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Macroinvertebrate community traits and nitrate removal in stream sediments

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SUMMARY

1. In-stream nitrate removal capacity may be used as a proxy for the ecosystem service of water quality regulation. It is well known that this natural function is driven by abiotic and biotic factors in running water environments. With regard to biotic drivers, most of the literature focuses on the microbial community influences, but there has been very little emphasis on the relationship with the benthic macroinvertebrate community. Since this community feeds on microbial assemblages (autotrophic and/or heterotrophic biofilms) that live on the streambed and in the hyporheic zone of the river, macroinvertebrates also have the potential to influence nitrate removal via its influences on microbiological processes.

2. The objective of this study was to examine the potential relationship between the macroinvertebrate communities and nitrate removal. A dataset of in-stream nitrate removal rates measured in nine third-order streams was analysed. The simultaneous influences of abiotic (hydromorphological, physical and chemical characteristics) and biotic (biofilm and macroinvertebrate) drivers were examined and together explained 56 % of the in-stream nitrate removal variance. An analysis of the independent contributions of each driver showed that abiotic drivers (e.g. ammonium, dissolved organic carbon, temperature and transient zone) contributed 40 % of this nitrate removal variance, while the macroinvertebrate community contributed 39 %.

3. The potential relationship between macroinvertebrates and nitrate removal was subsequently explored using trait-based approaches of the macroinvertebrate community. This method allows for the selection of trait modalities assuming a top-down control of microbial communities by macroinvertebrates, with in-stream abiotic conditions correlated to nitrate removal (assuming that environmental conditions affect macroinvertebrate community composition).

4. The main trait modalities positively correlated with nitrate removal were scraper (feeding habit), flagstones/boulders/cobbles/pebbles (substrate preference), crawler and interstitial (locomotion) and detritus (food). The main modalities negatively

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3 57 correlated with nitrate removal were silt and mud with microphytes (as substrate
4 preference), and with fine sediment with microorganisms, and dead animals (as food
5 sources). These results agreed with the hypothesis of top-down control and enhanced
6 understanding of the influence of hydromorphological factors on nitrate removal.
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11 5. This study highlights the involvement of the macroinvertebrate community in
12 in-stream nitrate processing, and demonstrates the usefulness of applying a functional
13 approach to explaining relationships between biodiversity and ecosystem function.
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20 **Introduction**

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23 Anthropogenic loading of nitrogen into freshwater ecosystems has increased by
24 more than one order of magnitude over the past two decades (Vitousek 1997;
25 Galloway *et al.*, 2004; Ruehl *et al.*, 2007). Streams and rivers are important sinks for
26 nitrogen (Grizzetti *et al.*, 2015), and about half of the nitrogen input is ultimately
27 removed by streams and rivers before flowing into coastal waters (Galloway *et al.*,
28 2004). In-stream nitrogen retention is the set of processes by which nitrogen is stored,
29 transformed and removed from the water column of streams and rivers or stored in
30 biota (Alexander *et al.*, 2000). This retention contributes to the regulation of
31 downstream nitrogen exports. Nitrate is one of the major forms of dissolved inorganic
32 nitrogen in rivers. Nitrate retention may be used as an ecosystem service indicator of
33 water quality regulation (Millennium Ecosystem Assessment 2005; Cardinale, 2011).
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45 A number of processes are involved in in-stream nitrate retention, including
46 abiotic processes such as hydrologic storage (Triska *et al.*, 1989 a, b) and biotic
47 retention (Gücker *et al.*, 2006). Biotic nitrate removal includes assimilatory processes
48 *via* uptake by aquatic plants, algae and fungi, and dissimilatory processes such as
49 denitrification and dissimilatory nitrate reduction to ammonium (DNRA) (Ranalli and
50 Macalady, 2010). These biotic processes are driven by microbial assemblages
51 (bacteria, fungi and algae) as well as by macrophytes in rivers (Pusch *et al.*, 1998;
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3 84 Sabater *et al.*, 2002; Battin *et al.*, 2003; Simon *et al.*, 2005; Ensign & Doyle, 2005;
4
5 85 Teissier *et al.*, 2007; Von Schiller *et al.*, 2008). In running-water ecosystems, these
6
7 86 processes mainly occur on the streambed and in the hyporheic zone (Triska *et al.*,
8
9 87 1989a; b; Fellows *et al.*, 2001; Marti *et al.*, 2004; Fischer, 2005; Argerich *et al.*,
10
11 88 2011). Nitrate removal can be estimated by conducting a pulse addition experiment
12
13 89 based on the nutrient spiralling concept (Newbold *et al.*, 1981; Stream Solute
14
15 90 Workshop, 1990).

17 91 In-stream biotic nitrate removal is controlled by abiotic factors such as nutrient
18
19 92 concentrations (Bernot & Dodds, 2005; Mulholland *et al.*, 2008) and the
20
21 93 hydromorphological features of the stream channel and the hyporheic zone (Gücker &
22
23 94 Boëchat, 2004; Ensign & Doyle, 2006). In-stream nitrate removal by biota is known to
24
25 95 be regulated by the bottom-up control of resources (such as carbon and nutrient
26
27 96 availability) and environmental conditions (Dodds *et al.*, 2002; Roberts & Mulholland,
28
29 97 2007). However, biotic nitrate removal could also be regulated by top-down controls
30
31 98 from consumers, such as macroinvertebrate communities feeding on biofilms
32
33 99 (Wallace & Webster, 1996; Mermillod-Blondin *et al.*, 2003; Covich *et al.*, 2004;
34
35 100 Karlson *et al.*, 2007; Stief, 2013). This top-down control has rarely been explored as a
36
37 101 biotic driver of in-stream nitrate removal (Lawrence *et al.*, 2002; Sabater *et al.*, 2002;
38
39 102 Law, 2011). The top-down control due to macroinvertebrate grazing could allow the
40
41 103 continuous growth of the microbial community in the hyporheic sediment and thus
42
43 104 help prevent clogging of the porous media of sediments where nitrogen
44
45 105 transformations take place. This influence should favour the transfer of surface water
46
47 106 and nutrients into the hyporheic zone and hence promote the interaction between
48
49 107 nitrate and microbial assemblages responsible for nitrate reduction
50
51 108 (Mermillod-Blondin *et al.*, 2000, 2003; Mermillod-Blondin & Rosenberg, 2006;
52
53 109 Mermillod-Blondin, 2011; Stief, 2013). Additionally, macroinvertebrate microhabitat
54
55 110 occupancy, burrowing and bioirrigation (the flushing of burrows with overlying water
56
57 111 by the peristaltic movements of the invertebrates) (Gerino *et al.*, 2003;
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3 112 Mermillod-Blondin *et al.*, 2003; Schaller *et al.*, 2014) in streambed sediments can
4
5 113 change the physical structure of the abiotic microenvironment and modify the spatial
6
7 114 distribution of the redox conditions in sediments (Aller, 2004; Meysman *et al.*, 2006).
8

9 The research referred to above has mainly been based on laboratory experiments,
10
11 116 and the relationship between macroinvertebrate communities and nitrate retention in
12
13 117 the field requires further exploration. There are two reasons why examination of the
14
15 118 macroinvertebrate community as a potential driver of in-stream nitrate removal is
16
17 119 warranted:

- 18
19 120 (i) There is a strong association of many macroinvertebrate taxa with the streambed
20
21 121 and hyporheic substrata, which are recognised as major sites of biogeochemical
22
23 122 reactions in streams (Giere, 2009)
24
25 123 (ii) the sensitivity of macroinvertebrates to in-stream environmental conditions
26
27 124 (Rosenberg & Resh, 1993; Statzner & Bêche, 2010; Menezes *et al.*, 2010) is likely to
28
29 125 affect in-stream nitrate retention *via* the community compositions.
30

31 126 Although taxonomy-based metrics are widely recognised as being useful as
32
33 127 bio-indicators, they are unlikely to adequately mirror ecosystem functioning (Sandin
34
35 128 & Solimini, 2009). Instead, a trait-based approach using multiple biological and
36
37 129 ecological traits of organisms (e.g. mobility, feeding type, size, lifespan of aquatic
38
39 130 macroinvertebrates) is more likely to reflect ecological processes and ecosystem
40
41 131 functioning (Hooper *et al.*, 2005; Bremner *et al.*, 2006; Colas *et al.*, 2013). This
42
43 132 approach allows (i) a description of macroinvertebrate community responses to many
44
45 133 abiotic and biotic stressors, (ii) a reduction in uncertainties related to seasonal effects
46
47 134 by integrating the variations in environmental conditions over time, such as seasonal
48
49 135 variability, and (iii) a more direct and easier detection of the mechanism by which the
50
51 136 community composition influences the ecosystem functioning. Furthermore, the trait
52
53 137 composition of macroinvertebrate communities is comparable across large spatial
54
55 138 scales, and even across ecoregions harbouring communities of a different taxonomic
56
57 139 composition (Dolédéc *et al.*, 2006; Feio *et al.*, 2010). Consequently, there is
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1
2
3 140 increasing interest in trait-based approaches to improve assessments of stream health
4
5 141 and link diversity to ecosystem functions (e.g. McKie *et al.*, 2008; Colas *et al.*, 2013;
6
7 142 2014; Frainer *et al.*, 2014).

8
9 143 The objective of this study was to explore the influence of both abiotic (i.e.
10
11 144 physico-chemical and hydromorphological) and biotic (i.e. biofilm and
12
13 145 macroinvertebrate) drivers on in-stream nitrate removal using a dataset from nine
14
15 146 third-order streams. A variance partitioning approach was used to assess the
16
17 147 independent contributions of biotic and abiotic drivers to nitrate removal, before
18
19 148 focusing on the potential relationship between macroinvertebrate and biotic nitrate
20
21 149 removal using trait-based approaches. It was hypothesised that the biological traits of
22
23 150 macroinvertebrate communities reflect a combination of environmental conditions
24
25 151 (examples of mobility and substrate preferences) and some functional relationships
26
27 152 between macroinvertebrates and biofilm microbes (examples of feeding habits and
28
29 153 food resources) that together influence the local nitrate retention rate. This
30
31 154 information should depict the processes that are controlled by macroinvertebrate
32
33 155 activity and related to nitrate removal according to the top-down control assumption.

34 35 36 156 **Methods**

37 38 39 157 **Data collection**

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41
42 158 The data were collected *in situ* as part of the EU-funded STREAMES project
43
44 159 (STream REAch Management, an Expert System,
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46 160 http://cordis.europa.eu/project/rcn/54747_en.html). The objective of this project was
47
48 161 to identify the relationships between in-stream nutrient retention capacity and
49
50 162 potential biotic and abiotic factors in a set of streams in different ecoregions. The
51
52 163 STREAMES project originally involved 11 third-order streams across seven European
53
54 164 countries plus Israel. In each stream, several *in situ* experimental measurements were
55
56 165 performed to cover contrasting hydrological conditions during 2002.
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4 166 For the specific objective of the present study, only the dates on which there
5
6 167 were simultaneous *in situ* records in the stream reaches of nitrate uptake and physical,
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8 168 chemical, hydrological, biofilm and macroinvertebrate characteristics were selected.
9
10 169 Only reaches located upstream from wastewater treatment plants (WWTP) were
11
12 170 chosen to avoid disturbance from local outflows into the river water. Finally, after this
13
14 171 data selection, 27 measurements from nine streams (Fig. 1) were included in this
15
16 172 study (see Table 1).

17
18 173 *In situ* and laboratory measurements followed common procedure guidelines,
19
20 174 ensuring comparability of all data (Gorden *et al.*, 1992; Clesceri, 1998; Gordon *et al.*,
21
22 175 2004; Vellido *et al.*, 2007; Morais *et al.*, 2009). During each *in situ* study, hydrology,
23
24 176 substrate and macroinvertebrate communities were measured in six equidistant
25
26 177 transects (with an average transect length of 3.5 m). Three water samples were
27
28 178 collected in each transect, immediately filtered through pre-combusted glass
29
30 179 microfibre filters (Whatman GF/F, 0.7 μ m) and stored in coolers before chemical
31
32 180 analysis. Three surface sediment samples in each transect were also collected (18
33
34 181 samples per reach) for quantification of biofilm chlorophyll a concentration after
35
36 182 pigment extraction from the sediment.

37 38 183 Nitrate removal measurements

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40
41 184 Nitrate addition experiments, using the slug addition technique, were conducted
42
43 185 to estimate the retention capacity for nitrate (NO₃-N) along each stream reach over a
44
45 186 short period of time, according to the procedure of Gorden *et al.* (1992) and applied
46
47 187 by Ruggiero *et al.* (2006) and Sánchez-Pérez *et al.* (2009). The estimation of the
48
49 188 stream nutrient retention rate for NO₃-N were made on each sampling date with a
50
51 189 solute addition of a known quantity of nutrient (NO₃-N) into the stream.
52
53 190 Simultaneously, a conservative element (Cl⁻) was added as a tracer to allow
54
55 191 downstream corrections for any dilution that may occur along the reach. Nitrate (as
56
57 192 NaNO₃ or KNO₃) and the conservative tracer (as NaCl) were added at the same time
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59
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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 ($\text{mg} \cdot \text{L}^{-1} \cdot \text{s}^{-1}$) of nitrate and NaCl were then used to
198 calculate the nutrient mass retained (mg). The nutrient uptake rate at experimental
199 level ($U_{\text{exp}}, \text{mg} \cdot \text{m}^{-2} \cdot \text{min}^{-1}$) 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 S_w (m) was the average distance travelled by a nutrient ion before
202 uptake, therefore estimated as:

$$203 \quad S_w = ([\text{Nut}]_b \cdot Q) / (U_{\text{exp}} \cdot w)$$

204 where $[\text{Nut}]_b$ is the nutrient background concentration ($\text{mg} \cdot \text{L}^{-1}$), Q is the discharge
205 ($\text{m}^3 \cdot \text{s}^{-1}$) and w (m) is the average stream width of the reach. The first-order uptake rate
206 coefficient (K_c, m^{-1}) was calculated by:

$$207 \quad K_c = v / S_w$$

208 where v is the stream water average velocity ($\text{m} \cdot \text{s}^{-1}$). Uptake velocity ($V_f, \text{m} \cdot \text{s}^{-1}$; the
209 vertical velocity at which nutrients move to the stream bottom) was estimated from:

$$210 \quad V_f = K_c \cdot d$$

211 where d is the mean stream depth. The gross nutrient uptake rate at ambient level (U,
212 $\text{mg} \cdot \text{m}^{-2} \cdot \text{min}^{-1}$) was calculated from:

$$213 \quad U = V_f \cdot [\text{Nut}]_b$$

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. " $U_{\text{NO}_3^- \cdot \text{N}}$ " 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).

221 Abiotic factors

222 Catchment factors

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

230 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 (A_s/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

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 (S_w) and this variable reflects the potential transient storage
249 zone effect (Morrice *et al.*, 1997).

250 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).

255 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).

262 Biotic factors

263 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

1
2
3 297 and deposit feeders for interstitial biofilm (Statzner & Bêche, 2010). The ‘food’
4
5 298 modalities supply additional information about the feeding habits, with the modalities
6
7 299 ‘fine sediment and micro-organisms’ being associated with deposit feeders that ingest
8
9 300 sediment. The modalities of microphytes are related to microscopic algae, such as
10
11 301 diatoms, that can live in surface biofilm covering large or fine particles and may be
12
13 302 ingested by scrapers or deposit feeders respectively. The ‘locomotion and substrate
14
15 303 association’ trait was selected to reveal the type of movement of macroinvertebrates
16
17 304 above the streambed or inside the hyporheic sediment. The ‘body size’ trait was also
18
19 305 included since large animals can have a more significant effect on ecosystem function
20
21 306 than smaller ones (Basset *et al.*, 2004; Badosa *et al.*, 2006; Brucet *et al.*, 2006; Gascón
22
23 307 *et al.*, 2009). The ecological trait ‘substrate preference’ was included to encompass
24
25 308 potential microhabitat occupancy by macroinvertebrates. All these macroinvertebrate
26
27 309 traits have already been reported as responding to chemical and hydromorphological
28
29 310 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

1
2
3 324 combined as predictors. A total of 11 predictors were used (i.e. Catch 1+ Catch 2 +
4
5 325 Phy-che 1+ Phy-che 2+ Sub 1 + Sub 2 + Hydro 1 +Hydro 2+ Biofilm Chl-*a* +
6
7 326 M.Inv1+ M.Inv2; see Table 3 and Fig. 2 for details). $U_{NO_3^-:N}$ as a dependent variable
8
9 327 was previously log-transformed to fulfil normality.

10
11 328 The D^2 of each model was calculated to account for the amount of deviance
12
13 329 according to the formula proposed by Guisan and Zimmermann (2000) (Eq. (1)):

14
15 330
$$D^2 = (\text{model}\$null \cdot \text{deviance} - \text{model}\$deviance) / \text{model}\$null \cdot \text{deviance} \quad (1)$$

16
17 331 The D^2 of GLMs is the equivalent of the R-squared value of linear models that
18
19 332 measures the proportion of variation accounted for by the model. Model checking
20
21 333 included homogeneity of variance and normal distribution of model residuals.

22
23 334 The relative importance of each predictor in the best-fitted model was then
24
25 335 examined using hierarchical partitioning (HP). A randomisation test, which was based
26
27 336 on the upper 0.95 confidence limit, was then run on the hierarchical partitioning
28
29 337 results to provide statistical significance (Nally, 2002). HP determined the
30
31 338 independent contribution of each predictor to the response variable and separated it
32
33 339 from the joint contribution resulting from the correlation with other variables. This
34
35 340 enabled a ranking of the importance of the covariates in explaining the response
36
37 341 variable independently of the other covariates.

38
39 342 For trait-based analyses, the mean functional trait profiles of the communities
40
41 343 were calculated from taxonomic data for each measurement using fuzzy-coded
42
43 344 biological and ecological traits (Chevenet *et al.*, 1994) described for each taxon from
44
45 345 the literature. Fuzzy coding uses positive scores (between 0 and 3 or 5) to describe the
46
47 346 affinity of a species for different modalities of a given trait, accounting for phenotypic
48
49 347 and ecological preference variability among taxa. The fuzzy coding procedure helps
50
51 348 to extract different types and levels of information available for different taxa
52
53 349 (Chevenet *et al.*, 1994), addressing spatial or temporal differences in the traits of a
54
55 350 given taxon (Statzner & Bêche, 2010). The mean weighted (by log-transformed
56
57 351 densities) trait profiles of community assemblages were then calculated for each

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2
3 352 measurement and expressed as relative density distributions of trait categories within
4
5 353 the assemblages (Thioulouse *et al.*, 1997). Partial least squares (PLS) regressions
6
7 354 (Abdi, 2003) were then carried out to identify macroinvertebrate trait modalities that
8
9 355 significantly predicted $U_{\text{NO}_3^--\text{N}}$. PLS was particularly suitable for this case because (i)
10
11 356 there were few replicates ($n=27$) and several predictors ($n=21$) and (ii) many
12
13 357 predictors showed high collinearity (Carrascal *et al.*, 2009). PLS reduced a set of
14
15 358 predictors to a few components that had maximum covariance with the response
16
17 359 variable. These components were defined as a linear combination of original variables,
18
19 360 so the original multi-dimensionality was reduced to a smaller number of components.
20
21 361 PLS analysis generated variable importance in projection (VIP) values, as well as the
22
23 362 variance (R^2) explained by each of the two components. VIP values reflect the
24
25 363 importance of each predicted variable of the model, with $\text{VIP} > 0.7$ indicating
26
27 364 important predictors (Eriksson, 1999).

28
29 365 For all steps in the statistical analysis, 'ade' (Chessel *et al.*, 2012), 'MASS',
30
31 366 'hier.part' (Walsh *et al.*, 2013) and PLS (Mevik & Wehrens, 2007) packages in R
32
33 367 software (R development Core Team, 2011) were used.

368 **Results**

369 Nitrate uptake rate ($U_{\text{NO}_3^--\text{N}}$) and environmental characteristics

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41
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43 370 Taking all the measurements into consideration, $U_{\text{NO}_3^--\text{N}}$ ranged from 0.04 to 10.75
44
45 371 $\text{mg}\cdot\text{m}^{-2}\cdot\text{min}^{-1}$, with a mean value equal to $1.64 \pm 2.39 \text{ mg}\cdot\text{m}^{-2}\cdot\text{min}^{-1}$ (standard
46
47 372 deviation) (Table 1). The catchment size of the investigated streams ranged from 11.2
48
49 373 to 480.0 km^2 (Table 1). The average catchment slope was 10 %. The studied
50
51 374 catchments included wide ranges of natural (20-87 %) and agricultural (11-79 %) land
52
53 375 uses. Urban land use accounted for less than 20 % in all the catchments (Table 1). The
54
55 376 first axis of the PCA performed on catchment variables accounted for 44 % of the
56
57 377 variance and indicated a gradient from natural to agriculture-dominated catchments.
58
59
60

1
2
3 378 The second axis (34 % of the variance) indicated a gradient from urban to
4
5 379 agriculture-dominated catchments (Table 3).

6
7 380 Concentrations (min - max) of phosphate ($0.003-8.2 \text{ mg PO}_4^{3-}\text{-P. L}^{-1}$), nitrate
8
9 381 ($0.05-8.98 \text{ mg NO}_3^-\text{-N. L}^{-1}$), dissolved organic carbon ($0.55-21.9 \text{ mg C. L}^{-1}$) and
10
11 382 ammonium ($0.003-0.18 \text{ mg NH}_4^+\text{-N. L}^{-1}$) spanned wide ranges. Water temperature
12
13 383 and conductivity ranged from 5.2 to 22.0 °C and 164 to 1258 uS.cm^{-1} respectively
14
15 384 (Table 2). The first axis (Phy-che 1) of the PCA, performed on the water
16
17 385 physicochemical variables and explaining 37 % of the variance, was negatively
18
19 386 related to $\text{NO}_3^-\text{-N}$, $\text{PO}_4^{3-}\text{-P}$ and conductivity. The second axis (Phy-che 2 explaining
20
21 387 24 % of the variance) was positively related to $\text{NH}_4^+\text{-N}$ (0.7), DOC (0.7) and
22
23 388 temperature (0.5) (Table 3).

24
25 389 Depth ($0.02-0.32 \text{ m}$), velocity ($0.02-0.50 \text{ m}^3. \text{ s}^{-1}$) and Froude ($0.03-0.43$) varied
26
27 390 by over an order of magnitude between the studied streams. Discharges ($0.001-0.267$
28
29 391 $\text{m}^3. \text{ s}^{-1}$), As/A ratios ($0.04-63.70$), HRF ($0.4-975.0$) and Reynolds ($358-73077$)
30
31 392 spanned wide ranges (Table 1). The first axis of the hydrological PCA (Hydro 1) was
32
33 393 representative (49 % of the variability) of a gradient of hydromorphological features
34
35 394 (Reynolds, velocity, Q and Froude). The second axis (Hydro 2), accounting for 24 %
36
37 395 of the variability, was negatively related with the transient storage zone variables (i.e.
38
39 396 HRF and As/A) (Table 3).

40
41 397 The substrata in these streams had different compositions (Table 1), as depicted
42
43 398 by the substrate size PCA. The first axis, which accounted for 40 % of the total
44
45 399 substrate variability, indicated the gradient of sediment granulometry (Table 3).

46
47 400 The third components in each PCA accounted for less than 20 % of the variance
48
49 401 and are not shown here.

50
51 402 Large variations in the concentration of biofilm chlorophyll a were observed
52
53 403 between the streams ($1 - 484 \text{ mg. m}^{-2}$) (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

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3 430 also had a negative loading in the CA results with some other taxa, it was concluded
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5 431 that the combination of these two negative correlations led to positive influences.
6
7 432 Thus the higher $U_{NO_3^-N}$ was positively related with the occurrence of taxa such as
8
9 433 Culicidae, Mesoveliidae, Nemouridae, Limnephilidae and Planorbidae. These results
10
11 434 suggested the occurrence of a particular influence of some macroinvertebrate taxa on
12
13 435 $U_{NO_3^-N}$ intensity. At the same time, it also showed the relative contribution of the
14
15 436 macroinvertebrate community in the nitrate retention capacities estimated by $U_{NO_3^-N}$,
16
17 437 with an influence comparable to that of the abiotic drivers (39 % and 40 %
18
19 438 respectively).

22 439 Relationship between $U_{NO_3^-N}$ and macroinvertebrate trait modalities

25
26 440 In the outcome of the PLS regression model, the first extracted component
27
28 441 accounted for 42 % of the variance of the macroinvertebrate functional profile and
29
30 442 contributed to 28 % of the variation in $U_{NO_3^-N}$ (Table 5).

31
32 443 The modalities identified as having a significant positive association with $U_{NO_3^-N}$
33
34 444 were coarse sediment (i.e. flagstones/boulders/cobbles/pebbles) for the substrate
35
36 445 preference trait, crawler and 'interstitial' for the locomotion trait, detritus and
37
38 446 microphytes for the food trait, and scraper for the feeding habit trait. The association of
39
40 447 these modalities is typical of a riverbed with large particle size sediment that forms the
41
42 448 hyporheic zone and allows interstitial invertebrates to colonise this macroporous zone.
43
44 449 Along with crawlers and scrapers preferentially live on the surface of this coarse
45
46 450 sediment where microphytes make up part of the autotrophic biofilm. The modalities
47
48 451 exhibiting a negative association with $U_{NO_3^-N}$ were silt or mud with microphytes
49
50 452 (substrate preference trait) and fine sediment and detritus (food trait). The association
51
52 453 of these modalities describes the habitat of riverbeds or stream beds with low
53
54 454 hydrodynamism, where fine sediment accumulates with microphytobenthos in the
55
56 455 upper layers and detritus make up part of the deposits.

57
58 456 The second component accounted for 36 % of the macroinvertebrate functional

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3 457 profile variance. This is not shown here since it selected similar important modalities as
4
5 458 predictors of $U_{NO_3^-N}$ as the first component. This second component explained 20 % of
6
7 459 the $U_{NO_3^-N}$ variation.

8
9 460 Modalities of the ‘maximum potential body size’ trait were not selected as
10
11 461 important predictors of $U_{NO_3^-N}$ (VIP < 0.7) and were consequently excluded from the
12
13 462 final PLS results.

14
15 463 These PLS results identified the macroinvertebrate trait modalities, related
16
17 464 positively and negatively to $U_{NO_3^-N}$, that allow identification of the community
18
19 465 functional profile and substrate composition that have significant influences on nitrate
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21 466 removal and thus on the nitrate removal efficiency of the water quality service.

22 23 24 467 **Discussion**

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26
27 468 The values of nitrate removal rates in the present stream study fall within ranges
28
29 469 that have previously been reported (Mulholland *et al.*, 2008). This includes streams
30
31 470 with mainly natural land uses and agriculture and few urban areas, which are the same
32
33 471 three land-use types that occur in the watersheds of the reaches in the present study.
34
35 472 The focus here was on predictors of gross nitrate uptake rate over a short period, and
36
37 473 thus the present study did not consider the specific effect of macroinvertebrate N
38
39 474 excretion on it nor the detailed influence of varying macroinvertebrate traits on N
40
41 475 release and net uptake rates.

42 43 44 476 **Relative contribution of biotic and abiotic drivers to nitrate removal**

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46
47 477 The slug addition method was used to quantify the *in situ* short-term nitrate
48
49 478 removal. It includes all the possible in-stream microbial processes that occurred
50
51 479 during the experimental period, such as short-term assimilative uptake and permanent
52
53 480 removal by denitrification and anammox. $U_{NO_3^-N}$ was found to be strongly regulated
54
55 481 by the combination of physicochemical and hydrological factors and by some
56
57 482 macroinvertebrate groups. Previous studies of $U_{NO_3^-N}$ drivers have demonstrated the
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3 483 influence of nitrate concentration, temperature and discharge, as well as the biotic
4
5 484 influence of microbial community composition and biomass (Simon *et al.*, 2005;
6
7 485 Mulholland *et al.*, 2008; Von Schiller *et al.*, 2008). However, none of these studies have
8
9 486 combined both types of biotic and abiotic influences together in the same analyses.
10
11 487 Furthermore, the biotic drivers generally considered the biofilm and macrophyte
12
13 488 composition of the biotic assemblages, without including macroinvertebrate
14
15 489 community composition. This study showed an additional independent and significant
16
17 490 contribution by the macroinvertebrate community to the microbial processes at the
18
19 491 source of the nitrate removal, as quantified by $U_{NO_3^-N}$. This offers a complementary
20
21 492 insight into the organisms possibly involved in relationships with the microbial
22
23 493 community responsible for nitrogen processing.

26 494 Abiotic drivers of nitrate removal

27
28
29
30 495 DOC and NH_4^+ concentrations and temperature were positively correlated with
31
32 496 $U_{NO_3^-N}$. DOC is an important source of carbon for stream heterotrophs and occasionally
33
34 497 for autotrophs (Bernhardt & Likens, 2011). Previous research has demonstrated how
35
36 498 DOC concentrations significantly control nitrate removal, in particular through the
37
38 499 in-stream denitrification process (Meyer *et al.*, 2005; Gücker & Pusch, 2006; Peyrard *et*
39
40 500 *al.*, 2011). High water temperature may accelerate metabolic processes and
41
42 501 consequently nitrate removal (Ortiz *et al.*, 2005).

43
44 502 Nitrate concentration was not selected as a predictor of $U_{NO_3^-N}$ by the final model,
45
46 503 probably due to the high NO_3^- concentrations in this study (2.2 mg.L^{-1} on average).
47
48 504 Under high nitrate concentrations, the microbial pool may become saturated with N,
49
50 505 resulting in decreased N-absorbing capacity (Garcia-Ruiz *et al.*, 1998; Kemp & Dodds,
51
52 506 2002; Arango *et al.*, 2008; Mulholland *et al.*, 2008). The nitrate concentrations in some
53
54 507 reaches in this study might therefore have been too high to allow the microbial
55
56 508 communities to sequester additional nutrients.

57 509 A wide range (0.04-63.7) and relatively high values of As/A (mean= 17.5) were

1
2
3 510 observed in this study across different streams. As/A and HRF were found to positively
4
5 511 influence $U_{NO_3^-N}$. The transient storage zone has been widely regarded as an important
6
7 512 geomorphological feature that has a positive influence on $U_{NO_3^-N}$ of streams (Valett *et*
8
9 513 *al.*, 1996; Gücker & Boëchat, 2004; Hall *et al.*, 2009). The spiralling process, which
10
11 514 characterises the water flow in the transient zone, facilitates the contact of reactive
12
13 515 solutes with a high biotic capacity for biogeochemical processing (Mulholland &
14
15 516 DeAngelis, 2000; Runkel, 2007). The significant contribution of transient storage to N
16
17 517 removal has also been demonstrated by modelling efforts at reach and watershed scales
18
19 518 (Stewart *et al.*, 2011). In contrast, several studies reported no relationship between
20
21 519 solute removal and transient storage parameters (e.g. Webster *et al.*, 2003; Niyogi *et al.*,
22
23 520 2004; Meyer *et al.*, 2005), probably due to the relatively low values and small range of
24
25 521 transient storage in these studies.

28 522 Biotic drivers of nitrate removal

31
32 523 No significant independent influence of Chl-*a* on $U_{NO_3^-N}$ was found, although
33
34 524 autotrophic biofilm organisms (macro- and microphytes) were likely to contribute to
35
36 525 this process. In the same studied reaches, heterotrophic biofilm biomass supported by
37
38 526 large transient storage zones may also suggest considerable heterotrophic
39
40 527 contributions to the nitrate retention process (Marmonier *et al.* 2012). Thus, it is
41
42 528 difficult to detect a probable relationship between surface and autotrophic biofilm
43
44 529 biomass alone and nitrate removal.

45
46 530 Hierarchical partitioning helped to identify the significant independent influences
47
48 531 of the macroinvertebrate community on $U_{NO_3^-N}$ in these streams. Previous laboratory
49
50 532 experiments (Mermillod-Blondin *et al.*, 2000; 2003; 2002; Mermillod-Blondin &
51
52 533 Rosenberg, 2006; Navel *et al.*, 2011) have already demonstrated a large decrease in
53
54 534 nitrate concentrations with sediment depth in down-welling flow-through columns
55
56 535 colonised with Oligochaetes or other interstitial macroinvertebrates. The fact that this
57
58 536 depletion was more accentuated in the columns with macroinvertebrates than in the

1
2
3 537 columns without fauna suggests the possible enhancement of microbial denitrification
4
5 538 under the influence of macroinvertebrate activities. These laboratory experiments
6
7 539 using intact macroinvertebrate and microbial assemblages from natural streams
8
9 540 sediments may reflect the set of processes that occur in the stream and involve
10
11 541 interactions between the two assemblages (Marshall & Hall, 2004). These previous
12
13 542 demonstrations allow the examination of the role of macroinvertebrates as possible
14
15 543 ecological engineers exerting top-down control on nitrate removal. Stief (2013)
16
17 544 explains the effect of benthic macrofauna on nitrate removal by animal-microbe
18
19 545 interactions due to sediment burrowing, grazing or symbiosis.

20
21 546 In the present study, which was undertaken in natural conditions, the direction of
22
23 547 the relationship between macroinvertebrate communities and nitrate removal is hard
24
25 548 to demonstrate and may occur simultaneously in several directions. The selected trait
26
27 549 profile of the macroinvertebrate community could provide a better understanding of
28
29 550 the links that relate the macroinvertebrate community to nitrate removal. These links
30
31 551 are summarised in a conceptual model shown in Figure 3 and imply the following:
32
33 552 (1) a direct influence of macroinvertebrates on nitrate removal *via* macroinvertebrate
34
35 553 feeding on autotrophic and heterotrophic biofilms, promoting top-down control on the
36
37 554 microbial community
38
39 555 (2) an indirect physical effect by the activities of macroinvertebrates dwelling on or in
40
41 556 the sediments, limiting sediment clogging and changing the abiotic interstitial
42
43 557 microenvironment
44
45 558 (3) an indirect link due to some abiotic conditions (including water quality) that may
46
47 559 influence both macroinvertebrate composition and nitrate removal.

48
49 560 This last indirect linkage could be the result of the initial improvement in water
50
51 561 quality due to natural water quality regulation. Furthermore, macroinvertebrate
52
53 562 diversity and nitrate removal may have been controlled by the same abiotic drivers,
54
55 563 such as discharge and pollutants, that lead to confounding effects on retention
56
57 564 processes and the related biodiversity.

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4 565 Relationships between macroinvertebrate trait profile and nitrate
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6 566 removal
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10 567 The macroinvertebrate community positively associated with $U_{NO_3^-N}$ was
11 568 characterised by relatively high densities of organisms that live in coarse sediment
12 569 and exhibit locomotion as crawlers or through interstitial movements in their habitats.
13
14 570 In these communities, the organisms positively related to nitrate removal were mainly
15
16 571 scrapers using autotrophic biofilm on the surface of the sediment, but also consuming
17 572 the heterotrophic biofilm and detritus of the interstitial matrix in the sediment column
18 573 as their main foods (Merritt & Cummins, 2007). These traits are illustrated by the
19 574 gastropod *Physella* with a positive loading on M.Inv 2 and nemourid stoneflies with a
20 575 negative loading on M.Inv 1. These results revealed a potential top-down control of
21 576 scraper macroinvertebrates on biofilm microbes. Indeed, scrapers primarily shear
22 577 attached algae from autotrophic biofilms, the main food source of macroinvertebrate
23 578 communities identified as microphytes. Furthermore the potential association of fine
24 579 and coarse detritus with all types of biofilm agreed with the occurrence of such food
25 580 sources in the diet of these macroinvertebrates. Both types of autotrophic and
26 581 heterotrophic biofilm consumption by macroinvertebrates were likely to influence
27 582 $U_{NO_3^-N}$, although it was difficult to draw conclusions about the main direction of the
28 583 cross-community relationships between microbial and macro-invertebrate
29 584 communities that probably exist in both directions: a top-down control of the
30 585 macroinvertebrates on the biomass and composition of the microbial community and,
31 586 inversely, a bottom-up effect of available microbial food for the macroinvertebrates.
32
33 587 Many arguments can be found to support the indirect contribution of scrapers on
34 588 nitrate removal by biofilm through the top-down aspect. Indeed, scraping can build
35 589 and maintain galleries in the biofilms matrix itself (Stief, 2013), as well as in the
36 590 interstitial sediments, and maintain the microbial diversity mosaic
37 591 (Mermillod-Blondin *et al.*, 2000; 2003; Law, 2011). Feeding on the biofilm
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3 592 components also prevents the biofilm from clogging the sediments' interstitial pores
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5 593 and fuels the productivity and activity of microbes by favouring nutrient penetration
6
7 594 in the whole sediment column (Covich *et al.*, 2004; Stief, 2013). It has been reported
8
9 595 that, to some degree, scraping effects may stimulate biofilm regrowth with a high
10
11 596 productivity and metabolism (Gasol *et al.*, 2002; Cheever *et al.*, 2011). This differs
12
13 597 from some studies in which intense scraping has been shown to substantially decrease
14
15 598 biofilm biomass and its nitrate removal capacity (Sabater *et al.*, 2002; Law, 2011).

16
17 599 The macroinvertebrate assemblages positively related with nitrate removal prefer
18
19 600 to live in coarse sediment instead of silt and muddy substrates. In contrast with coarse
20
21 601 sediment, fine sandy sediment and mud was negatively correlated with nitrate
22
23 602 removal. This can be explained by the fact that low hydraulic conductivity limits
24
25 603 opportunities for water exchange and is a supplementary source of interstitial clogging
26
27 604 (Morrice *et al.*, 1997). Moreover, coarse sediment suggests strong hydrological
28
29 605 connections between running surface water and interstitial water (advection-dominate
30
31 606 system). This allows macroinvertebrate colonisation at depth in the sediment column
32
33 607 and enhances the zone of biological influences (Gerino *et al.*, 2003;
34
35 608 Mermillod-Blondin & Rosenberg, 2006; Piscart *et al.*, 2011).

36
37 609 The locomotion trait modality associated with interstitial macroinvertebrates
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39 610 living in the hyporheic zone was also selected as an important predictor of $U_{NO_3^-N}$.
40
41 611 This agreed with previous studies, showing that hyporheic macroinvertebrate
42
43 612 assemblages have small but significant effects on nutrient and organic matter
44
45 613 processing by changing flow patterns in the interstitial water and associated solute
46
47 614 concentrations (Mermillod-Blondin *et al.*, 2000; 2001; Mermillod-Blondin & Gérino,
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49 615 2002; Marshall & Hall, 2004).

50
51 616 In summary, the results of this functional trait approach suggest a possible
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53 617 relationship between nitrate removal and macroinvertebrates *via* their top-down
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55 618 feeding control effect on biofilms as well as their dwelling activities in the hyporheic
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57 619 zone that influence microbial capacities. The substrate preferences of

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

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3 647 with coarse sediments would appear to be key habitats for biodiversity and the
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5 648 biogeochemical processes that support nitrate removal as a regulating ecosystem
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7 649 service in streams.
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9

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13
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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} ($mg \cdot m^{-2} \cdot min^{-1}$)	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 ($mg \cdot l^{-1}$)	0.003	0.039	0.18	0.048
	$NO_3^- -N$ ($mg \cdot l^{-1}$)	0.05	2.66	8.98	2.60
	$PO_4^{3-} -P$ ($mg \cdot l^{-1}$)	0.003	0.112	0.590	0.163
	DOC ($mg \cdot C \cdot l^{-1}$)	0.68	3.40	7.75	2.04
	Conductivity ($uS \cdot cm^{-1}$)	163.9	646.4	1257.5	323.6
	Temperature ($^{\circ}C$)	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

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 ($Q, m^3 \cdot s^{-1}$)	0.001	0.063	0.267	0.073
	Velocity ($m \cdot s^{-1}$)	0.02	0.17	0.50	0.13
	Froude	0.03	0.16	0.43	0.10
	Reynolds	358	17526	73077	18763
Biofilm	Chl- <i>a</i> ($mg \cdot m^{-2}$)	1.00	67.0	483.8	96.4
Denitrification rate *	$mg N_2O \cdot m^2 \cdot min^{-1}$	0	1.17	4.02	1.29
Macroinvertebr ates	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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4 1032 Table 2. Selected trait modalities (26 modalities of four biological traits and one
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6 1033 ecological trait) and the rationale (see detailed rationale in the text). Concerning the
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8 1034 modality trade-off, some rare or similar modalities were pooled together into the same
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10 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 (< 1 mm) Coarse detritus (> 1 mm) Microphytes Macrophytes Dead animal (≥ 1 mm) 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

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Macroinvertebrate traits and in-stream nitrate removal

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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4 1059 Table 3. Results of principal component analysis (PCA) for catchment,
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6 1060 physicochemical characteristics, substrate and hydrological factors. The percentage
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8 1061 values on each axis represent the amount of variance explained by each PCA
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10 1062 component. Only important factors are included (loading >0.5). See Table 1 for a
11
12 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)

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Macroinvertebrate traits and in-stream nitrate removal

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
 1070

Selected components	Step-GLMs		Hierarchical partitioning
	$D^2=0.56$ (n=27)		Independent influence (%)
	Estimate	P value	
Phy-che 2	0.54	0.03	18 *
Hydro 1	-0.19	>0.1	5
Hydro 2	-0.45	0.05	22 *
Biofilm Chl-<i>a</i>	-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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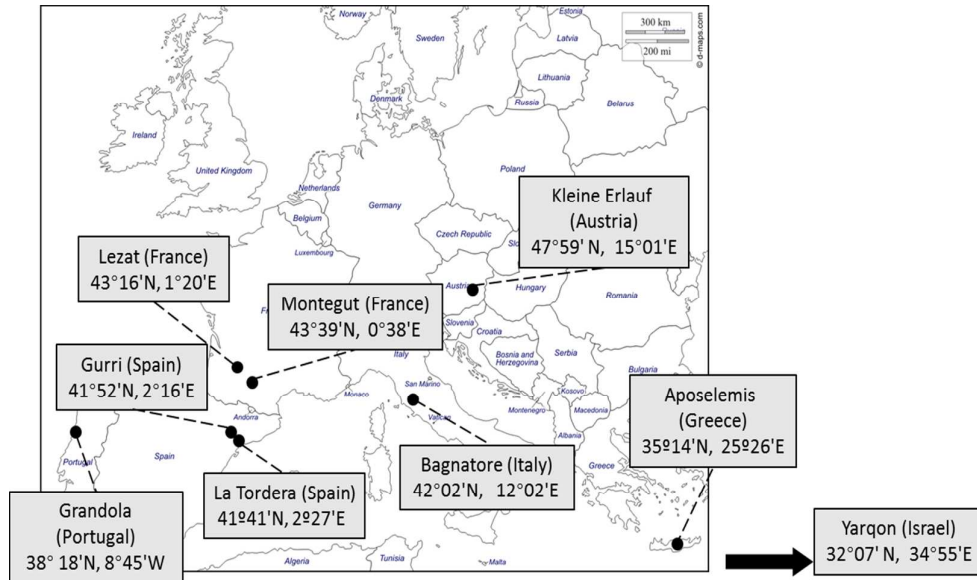
1
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4 1083 Table 5. Loadings of variable importance in projection (VIP) of the first component
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6 1084 extracted from partial least squares (PLS) regression analysis performed for the nine
7
8 1085 streams (n=27), with $U_{NO_3^-N}$ as dependent variables and selected modalities of
9
10 1086 macroinvertebrates as independent variables (i.e. 21 modalities of four traits).
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12 1087 Y-weights correspond to loadings of $U_{NO_3^-N}$. VIP >0.7 are in bold. Italicised values
13
14 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	<i>Flagstones/boulders/cobbles/pebbles</i>	0.89	0.20
(preference)	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	<i>Crawler</i>	2.15	0.47
and	Burrower	0.34	0.07
substrate	<i>Interstitial</i>	2.11	0.46
association			
Food	Fine sediment +microorganism	1.12	-0.24
	<i>Detritus</i>	0.92	0.20
	<i>Microphytes</i>	1.17	0.25
	Macrophytes	0.19	0.04
	Dead animals	2.07	-0.45
	Macroinvertebrates, macro- invertebrates and vertebrates	0.10	0.02
Feeding	Deposit feeder	0.42	0.09
habits	Shredder	0.63	-0.14
	<i>Scraper</i>	0.74	0.16
	Filter-feeder	0.42	0.09
	Predator	0.06	0.01

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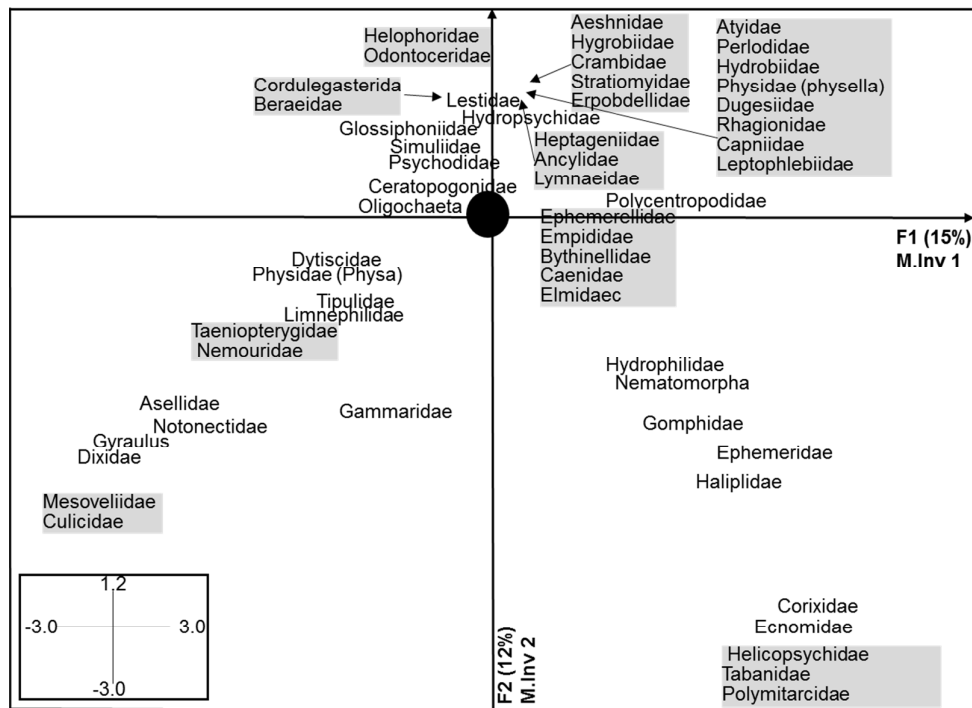
1090 **Figures**



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1092 Figure 1. The locations of the nine streams investigated

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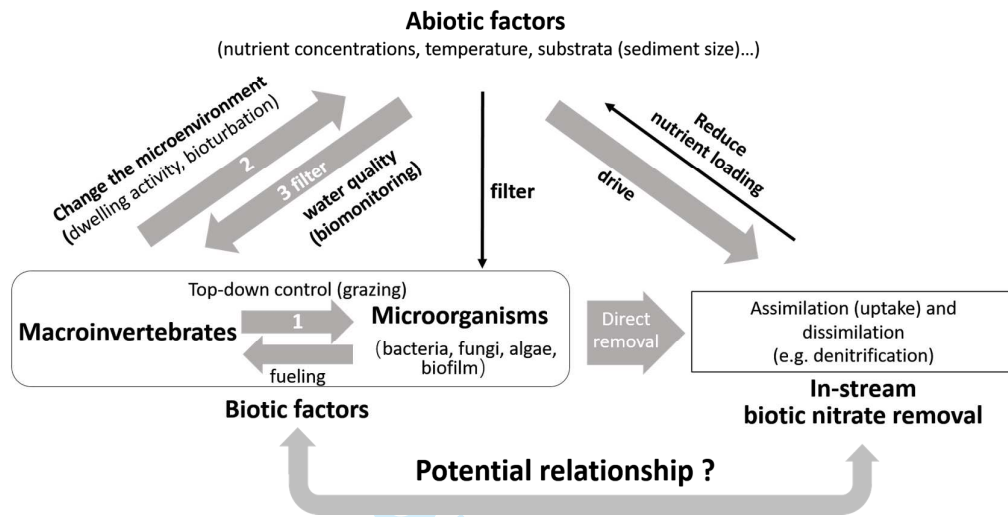
1095 Figure 2. Factorial plane of correspondence analysis (CA) performed on

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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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)

1106

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	Dugesidae	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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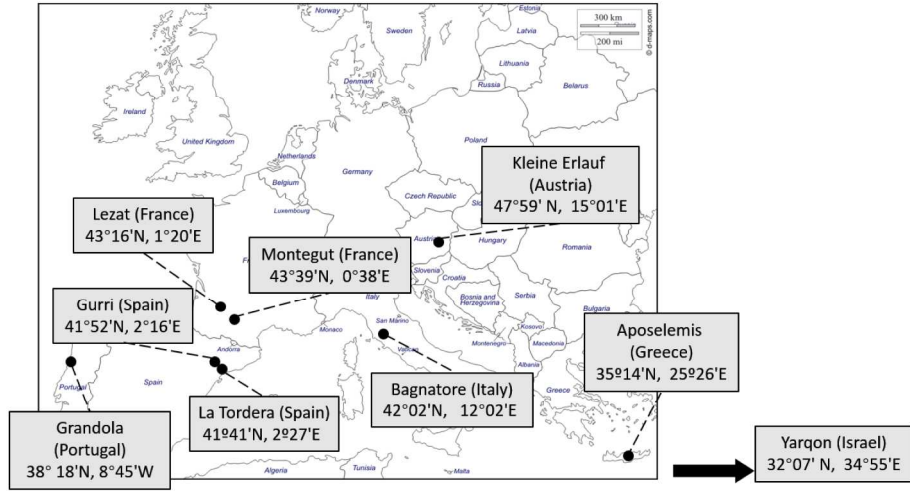


Figure 1. The locations of the nine streams investigated

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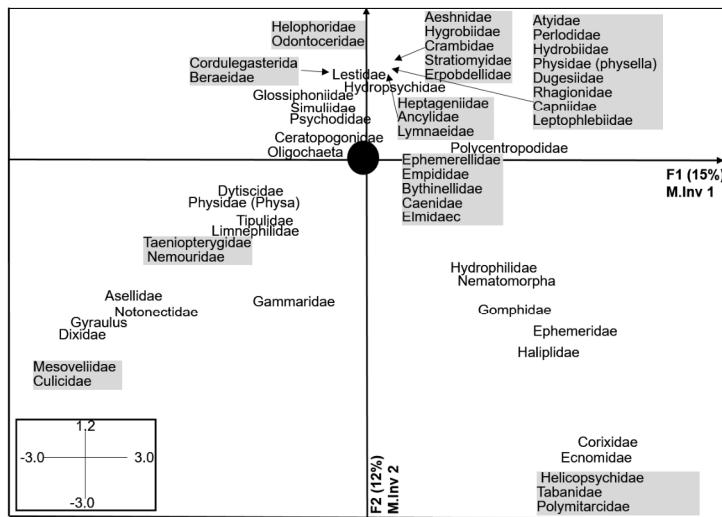


Figure. 2. Factorial plane of correspondence analysis (CA) performed on log-transformed densities. Only species with loadings > 0.5 are shown in this figure. The black circle represents taxa with loadings below 0.5 in axes 1 and 2. Grey rectangles group different taxa with similar loadings. The inset box shows the axes scales. See detail in the appendix

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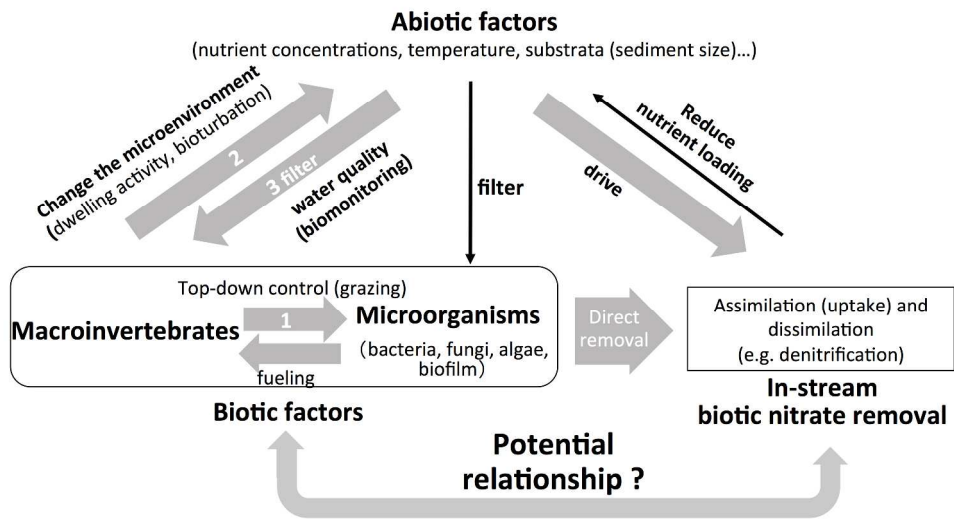


Figure 3. Schematic of the potential links between macroinvertebrates and in-stream nitrate removal (the lines in grey are explored in this study)

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