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Macrophyte landscape modulates lake ecosystem-level nitrogen losses through tightly coupled plant-microbe interactions

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Abstract

Root functional diversity of submerged vegetation exerts a major effect on nitrogen (N) cycling in lake sediments. This fact, however, is neglected in current N-balance models because the links between the engineering role of plants and in situ microbial N cycling are poorly understood. We hypothesized that macrophyte species with high root oxygen loss (ROL) capacity promote the highest denitrification because of a higher abundance of ammonia oxidizers and tighter coupling between nitrifiers and denitrifier communities. We sampled five small ultraoligotrophic shallow lakes with abundant macrophyte cover including sediments dominated either by *Isoetes* spp. (high ROL), mixed communities of natopotamids (low ROL), and unvegetated sandy sediments. At each site, we quantified denitrification (DNT) rates and proxies for the abundance of denitrifiers (*nirS* and *nirK* genes), and both ammonia oxidizing archaea (AOA) and ammonia oxidizing bacteria (AOB) and the diversity of *nirS*-harboring bacteria. Vegetated sediments showed significantly higher abundances of N-cycling genes than bare sediments. Plant communities dominated by *Isoetes* generated sediments with higher redox and NO_3^- concentrations and significantly higher DNT rates than natopotamids-dominated landscapes. Accordingly, increasing DNT rates were observed along the gradient from low ROL plants-bare sediments-high ROL plants. Significantly higher abundance of the archaeal *amoA* gene was recorded in sediments colonized by high ROL plants unveiling a key biogeochemical role for AOA in coupling macrophyte landscape and ecosystem denitrification.

The global nitrogen cycle has been strongly modified by massive industrial fixation of nitrogen gas (N_2) for human use and by fossil-fuel combustions (Gruber and Galloway 2008). Current concentrations of bio-available nitrogen (N) forms are higher than ever in the human era (Fowler et al. 2013). Terrestrial and aquatic denitrification (DNT) and anammox processes are of special interest because they represent the only permanent removal pathway whereby bioavailable N is returned to inert N_2 gas (Rockström et al. 2009). The presence and specific composition of rooted plants is a major factor influencing DNT rates of soils and sediments (Risgaard-Petersen and Jensen 1997). Plants can alter physicochemical factors known to control denitrification rates such as pH, oxygen, carbon sources and nitrate concentrations (Griffiths et al. 1997; Gacia et al. 2009). These modifications, in turn, influence the activity, diversity and abundance of rhizosphere nitrifiers and denitrifiers populations although few studies have reported quantitative evidences of these plant-microbial interactions in lake ecosystems (Kofoed et al. 2012). Commonly, nitrification (NT) produces NO_3^- under oxic conditions after ammonification while DNT requires suboxic conditions and is highly dependent on both NO_3^- transport from aerobic to anaerobic zones and changes of the in situ redox conditions (Seitzinger et al. 2006).

Submersed aquatic plants (SAV) promote the coupling between NT and DNT through the combined effect of amplifying oxygen gradients (i.e., Wang et al. 2013), and increasing the organic matter load (Reddy et al. 1989; Bodelier et al. 1996; Maltais-Landry et al. 2009) at the water-sediment

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interface and beyond. Different SAV functional typologies coexist in situ and may influence differentially the ecosystem-level DNT potential. This is particularly relevant in sediments colonized by isoetids, a cosmopolitan functional group of aquatic plans common in oligo-mesotrophic lakes of North America and Central and Northern Europe. Isoetids have highly porous roots with high underground biomass and large surface area to efficiently capture sediment CO_2 that supplies photosynthesis in C-limited soft-waters (Rascio 2002). As a byproduct, roots release significant amounts of photosynthetic oxygen into the rhizosphere (i.e., high root oxygen loss [ROL]; Sand-Jensen et al. 1982; Pedersen et al. 1995), promoting organic matter mineralization, ammonia oxidation and nitrate (NO_3^-) accumulation in pore-waters (Olsen and Andersen 1994). Conversely, sediments colonized by low ROL functional groups such as elodeids or natopotamids (the most common freshwater macrophytes throughout) with low ROL are mostly anoxic and rich in ammonia (Gacia et al. 2009).

Genetic techniques may help pinpoint the plant-mediated controls on microbiological communities. The abundance of N-cycling functional genes has been used to trace and assess the potential importance of nitrogen biogeochemical processes in situ (e.g., Petersen et al. 2012; Vila-Costa et al. 2014). Nitrification is initiated by archaeal (AOA) and bacterial ammonia oxidizers (AOB) through the ammonia monooxygenase AMO enzyme (Fernàndez-Guerra and Casamayor 2012). Whereas some studies report dominance of AOB over AOA along with increasing NH_4^+ concentrations in the rhizosphere of different estuarine macrophytes (Trias et al. 2012), AOA outnumbered AOB in sediments colonized by the isoetid *Littorella uniflora* (Herrmann et al. 2008, 2009) in comparison to sediments colonized by other typologies of aquatic plants or devoid of vegetation. Whether these abundances are related to DNT activities of the rizosphere remains poorly studied, even less through cultivation-independent techniques (but *see* Kofoed et al. 2012). A key step of denitrification, the conversion of NO_2^- to NO^-, is carried out via nitrite reductases encoded by the *nirK* or *nirS* genes (Kraft et al. 2011). Preference of one or the other *nir* gene is scarcely understood, although several studies have inferred that *nirK* abundance is larger and more widely distributed among different physiological groups (Wallenstein and Vilgalys 2005; Jones and Hallin 2010).

In this study, we aimed to go one step further previous approaches by looking at the coupling between nitrification and denitrification at gene level and their relationship to DNT activities in different sediment depth—levels of macrophyte-dominated areas. We selected high-altitude macrophyte communities in the Central Pyrenees as a model to study coupled plant-microbe and microbe-microbe interactions because (1) the ultraoligotrophic nature of soft-water lakes and ponds suggests tightly coupled nitrification/denitrification (Seitzinger et al. 2006), (2) isoetid species are an abundant component of the underwater flora forming either monospecific or mixed meadows together with soft-water natopotamids, and (3) we can predict the in situ sediment redox potential in the different plant communities based on extensive previous knowledge (Gacia et al. 2009). The presence of the two key species *Isoetes lacustris* and *Isoetes echinospora* is related to positive sediment redox potential, wide variability in redox values, and high NO_3^- in pore-water. Conversely, macrophyte communities dominated by natopotamids (*Myriophyllum* and *Potamogeton* species) or sediments lacking vegetation, result in a negative average sediment redox, low variability and dominance of NH_4^+ as DIN form. We therefore predict the rhizosphere of *Isoetes* species hold higher abundance of ammonia oxidizers and tight coupling nitrifiers-denitrifiers, whereas sediments holding low ROL aquatic plants would show significant abundances of NT and DNT genes only in the upper sediment layer (i.e., sediment water interphase) as compared with areas without vegetation. Accordingly, the macrophyte landscape may modulate lake ecosystem-level nitrogen losses through differential species specific root surface area and oxygen and dissolved organic carbon release.

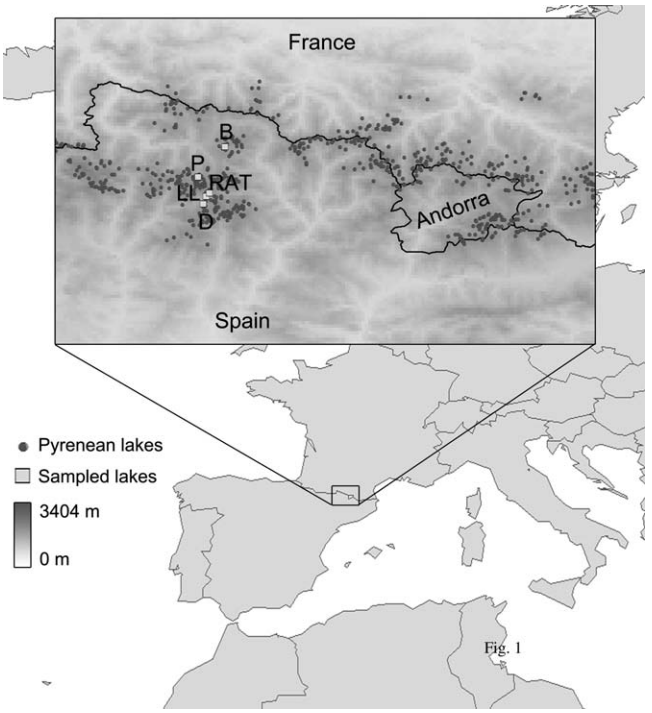

Fig. 1. Study site map. Lake names are abbreviated as RAT, Redó; LL, Llong; D, Delluí; B, Baciver petit and P, Plan.

Materials and methods

We sampled five small ultraoligotrophic high altitude lakes, located between 2000 m and 2300 m. a.s.l., in the Spanish Pyrenees (42°41′N, 0°59′E) in August 2011 (Fig. 1). The lakes were shallow (<13 m depth) and with abundant

Table 1. Physical descriptors of the lakes and macrophyte community composition. The area of each unit is calculated based on the vegetation maps provided as Supporting Information (Fig. S1). Geographic location is provided in the UTM coordinate system (ED50).

Lake		RAT	D	LL	P	B
Altitude (m)		2114	2315	2000	2189	2307
Latitude (x)		332557	331248	331849	330254	336086
Longitude (y)		4716498	4713465	4715653	4721070	4729340
Lake area (ha)		6.34	1.08	7.1	5.2	0.97
ISO area (ha)		1.91	0.003	0	1.10	0.46
ISO'area (ha)		0	0	0	0	0.27
MIX area (ha)		0	0.05	1.43	1.12	0
SED area (ha)		4.43	0.86	0	0	0
GRAVEL area (ha)		0	0	5.98	2.95	0
Num. of sampled patches		2	3	2	3	2
Species coverage per patch (%)						
ISO	*I.lacustris*	100	100	-	100	100
ISO'	*S.angustifolium*	-	-	-	-	29.1
	S.aquatica	-	-	-	-	1.4
	I.echinospora	-	-	-	-	69.5
MIX	*M.alterniflorum*	-	45	76.6	90	-
	P.alpinus	-	45	15.7	-	-
	Nitella sp.	-	-	7.8	-	-
	Isoetes sp.	-	10	-	10	-
SED		100	100	-	-	-
GRAVEL		-	-	100	100	-

ISO, *Isoetes lacustris*-dominated sediments; ISO', *Isoetes echinospora*-dominated sediments; MIX, *Myriophyllum*-dominated sediments; SED, sandy sediments; GRAVEL, gravel sediments; RAT, lake Redó d'Aigüestortes; D, lake Delluí; LL, lake Llong; P, lake Plan; B, lake Baciver petit; *S. angustifolium*, *Sparganium angustifolium*; *S. aquatica*, *Subularia aquatica*; *M. alterniflorum, Myriophyllum alterniflorum*; *P. alpinus*, *Potamogeton alpinus*.

macrophyte cover (between 6% and 76% of lake area). Two to three sites were sampled per lake. Sediments dominated either by *Isoetes* spp. (pure *I. lacustris* communities or mixed communities of *I. echinospora*, ISO hereafter) or mixed communities of *natopotamids* (MIX), and unvegetated sandy sediment (SED) or gravel (GRAVEL) were characterized (Table 1). Samples were collected around noon (±30 min) on sunny days to reduce variability associated with potential daily fluctuations in plant activity.

A total of six sediment cores were collected per site for sediment redox measurements, pore-water extraction, physicochemical and DNA analyses, respectively. Vertical redox profiles were performed in situ in triplicated cores with lateral holes every 2 cm using a redox electrode (238145, Liqglass orp, Hamilton) coupled to a portable meter (SP90M5, VWR symphony) calibrated with a standard solution (Crison 475 mV at 25°C) (*see* Gacia et al. 2009). For pore-water extraction three horizons, based on variations in the in situ redox profiles, were sampled per core: horizon 1 (2–4 cm; H1), horizon 2 (6–10 cm; H2) and horizon 3 (11–18 cm; H3) and water was extracted using rhizons CSS with 3.5 cm of porous connected to syringes. Extracted pore-water samples were kept in a portable refrigerator in sterile tubes and analyzed in the laboratory for NO_3^-, NO_2^-, NH_4^+ and PO_4^- concentrations using standard colorimetric methods (APHA 1995). Samples for DNA were stored at −20°C. Sediment percentage of organic matter (%OM), water content (WC), density (DEN), percentage of total C and N (%C and %N) were obtained as in Gacia et al. (2002). The potential biological O_2 demand (BOD) was measured at the lab by incubating 15 mL of fresh sediment in 140 mL vials in the dark in artificial floodwater initially at air equilibrium. Sediment samples were first preincubated for 5 h in 20 mL of water bubbled with air to eliminate chemical O_2 demand. The vials were then filled with air-bubbled artificial floodwater, closed, and incubated at 20°C in the dark on a shaker for a known amount of time. Oxygen concentrations were measured before and after incubation using an O_2 minielectrode (OX500, Unisense, Aarhus, Denmark). Potential biological oxygen demand was calculated as O_2 consumption per sediment volume and unit time (nmol O_2 L^{-1} s^{-1}) following Pulido et al. (2012). DNA was extracted from 0.45 g to 1.0 g of wet sediment using FastDNA® SPIN Kit for Soil (Qbiogene, Carlsbad, California). N-cycling marker genes *nirK*, *nirS*, *amoA*-AOA, and *amoA*-AOB were quantified and analyzed by qPCR as recently reported (Vila-Costa et al. 2014, Table S1

Supporting Information). Unsuccessful amplification of anammox bacteria was attempted using the primer pairs described in Table S1 Supporting Information. Sediment gene abundances were normalized to g-dry sediment. *NirS*-gene-bearing community composition was analyzed by TRFLP with the primer pair cd3aF- R3cd using 6-FAM-fluorescent labeled forward primer slightly modifying the protocol of Throbäck et al. (2004). Amplifications were improved experimentally by adding 1:5 concentration of forward (0.4 μmol L^{-1} final conc.) vs. reverse primers (2 μmol L^{-1} final conc.) and aliquots of 20 ng of PCR fragments were digested with two enzymes Hae III and BstUI (Fermentas) following manufacturer instructions. Data analyses were performed using the software SoftGenetics LLC (version 1.90). Peaks below 50 relative fluorescence units (FU) were discarded. A table merging Hae III and BstuI fragments was created using the normalized area of each peak. Alpha-diversity was measured by the abundance-based estimators Chao1 and ACE, which yielded identical numbers, and the Shannon indexes. Diversity analyses were carried out in R (http://www.r-project.org/) with the vegan package version 1.15 (Oksanen et al. 2009).

In situ DNT rates and potential DNT rates (Denitrification Enzyme Assays, DEA hereafter) were measured using the acetylene inhibition technique (Yoshinari and Knowles 1976). Sediments from three cores collected were homogenized and six replicates (three replicates for DNT, three replicates for DEA) of 100 g each were slurred with 60 mL (DNT) or 50 mL (DEA) ultrapure water. DEA samples were amended with both 5 mL of glucose solution (0.44 mol L^{-1}) and 5 mL of potassium nitrate (14.3 mmol L^{-1}) to avoid C and N limitations, respectively. Incubations were carried out at in situ temperature in the dark. Gas subsamples of 5 mL were taken over a time course from 0 h to 8 h (a minimum of three time points per sample). Samples were analyzed for N_2O using an Agilent Technologies 7820A gas chromatograph with an electron-capture detector. DNT and DEA rates were obtained from the slope of the accumulation of N_2O and corrected by sediment dry mass. The percent of inhibition of DNT activity was calculated as "(100-DNT rate constant/DEA rate constant) $\times$ 100)".

Statistical analysis

Cumulative distributions of all variables were compared against normal-distribution function using the Shapiro–Wilk test. Non-normal variables ($p < 0.05$) were log transformed. Principal correspondent analysis (PCA) was used to summarize the physicochemical structure of the sediments. Pearson correlations and spearman rank-order correlation coefficients were determined for pairwise comparisons of physicochemical (dataset values and PC1, PC2 scores) and biological variables. Alpha was set at $p < 0.05$. To correlate these variables to DNT and DEA rates, averaged values from the three horizons from each core were used. PERmutational Multivariate ANOVA [PERMANOVA (Mcardle and Anderson 2001)] was applied to elucidate the factors (i.e., lake, zone or horizon) that significantly structured the sediments and comparison of composition of *nir* S community among lakes and sediments. We used ANOVAs and Tukey's HSD post-hoc test and, for non-normal distributed data, a Krustal–Wallis test with Bonferroni correction, to determine whether variables exhibited significant variation between sediment types (ISO, MIX, SED, and GRAVEL), horizons or lakes. If only two groups were compared (for instance, ISO vs. all the other sediments together), a Mann–Whitney *U*-test was used. Analyses were performed using PRIMER 6.0 (for PCA) and the Statistica software package (StatSoft, Tulsa, Oklahoma).

Vegetation maps

All five lakes were studied in detail in a previous fieldwork during summer 2005. An expert snorkeler prospected the whole bottom of the lake and mapped the aquatic macrophyte communities and substrate types. Aquatic macrophyte communities were characterized by phytosociological relevés (using both the Braun-Blanquet method and the plant biomass method, *see* Gacia et al. 2009 for more information). No major changes were observed in vegetation cover during the fieldwork in summer 2010. Maps were digitalized in ArcView 3.0 and the total area covered by each community calculated using the same software. Denitrification rates were upscaled to the ecosystem level based on these maps that are available as Supporting Information (*see* Fig. S1).

Results

The main drivers for both physicochemical structure and abundance of DNT and NT genes were plant community type (ISO and MIX) and lack of plants (SED and GRAVEL) and not the factors "lake" neither "horizons" (PERMANOVA analysis, $R^2 = 0.6$, $p < 0.005$). Those variables accounting for the maximal physicochemical variability were WC, %OM, %C, and %N (PC1, 38.5% of the variability) and redox values and nutrients (NO_3^-, NH_4^+, NO_2^-, and PO_4^-) concentration; (PC2, 23.6% of the variability; Table S2 Supporting Information). Highly porous sediments had low organic matter content which segregated GRAVEL sediments from the remaining types (PC1 scores, Fig. 2). Oxygenated sediments showed significantly higher NO_3^- and lower NH_4^+ concentrations (Fig. 3). Depth-averaged redox potentials were significantly higher in ISO and GRAVEL sediments (averaging 369.2 ± 66.3 mV and 346.3 ± 34.2 mV, respectively) than in MIX and SED sediments (averaging 223.9 ± 48.6 mV and 196.4 ± 27.2 mV, Fig. 3). Overall, we found higher concentrations of genes *nirK* ($1.2 \pm 1.5 \times 10^{10}$ g^{-1} dry sediment) and *amoA*-AOA ($1.7 \pm 2.8 \times 10^8$ g^{-1} dry sediment) than of *nirS* ($3.3 \pm 3.7 \times 10^8$ g^{-1} dry sediment) and *amoA*-AOB ($1.3 \pm 1.8 \times 10^6$ g^{-1} dry sediment). According to that, *nirK*-harboring bacteria would dominate the denitrifying assemblage (>95% of the *nir* genes) and AOA the nitrifiers (>90%

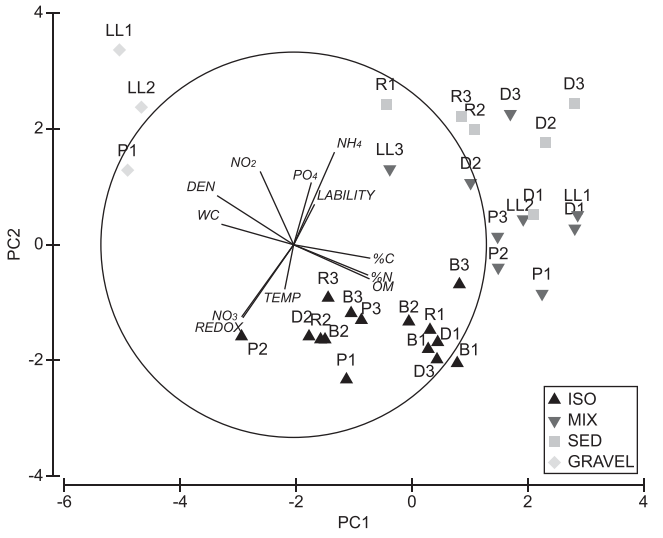

Fig. 2. Principal component analysis (PCA) of physicochemical descriptors of the sediment. Values were log-transformed. Each point is labeled with a capital letter indicating the lake (R, Redó; LL, Llong; D, Delluí; B, Baciver petit; P, Plan) and a number indicating the horizon sampled (1: horizon 1 (2–4 cm depth), 2: horizon 2 (6–10 cm depth), 3: horizon 3 (11–18 cm depth)). ISO, *Isoetes*-dominated sediments; MIX, *Myriophyllum*-dominated sediments; SED, sandy sediments; GRAVEL, gravel sediments. See Table 1 for more details and check Materials and Methods section for environmental variables abbreviations.

of total *amoA* genes). Vegetated sediments promoted significantly higher abundances of all N-cycling genes (one-way ANOVA, $p < 0.05$). Significantly higher abundances (fourfold) of *amoA*-AOA were recorded in ISO sediments than in MIX sediments, and 120- and 105-fold higher than in SED and GRAVEL sediments, respectively (Fig. 3). Regarding diversity of *nirS* denitrifiers, communities were structured by lake ($R^2 = 0.2$, $p < 0.005$) and by zone (nested PERMANOVA, $R^2 = 0.2$, $p < 0.01$). MIX sediments presented the highest diversity and richness of *nirS*-bearing community (Krustal–Wallis test, $p < 0.05$) and showed maximum similarity within lakes (42%), followed by the non-vegetated sediments (32%) and finally the ISO sediments (28%). ISO sediments differed the most from the rest and particularly with the non-vegetated sediments (74%).

Nitrification is a non-facultative process and gene abundances can be taken as a proxy of potential NT activities whereas DNT is a facultative process that requires quantification (Le Roux et al. 2013). The highest DNT rates were recorded in ISO sediments, ranging from 0.017 μmol N Kg^{-1} h^{-1} to 0.4 μmol N Kg^{-1} h^{-1}, and were significantly higher than in the remaining sediments (Mann–Whitney *U*-test, $p < 0.05$). Specifically, from 40- to 173-fold lower DNT rates were observed in MIX sediments and in sediments devoid of vegetation (averaging 0.002 ± 0.002 μmol N Kg^{-1} h^{-1}, Fig. 2) than in ISO sediments (Fig. 4). After glucose and NO_3^- addition, a 374-fold increase in DNT rates was observed in MIX sediments, contrasting to a 68-, 13- and 2.4-fold increases

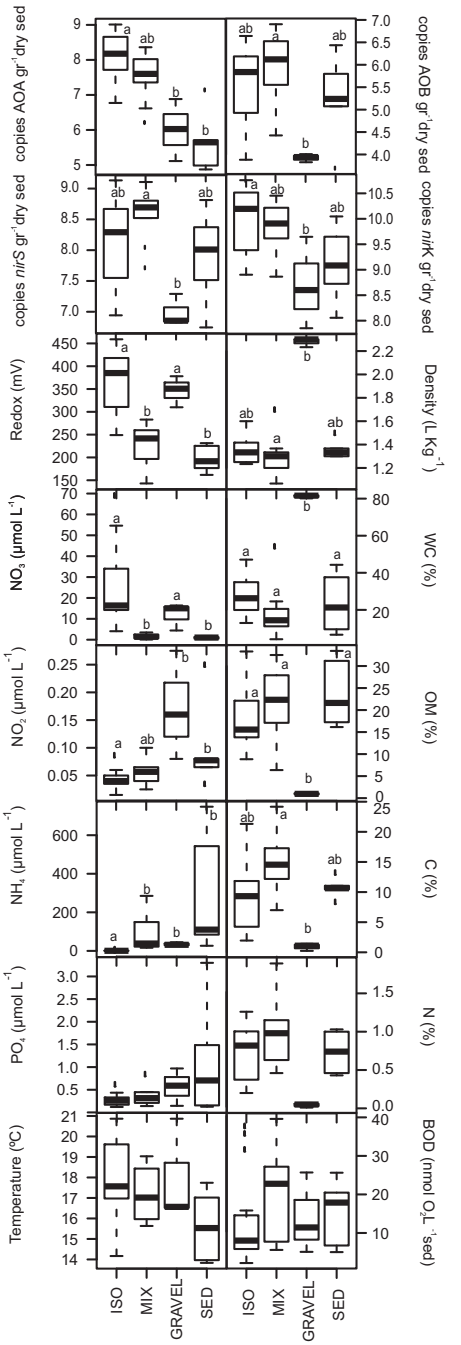

Fig. 3. Comparison of physicochemical parameters of the sediments and gene copy numbers of denitrifying bacteria (*nirS* and *nirK*) and ammonia-oxidizing archaea (*amoA*-AOA) and bacteria (*amoA*-AOB) between sampled zones. Horizon values ($n = 3$ to 9) were averaged. Error bars represent the standard deviation of all lakes from triplicate analyses/lake. Significant differences ($p < 0.05$) of mean values were analyzed using 1-way anova (for parametric data) and Krustal–Wallis test (for nonparametric data) followed by a post-hoc Tukey HSD test and labeled in the graph. Outliner numbers of BOD in ISO sediments correspond to lake RAT. ISO, *Isoetes*-dominated sediments; MIX, *Myriophyllum*-dominated sediments; SED, sandy sediments; GRAVEL, gravel sediments. Notice the log scale of the *y*-axes of gene copy numbers.

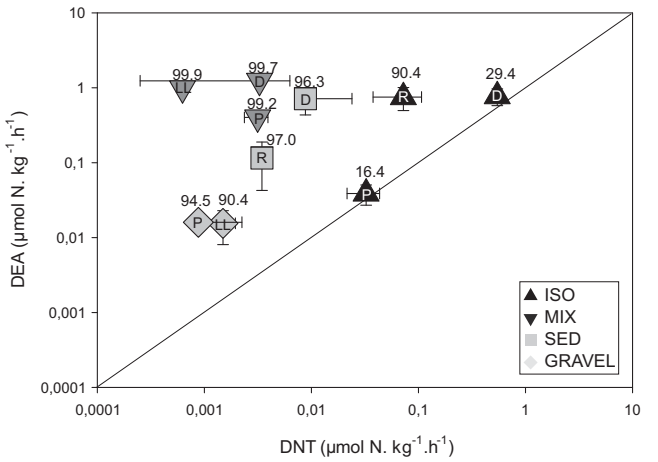

Fig. 4. Denitrifying enzyme activity rate (DEA, potential activity) and denitrification rate (DNT, in situ activity) in the different sampled zones. The percentage of inhibition of DNT rate is written on the top of each symbol. Values on the 1 : 1 line indicate samples with no DNT inhibition. Note the log-scale of the axes. Error bars indicate standard deviation of triplicate analyses. Lakes are labeled inside the symbols (R, Redó; LL, Llong; D, Delluí; P, Plan. ISO, *Isoetes*-dominated sediments; MIX, *Myriophyllum*-dominated sediments; SED, sandy sediments; GRAVEL, gravel sediments.

observed in SED, GRAVEL and ISO sediments respectively. DEA rates in MIX sediments reached values similar or slightly higher than those of ISO sediments (Fig. 4). DNT inhibitions > 90% were observed in all samples but in ISO (Fig. 4). DNT and DEA rates did not correlate. Significant negative correlation was observed between DNT and PC2 scores whereas DEA correlated positively to PC1 (Table S2 Supporting Information), suggesting a primary dependence of DNT on NO_3^- availability but also high DNT potential in those sediments with high OM content and low density.

Interestingly, *nirS* gene abundances correlated to PC1 and DEA rates, whereas *nirK* correlated to PC2 and DNT rates, indicating different environmental drivers for both genes and suggesting different active players in DNT (Table 2). Abundance of *nirS* rather depended on the quantity and quality of OM-content whereas *nirK* inversely correlated to NO_2^- concentrations (Table 2). The highest number of correlations between biological and physicochemical data was found for *amoA*-AOA genes, i.e., with PC2 (and specifically to NO_3^-, NO_2^-, NH_4^+, redox), to DNT rates, to *nirK, nirS*, and *amoA*-AOB gene abundances and to *nirS* diversity indices (Table 2; Fig. 5). Overall, results suggest that environmental factors that promoted abundance of *amoA*-AOA genes matched conditions for denitrification and promoted higher diversity among *nirS* denitrifiers.

Discussion

The isoetid-like functional group of macrophytes can be found worldwide in oligo-mesotrophic lakes (mainly in Northern and Central Europe and in the littoral of great lakes of North America), and in vernal and rock pools of temperate and arid regions. Unfortunately, such fragile and often endangered species and ecosystems are declining world-wide due to eutrophication (Sass et al. 2010; Sand-Jensen and Møller 2014). One of the most relevant properties of isoetids is the release of photosynthetic oxygen in the rhizosphere which can enhance nitrification rates (Smolders et al. 2002) leading to higher coupled nitrification-denitrification and overall high N losses. In our study, we show that macrophyte landscape is the main factor determining the presence of N-cycle functional genes in oligotrophic lake sediments and that denitrification rates are, as predicted, linked to the underwater plant functional typology. In agreement, we observed ISO sediments with high redox potentials, high NO_3^- and low NH_4^+ concentrations, indicative of mineralization and nitrification activities (Gacia et al. 2009). DNT rates were significantly higher in ISO sediments than in the other sediments. However, ISO rhizosphere was not significantly enriched with denitrifiers neither the *nirS*-harborig bacteria composition was significantly different to other vegetated sediments. Conversely,

Table 2. Correlation coefficients between physicochemical and biological parameters of the sediments (gene abundances of *nirS*, *nirK*, *amoA*-AOA, and *amoA*-AOB, *nirS*-harboring bacteria diversity (Shannon index (*nirS*-Shan)) and Chao1 richness (nirS-Richn)) and denitrification activity measurements (DNT and DEA rates). Horizon values ($n = 3$ to 9) were averaged in the correlations with DNT and DEA. Only significant correlated parameters ($p < 0.05$) are shown.

	nirK	*nirS*	*amoA*-AOA	*amoA*-AOB	DNT	DEA
PC1	ns	0.5	ns	ns	ns	0.7
PC2	−0.4	ns	−0.7	ns	−0.7	ns
NO_3	ns	ns	0.5	ns	0.7	ns
NO_2	−0.4	ns	−0.5	ns	ns	ns
NH_4	ns	ns	−0.7	ns	ns	ns
PO_4	ns	ns	ns	−0.4	ns	ns
OM	ns	0.4	ns	ns	ns	0.8
WC	−0.5	−0.6	ns	−0.5	ns	−0.7
DENS	−0.5	−0.6	ns	−0.5	ns	−0.7
Redox	ns	ns	0.5	ns	ns	ns
Temp	ns	ns	ns	ns	ns	ns
BOD	ns	ns	ns	ns	ns	ns
%N	ns	0.4	ns	ns	ns	ns
%C	ns	ns	ns	ns	ns	0.6
nirS-Shann	ns	ns	0.39	ns	ns	ns
nirS-Richn	ns	ns	ns	ns	ns	ns
nirK	-	0.8	0.7	0.8	0.7	ns
nirS	-	-	0.6	0.8	ns	0.9
amoA-AOA	-	-	-	0.6	0.7	ns
amoA-AOB	-	-	-	-	ns	0.7

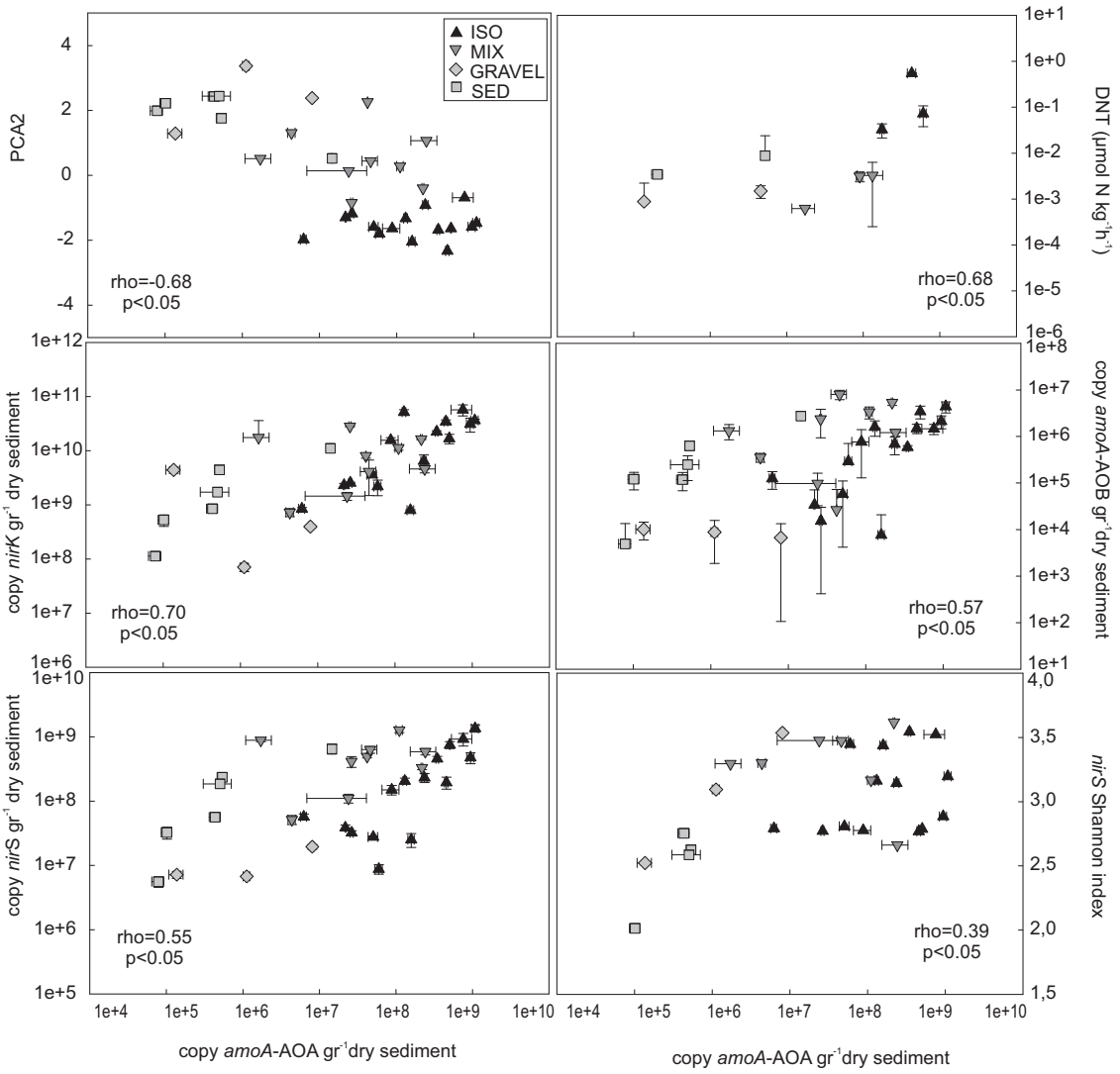

Fig. 5. Correlations between *amoA*-AOA gene abundances and physicochemical variation of the sediments measured by PC2 scores from Fig. 1, DNT rates, *nirS*- *nirK* and *amoA*-AOB copy numbers (corrected by dry sediment mass) and *nirS*- Shannon diversity indices. PC2 explained 21.3% of the variability and it was mainly weighted by redox and N-species concentrations (NO_3^-, NH_4^+, NO_2^-). Correlations are given as Spearman's rank-order correlations (rho). Significant values are set at $p < 0.05$.

the community was clearly enriched in AOA that significantly correlated to DNT rates, to porewater DIN forms (NO_3^-, NH_4^+, and NO_2^- concentrations), to the abundance of denitrifiers and to the diversity of *nirS*-denitrifier community, when analyzing the sediments altogether. Overall, these results suggest that the tight coupling between nitrification and denitrification is fuelled through the presence of AOA. To the best of our knowledge, this is the first study that assigns a key role for AOA in shaping denitrification potential at the ecosystem level. Since high abundance of AOA-*amoA* genes had also been observed in the rizosphere of other freshwater isoetids (i.e., *Litorella uniflora*, *Juncus bulbosus* and *Eleocharis* spp.) compared with sediments overgrown by other functional typologies of aquatic plants (i.e., *Typha angustifolia* and *Myriophyllum alterniflorum*; *see* Herrmann et al. 2008, 2009; Beier et al. 2010; Kofoed et al. 2012) probably this key role shaping system-level N losses may be extended to additional macrophyte landscapes.

We observed that AOA outnumbered AOB in all sediments, independently if they were populated by macrophytes or not, although higher abundances were observed in plant sediments (where more organic matter was present) and significantly more in ISO sediments. A similar trend was observed in the sediments of the Fitzroy river estuarine (Abell et al. 2010) although AOA dominance in sediments is not a widespread feature (Trias et al. 2012). It was intriguing to observe that high abundances of AOA in MIX rhizosphere were not coupled to high DNT rates which were even lower

than in bare sediments. One possible explanation is that AOA activities could be fuelling other N removal anaerobic processes such as providing nitrite to anaerobic ammonium-oxidizing (anammox) bacteria (Yan et al. 2012). However, we were unable to amplify anammox genes in our samples and we did not detect a significantly decrease in NH_4^+ from MIX sediment as expected from anammox bacteria N_2 production. DEA rates were higher in MIX sediments than in ISO sediments, indicating a very high potential for denitrification. Thus, low DNT rates may be related to unknown factors possibly related to limitations of AOA activities in MIX sediments. AOA have extremely high affinity to NH_4^+ ($K_s = 133$ nmol L^{-1}; Martens-Habbena et al. 2009) that allow them to outcompete AOB under low NH_4^+ concentrations. However, if this was the main factor, we should have found higher abundance of AOB over AOA in MIX sediments. Rather, we observed that BOD was higher in MIX than in ISO sediments indicating higher heterotrophic carbon mineralization. Interestingly, we recorded DNT inhibitions close to the ones in MIX sediments (90%) in ISO sediments of lake RAT that showed the highest BOD (similar to those observed in MIX sediments) among all samples. More respiration implies higher CO_2 concentrations that favor autotrophy. Although both AOB and AOA have an autotrophic lifestyle, it has been recently found that AOA can also grow on OM (e.g., Alonso-Sáez et al. 2012). Our results suggest that AOA found in Pyrenees ultraoligotrophic waters might rely more on a mixotrophic lifestyle than previously thought. If so, factors such as the quality and quantity of root exudates and competition with pure heterotroph might be relevant factors not only for nitrification activities as previously suggested (Herrmann et al. 2008) but also as rate-limiting factors of the denitrification potential in sediments.

Differences in abundances of *amoA* and *nir* genes were not significant among sediment horizons from −2 down to 20 cm depth in bare and MIX sediments, as expected from the lack of root structuring elements (unvegetated) or the presence of fine anchorage roots (i.e., MIX sediments) with very low ROL. We observed, however, lower *amoA* abundances below the *Isoetes* root limits, in agreement with the expected tight coupling between root activity and AOA.

We hypothesized that sediments with the best conditions for DNT activity would harbor the highest abundance of *nir* genes. This was only observed for *nirK* genes, which correlated to in situ porewater NO_3^- concentrations, whereas *nirS* abundances correlated to DEA rates and to porosity and organic content of the sediment. This result agrees with previous studies in soils and aquifers that showed different habitat selectivity for denitrifiers (Throbäck et al. 2004; Santoro et al. 2006). Although most studies show no correlation between DNT rates and denitrifier abundances (Abell et al. 2010; Dandie et al. 2011), good correlations between DNT and *nirS* and *nirK* abundances have been observed in different trophic stream sediments (Graham et al. 2010) indicating that these genetic markers better traced the overall functional potential as also seen in our study.

DNT rates in Pyrenean lakes were in the range of those measured in low-N deposition Norwegian lakes and orders of magnitude lower than those from high-N deposition regions (Mccrackin and Elser 2010). Using the acetylene blocking technique is discouraged in systems with a tight coupling between nitrification and denitrification such in our samples because acetylene also inhibits nitrification thus potentially modifying DNT rates (Dodsworth et al. 2011). In a comparison study, acetylene DNT rate estimates were 50% lower than those measured through isotopes approach (Piña-Ochoa and Álvarez-Cobelas 2006). Thus, despite that we did not add chloramphenicol to inhibit de novo synthesis of nitrate reductase enzymes (Murray and Knowles 1999), DNT rates measured in this study possibly represent underestimates of the actual rates.

DNT rates measured in ISO sediments were 8- to 100-fold higher than in bare sediments and 18- to 370-fold higher than in MIX-sediments. These increases are higher than the values measured in other lake sediments after comparing ROL vs. non vegetated sediments, i.e., within the range 0.1–11.7 increase (Bodelier et al. 1996; Risgaard-Petersen and Jensen 1997; Karjalainen et al. 2001). Differences appear to be higher under nutrient-limitation conditions as is the case of the ultraoligotrophic waters in the Pyrenean area. Interestingly, differences of DEA rates between sediment units were much lower than for DNT rates (from 0.7 to 3.7-fold between ISO and non-vegetated and 0.2 to 0.9-fold between ISO and MIX sediments). The high DEA rates observed in MIX sediments mirror the high degree of NO_3^- limitation of these sediments that hold more abundant and more labile organic matter than in the remaining sediment types. After DNT rates were calculated at the whole lake level using community coverage areas (*see* Supporting Information Fig. S1 for detailed vegetation maps), we observed a range of variability from a 19% decrease respect to lake DNT rates taking only bare sediment in Lake Llong (where only MIX macrophytes can be found) to 767% increase in Lake RAT that holds the most extended area of ISO sediments (Table 3). The increase on whole-lake DNT rates including specifically the vegetated sediment DNT rates was therefore proportional to the ISO-covered lake area.

Overall, these results highlighted that the enhanced DNT rates observed at ecosystem level in the presence of *Isoetids* (up to 7.7 times increase in vegetated sediments over non-vegetated sediments) should be taken into account for more accurate modeling N-processing in lakes. Small water bodies are abundant and more active than large aquatic systems worldwide (Downing et al. 2006), and contribute disproportionately to global carbon budgets (Cole et al. 2007) and to global N losses (Mccrackin and Elser 2012). The different regulation mechanisms arising from this study (high lability of organic matter potentially inhibiting AOA in sediments

Table 3. Averaged denitrification rates per lake presuming no aquatic macrophytes in the lake (estimated from DNT rates measured in SED and GRAVEL sediments and the area of the lake) and considering the real contribution of the different macrophyte-dominated areas (using community coverage areas from Table 1). RAT, lake Redó d'Aigüestortes; D, lake Delluí; LL, lake Llong; P, lake Plan; B, lake Baciver petit.

DNT rates (μmol N h^{-1})	RAT	D	LL	P
Presuming total absence of vegetation	1127.9	360.4	5001.1	1868.7
Considering macrophytes-dominated sediments	8655.3	462.5	4046.7	4360.3
Fold-change DNT rates when macrophytes are considered	7.7	1.38	0.8	2.3

colonized by ISO species, and NO_3^- as a limiting factor for DNT in sediments colonized by elodeids) may be useful information to enhance DNT in aquatic systems and to ameliorate water quality status of lakes, ponds and pools. These results should be considered in catchment and regional assessments of N-cycle budgets for continental waters, including conservation policies based on the potential of vegetated water bodies to counteract the effects of global change, especially perturbations related to the increase in diffusive N loads.

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