebrates with specific trait

641   modalities, such as scraping and living in coarse sediment, are likely to make the

642   greatest contribution to this process.

643   The present study suggests that in-stream biodiversity, which contributes to the

644   processes underpinning the ecosystem service of water purification, should not only

645   be confined to the microbial community, but should be seen as a consortium of

646   ecological engineers comprising microbes and macroinvertebrates. Hyporheic zones

647 with coarse sediments would appear to be key habitats for biodiversity and the

648 biogeochemical processes that support nitrate removal as a regulating ecosystem

649 service in streams.

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1009 **Tables**

1010   Table 1. Values of $U_{NO_3^--N}$ and the main characteristics of the study sites, including

1011   catchment, physicochemical characteristics, substrate, hydrology and biofilm factors.

1012   † Percent data were normalised prior to analysis by arcsine $\sqrt{(x)}$ transformation.

1013   * For the denitrification rate, only 15 out of 27 measurements had data records. This is

1014   shown here as a reference, but was not used in the subsequent analysis

		9 streams (n=27)			
		Minimum	Mean	Maximum	Standard deviation
Nitrate uptake rates	$U_{NO_3^--N}$ $(mg.m^{-2}.min^{-1})$	0.04	1.64	10.75	2.39
Catchment†	Catchment area (Km^2)	11.2	53.2	480.0	88.3
	Slope (%)	0.0	11	24	10
	Natural (%)	20.0	51.7	87.4	21.7
	Agricultural (%)	10.8	45.6	79.0	20.4
	Urban (%)	0.0	2.7	20.0	3.9
Physicochemical characteristics	NH_4^+-N $(mg\ N.l^{-1})$	0.003	0.039	0.18	0.048
	NO_3^--N $(mg\ N.l^{-1})$	0.05	2.66	8.98	2.60
	$PO_4^{3-}-P$ $(mg\ P.l^{-1})$	0.003	0.112	0.590	0.163
	DOC $(mg\ C.l^{-1})$	0.68	3.40	7.75	2.04
	Conductivity $(uS.cm^{-1})$	163.9	646.4	1257.5	323.6
	Temperature (°C)	5.20	13.19	22.30	4.13
Substrate size†	Boulders (%)	0	23.5	64.8	18.1
	Cobbles (%)	0	17.0	42.5	13.0
	Pebbles (%)	0	14.1	35.0	11.4
	Gravel (%)	0	29.0	81.5	21.6
	Sand (%)	0	10.1	45.0	11.9
	Silt and mud (%)	0	6.3	76.5	14.8
Hydrological	Depth (m)	0.02	0.11	0.32	0.07

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Macroinvertebrate traits and in-stream nitrate removal

characteristics	As/A	0.04	17.45	63.70	17.37
	HRF	0.39	188.16	974.96	220.57
	Discharge (Q, $m^3.s^{-1}$)	0.001	0.063	0.267	0.073
	Velocity ($m.s^{-1}$)	0.02	0.17	0.50	0.13
	Froude	0.03	0.16	0.43	0.10
	Reynolds	358	17526	73077	18763
Biofilm	Chl-*a* (mg. m^{-2})	1.00	67.0	483.8	96.4
Denitrification rate *	mg $N_2O.m^2.min^{-1}$	0	1.17	4.02	1.29
Macroinvertebrates	Total density (number of individuals per m^2)	548	9205	64912	12955
	Richness	5	17	38	8
	Shannon index	0.35	1.24	2.5	0.58

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1032   Table 2. Selected trait modalities (26 modalities of four biological traits and one

1033   ecological trait) and the rationale (see detailed rationale in the text). Concerning the

1034   modality trade-off, some rare or similar modalities were pooled together into the same

1035   trait (as example '≤0.25 cm' and '>0.25-0.05 cm' were pooled in '≤0.5 cm')

Trait	Modalities	Rationale
Feeding habits	Deposit feeder Shredder Scraper Filter-feeder Predator	Indicating top-down controls of invertebrates on micro-organisms (mostly inside autotrophic and heterotrophic biofilm) or other invertebrates.
Food	Fine sediments and microorganisms Fine detritus (< 1mm) Coarse detritus (> 1mm) Microphytes Macrophytes Dead animal (>= 1mm) Microinvertebrates macroinvertebrates + vertebrates	Indicating main food sources and adding complementary information on the top-down control mechanism. Fine and coarse detritus, as FROM and CPOM, are grouped together with "dead animal" under the general term of detritus in the text. Microphytes are microscopic algae that grow at the top of the sediment.
Maximum potential size	≤0.5 cm > 0.5-1 cm > 1-2 cm > 2-4 cm > 4 cm	The size for consideration corresponds to the final step in the life cycle when the invertebrate is still in the aquatic environment
Locomotion and substrate association	Crawler Burrower Interstitial	Describes dwelling activities: crawler moves slowly with thin legs, more likely on the surface of the sediment, while burrowers dig permanent galleries and live there with bioirrigation. Interstitial refers to one organism that lives and moves in the interstitial medium of the sediment without digging permanent galleries.
Substrate (preference)	Flagstones/boulders/cobbles/pebbles Gravel Sand	Indicating microhabitat compositions, with microphytes being living microscopic algae

Silt and mud	that develop on the surface of
Macrophytes	the sediment layer as
Microphytes	microphytobenthos when
Organic detritus/litter	associated with fine sediment or
	biofilm when associated with
	coarser particles such as
	boulders.

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1059   Table 3. Results of principal component analysis (PCA) for catchment,

1060   physicochemical characteristics, substrate and hydrological factors. The percentage

1061   values on each axis represent the amount of variance explained by each PCA

1062   component. Only important factors are included (loading >0.5). See Table 1 for a

1063   more detailed description of the factors included in each PCA

Extracted component	Code	Variance explained	Positive (+) loading	Negative (-) loading
Catchment axis 1	Catch 1	44 %	Agricultural (0.8), urban (0.7), catchment area (0.5)	Natural (-0.9)
Catchment axis 2	Catch 2	34 %	Catchment area (0.7), urban (0.5)	Slope (-0.6), agricultural (-0.6)
Physicochemical axis 1	Phy-che 1	37 %	Temperature (0.5)	NO_3^--N (-0.9), PO_4^{3-}-N (-0.8), conductivity (-0,6)
Physicochemical axis 2	Phy-che 2	24 %	NH_4^+-N (0.7), DOC (0.7), Temperature (0.5)	
Substrata axis 1	Sub 1	40 %	Silt (0.9), Sand and mud (0.8)	Cobbles (-0.8)
Substrata axis 2	Sub 2	24 %	Pebbles (0.6)	Gravel (-0.9)
Hydrological axis 1	Hydro 1	49 %		Reynolds (-1.0), velocity (-0.9), Q (-0.8), Froude (-0.8)
Hydrological axis 2	Hydro 2	24 %		HRF(-0.9), As/A (-0.9)

1064 Table 4. Step-GLM coefficients and P-values for seven components selected from 11

1065 initial components resulting from PCA and CA analyses. This outcome model is based

1066 on the most representative components that were used as independent variables and on

1067 $U_{NO_3^--N}$ as a dependent variable. D^2 is the total variance of $U_{NO_3^--N}$ explained by this

1068 model. Hierarchical partitioning quantifies the independent influences of each selected

1069 component on $U_{NO_3^--N}$; * indicates statistically significant influences of HP results

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Selected components	Step-GLMs D^2=0.56 (n=27)		Hierarchical partitioning
	Estimate	P value	Independent influence (%)
Phy-che 2	0.54	0.03	18 *
Hydro 1	-0.19	>0.1	5
Hydro 2	-0.45	0.05	22 *
Biofilm Chl-*a*	-2.61	0.06	14
M.Inv 1	-1.0	0.02	16 *
M.Inv 2	0.81	0.07	23 *

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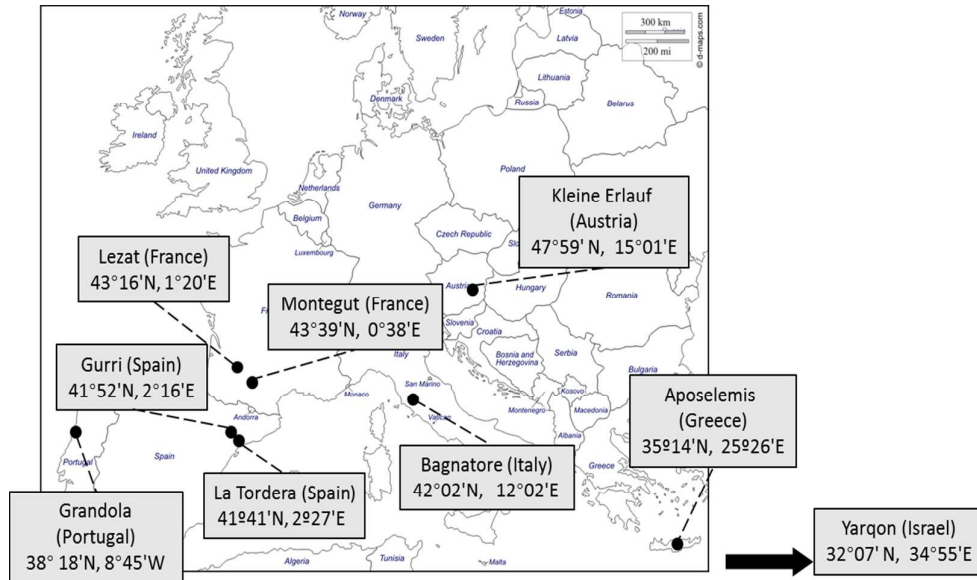
1083 Table 5. Loadings of variable importance in projection (VIP) of the first component

1084 extracted from partial least squares (PLS) regression analysis performed for the nine

1085 streams (n=27), with $U_{NO_3^--N}$ as dependent variables and selected modalities of

1086 macroinvertebrates as independent variables (i.e. 21 modalities of four traits).

1087 Y-weights correspond to loadings of $U_{NO_3^--N}$. VIP >0.7 are in bold. Italicised values

1088 were correlated positively with $U_{NO_3^--N}$

		Loading VIP	Component 1 (R^2 =42%)
Y-weights	+0.28		
Traits	**Variables** **Selected modalities**		
Substrate (preference)	*Flagstones/boulders/cobbles/pebbles*	*0.89*	*0.20*
	Gravel	0.31	0.07
	Silt and mud	**0.93**	**-0.20**
	Sand	0.30	0.06
	Macrophytes	0.55	0.12
	Microphytes	**0.76**	**-0.17**
	Organic detritus/litter	0.13	-0.03
Locomotion and substrate association	*Crawler*	*2.15*	*0.47*
	Burrower	0.34	0.07
	Interstitial	*2.11*	*0.46*
Food	**Fine sediment +microorganism**	**1.12**	**-0.24**
	Detritus	*0.92*	*0.20*
	Microphytes	*1.17*	*0.25*
	Macrophytes	0.19	0.04
	Dead animals	**2.07**	**-0.45**
	Microinvertebrates, macro-invertebrates and vertebrates	0.10	0.02
Feeding habits	Deposit feeder	0.42	0.09
	Shredder	0.63	-0.14
	Scraper	*0.74*	*0.16*
	Filter-feeder	0.42	0.09
	Predator	0.06	0.01

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1090   **Figures**

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1092   Figure 1. The locations of the nine streams investigated

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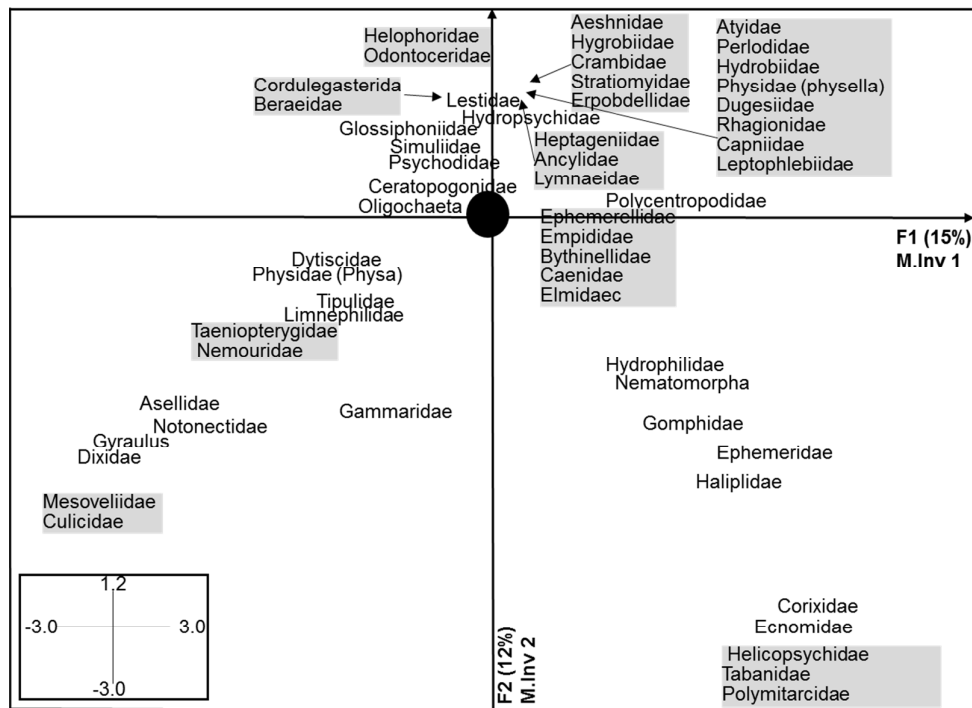
1095   Figure 2. Factorial plane of correspondence analysis (CA) performed on

1096   log-transformed densities. Only species with loadings > 0.5 are shown in this figure.

1097   The black circle represents taxa with loadings below 0.5 in axes 1 and 2. Grey

1098   rectangles group together different taxa with similar loadings. The inset box shows the

1099   scales of the axes. See detail in the appendix

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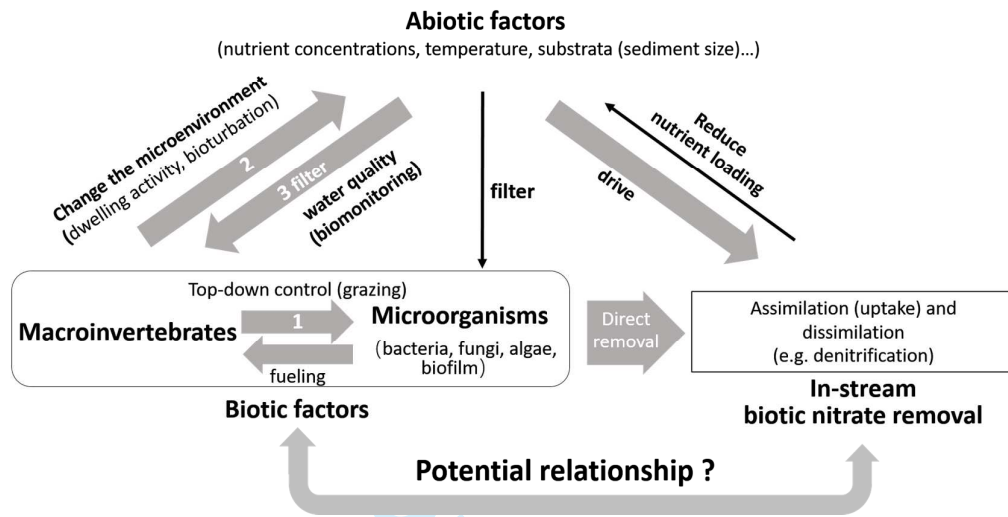
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1104   Figure 3. Schematic of the potential links between macroinvertebrates and in-stream

1105   nitrate removal (the lines in grey are explored in this study)

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1107 **Appendix**

1108 Table 1A. Results of the correspondence analysis (CA) for macroinvertebrate species;

1109 the first component of CA explained 15 % of the variance and the second component

1110 of CA explained 12 % of the variance; only important species are included

1111 (loading >0.5)

Order	Family	Genus/species	Axis 1 loading
Diptera	Culicidae		-2.40
Hemiptera	Mesoveliidae		-2.40
Diptera	Dixidae		-2.26
Gastropod	Planorbidae	Gyraulus sp.	-2.19
Isopod	Asellidae		-1.93
Hemiptera	Notonectidae		-1.74
Plecoptera	Nemouridae		-1.55
Plecoptera	Taeniopterygidae		-1.53
Gastropod	Physidae	Physa sp.	-1.20
Trichoptera	Limnephilidae		-1.04
Coleoptera	Dytiscidae		-0.93
Diptera	Tipulidae	Tipula	-0.86
Amphipod	Gammaridae		-0.67
Oligochaeta			-0.59
Diptera	Empididae		0.51
Ephemeroptera	Ephemerellidae		0.51
Gastropod	Bythinellidae		0.61
Ephemeroptera	Caenidae		0.65
Coleoptera	Elmidae		0.67
Trichoptera	Polycentropodidae		0.88
Coleoptera	Hydrophilidae		1.14
Nematomorpha			1.18
Odonata	Gomphidae		1.30
Coleoptera	Haliplidae (larvae)		1.57
Ephemeroptera			1.63
Trichoptera	Helicopsychidae		1.73
Diptera	Tabanidae		1.73
Ephemeroptera	Polymitarcidae		1.73
Trichoptera	Ecnomidae		1.84
Heteroptera	Corixidae		1.91

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Order	Family	Genus/species	Axis 2 loading
Trichoptera	Helicopsychidae		-2.70
Diptera	Tabanidae		-2.70
Ephemeroptera	Polymitarcidae		-2.70
Trichoptera	Ecnomidae		-2.51
Heteroptera	Corixidae		-2.38
Diptera	Culicidae		-1.88
Hemiptera	Mesoveliidae		-1.88
Coleoptera	Haliplidae (larvae)		-1.63
Diptera	Dixidae		-1.47
Ephemeroptera	Ephemeridae		-1.45
Gastropod	Planorbidae	Gyraulus sp.	-1.40
Odonata	Gomphidae		-1.28
Hemiptera	Notonectidae		-1.27
Amphipod	Gammaridae		-1.20
Isopod	Asellidae		-1.14
Nematomorpha			-1.05
Coleoptera	Hydrophilidae		-0.92
Plecoptera	Nemouridae		-0.76
Plecoptera	Taeniopterygidae		-0.73
Trichoptera	Limnephilidae		-0.59
Diptera	Tipulidae	Tipula	-0.57
Gastropod	Lymnaeidae		0.53
Ephemeroptera	Heptageniidae		0.57
Gastropod	Ancylidae	Ancylus sp.	0.59
Trichoptera	Hydropsychidae		0.59
Ephemeroptera	Leptophlebiidae	Leptophlebiidae Gen. sp.	0.60
Plecoptera	Capniidae	Capnioneura	0.61
Diptera	Rhagionidae	Rhagionidae	0.62
Turbellaria	Dugesiidae	Dugesia	0.62
Gastropod	Physidae	Physella acuta	0.63
Gastropod	Hydrobiidae	Potamopyrgus	0.64
Trichoptera	Beraeidae	Beraea sp.	0.64
Odonata	Lestidae	Lestes sp	0.65
Gastropod	Hydrobiidae	Bythiospeum	0.66
Plecoptera	Perlodidae	Perlodidae	0.67
Odonata	Cordulegasteridae	Cordulegaster	0.67

Macroinvertebrate traits and in-stream nitrate removal

Crustacean	Atyidae	Atyaephyra desmarestii	0.67
Hirudinea	Erpobdellidae		0.71
Diptera	Stratiomyidae		0.75
Lepidoptera	Crambidae	Cataclysta sp	0.79
Coleoptera	Hygrobiidae	Hygrobia sp	0.82
Odonata	Aeshnidae	Boyeria irene	0.85
Trichoptera	Odontoceridae	Odontocerum albicorne	0.90
Coleoptera	Helophoridae	Helophorus sp.	0.90

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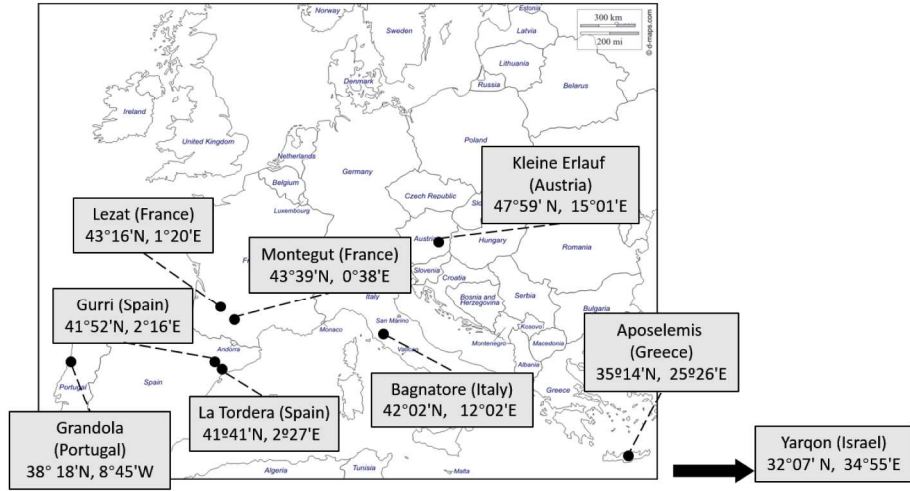
Figure 1. The locations of the nine streams investigated

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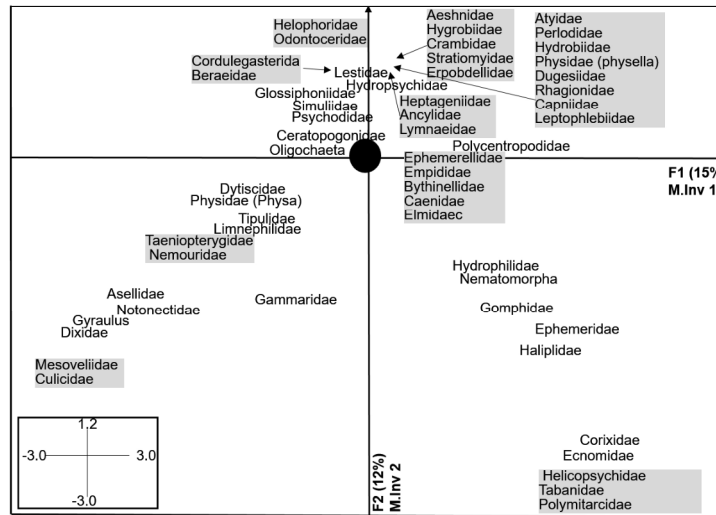50
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Figure. 2. Factorial plane of correspondence analysis (CA) performed on log-transformed densities. Only species with loadings > 0.5 are shown in this figure. The black circle represents taxa with loadings below 0.5 in axes 1 and 2. Grey rectangles group different taxa with similar loadings. The inset box shows the axes scales. See detail in the appendix
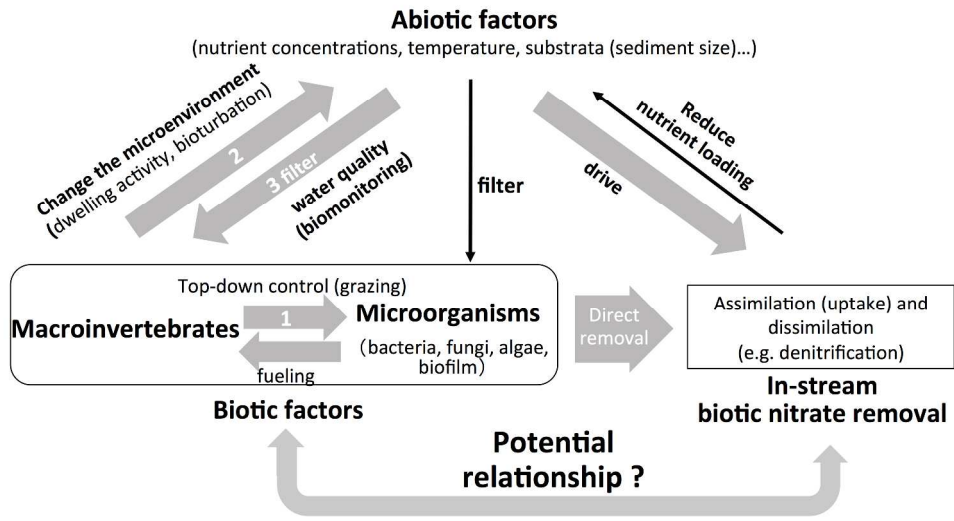
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Figure 3. Schematic of the potential links between macroinvertebrates and in-stream nitrate removal (the lines in grey are explored in this study)

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