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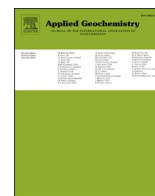
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# Spatial variation of denitrification and key controlling factors in streams and ponds sediments from a critical zone (southwestern France)

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## ABSTRACT

Numerous ponds were constructed along streams for local water supply in agricultural areas of south-western France. Although the water quality of streams and ponds has been surveyed for nitrate (NO<sub>3</sub><sup>-</sup>), the role of ponds in NO<sub>3</sub><sup>-</sup> mitigation at a catchment scale has rarely been investigated. Since denitrification in sediments is a key NO<sub>3</sub><sup>-</sup> pathway, the denitrification variability in stream and pond sediments and its controlling factors, particularly the pond position and characteristics are important aspects to investigate. Potential denitrification rate (PDR), physico-chemical factors, and denitrification functional genes were quantified in sediments from nine ponds located in three adjacent agricultural catchments in spring period. PDR showed a great spatial variation (both horizontal and vertical) and was mainly driven by the availability of water NO<sub>3</sub><sup>-</sup> and sediment organic carbon. Stream discharge and pond hydraulic retention time can also affect PDR in stream and pond sediments, respectively. Although high PDR was observed in stream sediments, stream discharge diluted NO<sub>3</sub><sup>-</sup> and can indirectly lower PDR downstream. The function of denitrifiers was revealed by the abundance ratio of *nirK*/*16S rRNA*, but no significant relationship was found between PDR and abundance of functional genes. N<sub>2</sub>O emission rate was positively related to PDR, which should be aware of in the upstream PDR hotspot ponds. Simple empirical models based on pond and sediment characteristics were good predictors of PDR, which could also help a better management of ponds by policy makers with the aim to improve NO<sub>3</sub><sup>-</sup> mitigation and water quality.

## 1. Introduction

Nitrate (NO<sub>3</sub><sup>-</sup>) contamination in surface waters and groundwaters is a serious global environmental problem, especially in areas of intensive agriculture (Verhoeven et al., 2006). The extensive application of synthetic fertilizer contributes to high NO<sub>3</sub><sup>-</sup> loadings into agricultural surface waters because of soil leaching, soil erosion, and surface runoff (Verhoeven et al., 2006; Zak et al., 2018). A high NO<sub>3</sub><sup>-</sup> level can induce water eutrophication and health problems in human beings (Kapoor et al., 1998). In agricultural areas like southwestern France, the NO<sub>3</sub><sup>-</sup> concentration in many streams and rivers was close to or exceeded the potability threshold (50 mg L<sup>-1</sup> of NO<sub>3</sub><sup>-</sup>) for European rivers set by the European Water Framework Directive in order to control and regulate the NO<sub>3</sub><sup>-</sup> level in water bodies (Ferrant et al., 2013; Boithias et al., 2014).

Constructed ponds are very common in southwestern France (an agricultural region for decades) and there can be several in a chain in a given agricultural catchment. They were mainly set up historically by local farmers for the purpose of water storage for cattle farming and crop irrigation (Carlier et al., 2016). Nowadays, they are used for water

irrigation or for private landscaping. Although the quality of stream and river water has been surveyed, the role of such ponds in the quality of downstream water and sediments has rarely been investigated.

Such ponds are biogeochemical reactors, which may influence nitrogen behaviour as they were found to be effective in mitigating NO<sub>3</sub><sup>-</sup> in the stream waters draining agricultural catchments (Vymazal, 2007; Tournebize et al., 2017). The denitrification process, a microbial process which reduces NO<sub>3</sub><sup>-</sup> to molecular nitrogen gas (N<sub>2</sub>) in four steps (NO<sub>3</sub><sup>-</sup> → NO<sub>2</sub><sup>-</sup> → NO → N<sub>2</sub>O → N<sub>2</sub>) (Tiedje, 1994), is one of the main processes contributing to the removal of NO<sub>3</sub><sup>-</sup> from the aquatic system (Burgin and Hamilton, 2007). It may cause the ecosystem to be N-limiting when N is not in excess, but in an agricultural context with significant NO<sub>3</sub><sup>-</sup> loading due to fertilizer inputs, it can remove excessive NO<sub>3</sub><sup>-</sup> and participate in NO<sub>3</sub><sup>-</sup> mitigation in streams (Fisher and Acreman, 2004). Nevertheless, the reaction of this process may not be complete and may lead to the production of N<sub>2</sub>O, a harmful intermediate greenhouse gas that can contribute to the global warming phenomenon (Garnier et al., 2010). Studies have shown that the magnitude of N<sub>2</sub>O production via incomplete denitrification is in response to changes in

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dissolved oxygen (DO), ammonium, and nitrite concentrations (Rassamee et al., 2011).

As mentioned by several authors, it is important to investigate both assessment of the denitrification process (Piña-Ochoa and Álvarez-Cobelas, 2006) and the development of modelling of nitrogen catchment export (Álvarez-Cobelas et al., 2008) at regional scale. Although the spatial variability of the denitrification process at catchment scale has been investigated, it has been less well studied in ponds (Scaroni et al., 2010; Song et al., 2012; Bernard-Jannin et al., 2017). Indeed, with increasing climate change pressure, particularly where water resources are limited, the use of ponds as tools of water storage will probably be extended in many cultivated places around the world. Investigations of nitrogen behaviour and particularly the spatial variability of the denitrification process according to environmental conditions are thus a major issue to be evaluated at regional scale, especially where a number of constructed ponds exists.

The factors influencing the denitrification process have been investigated by a large number of studies. Nitrate and organic carbon can influence the denitrification rate as they are two key reactants in the process (Groffman et al., 2006; Arango et al., 2007; Saeed and Sun, 2012; Saggat et al., 2013). Meanwhile, the abundance of denitrifiers and other water and sediment characteristics (pH, water content, redox potential, etc.), namely distal and proximal controllers (Wallenstein et al., 2006), may also play an important role (Oehler et al., 2007; Attard et al., 2011; Luo et al., 2012; Iribar et al., 2015).

Works combining geochemical investigation of the denitrification process with examination of the *in situ* microbial genus at ecosystem scale are not often found in the literature (Camargo Valero et al., 2010; Blaszcak et al., 2018). The denitrification rate has been shown to be related to specific gene abundance (Braker et al., 2000), while some studies have found some physicochemical parameters of sediments (e.g.  $\text{NO}_3^-$  concentration, organic carbon content, and/or sediment texture) to be better explanatory variables of denitrification rates than the abundance of denitrifiers at a small regional scale (Attard et al., 2011, with a similar sampling date to this study); Shrewsbury et al. (2016).

Although a great number of studies have shown the relationship between denitrification and some water and sediment properties, the power of environmental factors (*i.e.*, distance to stream source, stream hydrology, pond size, etc.) to influence denitrification has not been well identified in the literature. Moreover, Fisher and Acreman (2004) pointed out that effective attributes of wetlands are important to reduce nitrogen and phosphorous loadings efficiently. Developing simple empirical models based on pond and sediment physicochemical characteristics represents an interesting challenge for a regional-scale approach but requires thorough field data to be robust. This may also help identify the most appropriate pond characteristics for policy makers in charge of water quality management.

Considering the current situation mentioned above, the main objectives of this study were: (1) to investigate the spatial variability of denitrification rates in sediments from several streams and ponds considering their locations and characteristics; (2) to study the main controlling factors that regulate the denitrification process; (3) to draw some lessons and deduce recommendations about pond management to control the impact of  $\text{NO}_3^-$  in such agricultural environments.

Since denitrification is a key pathway for  $\text{NO}_3^-$ , the denitrification magnitude could be higher in those sites with more agricultural pressure with higher input of inorganic fertilisers. It could present a spatial variation according to the controlling factors even at a catchment scale.

Therefore, in this study, we measured the physicochemical characteristics of water and sediment samples from various streams and ponds collected at the same time in three small agricultural catchments located in a traditionally agricultural region (the Save basin, southwestern France). The denitrification magnitude has been quantified and the denitrification functional genes were investigated for some sediments representing the denitrification magnitude. Empirical models using some easily measured characteristics ( $\text{NO}_3^-$  concentration, temperature,

pH, etc.) were attempted, and environmental catchments and pond characteristics were considered to facilitate better pond management for  $\text{NO}_3^-$  removal.

## 2. Materials and methods

### 2.1. Site description

#### 2.1.1. Catchment characteristics

Three adjacent agricultural catchments drained by tributaries of the middle Save river (southwestern France) were considered: the Montoussé catchment (MON) and the Mican catchment (MIC, crossing the small village of Auradé, close to one another located on the right bank, and the Nuguet catchment (NUG, crossing Marestaing village) on the left bank (NUG) (Fig. 1 and Table 1) in the same latitude segment.

The regional climate is oceanic (Köppen Climate Classification) with an average annual precipitation and air temperature of 620 mm and 13.9 °C over the last 32 years, respectively (Ponnou-Delaffon et al., 2020). The three catchments were mainly cultivated with wheat and sunflower in a two-year rotation strategy. During the sampling period of March, wheat was the dominant crop. Local farmers applied fertilizers on soils, which were mainly synthetic fertilizers of the N–P–K type (N,  $\text{P}_2\text{O}_5$ , and  $\text{K}_2\text{O}$ ), fertilizers containing ammonium nitrate ( $\text{NH}_4\text{NO}_3$ ) and a limited number of urea and ammonium sulphate ( $\text{NH}_4)_2\text{SO}_4$  (Perrin et al., 2008) fertilizers. The amount of fertilizers applied corresponds to the crop demands, and the application for wheat usually occurs between the middle of January and March, with an optional application in April (Paul et al., 2015).

The three agricultural catchments integrate representative ponds in terms of size and volume, but with different shapes, management, and positions in the catchment (Fig. 1 and Table 1). They were found to be representative of the 3000 ponds in the area (as referenced by the Direction Départementale des Territoires, DDT, <http://www.haute-garonne.gouv.fr/Services-de-l-Etat/Agriculture-environnement-amenagement-et-logement/Direction-Departementale-des-Territoires>) (Wu and Probst, 2021).

The catchment areas are of the same order of magnitude (a hundred of ha) with slopes falling in the range of 4–10% (as observed in the area, Perrin et al., 2008), although slopes are steeper in MON and MIC than in NUG (Table 1).

#### 2.1.2. Pond characteristics

Nine ponds were sampled in the three catchments: two ponds in MON, four in MIC, and three in NUG (NUG4 was not sampled, see below; Table 1). Table 1 provides detailed information about the sampling ponds.

MON1 is a very small, isolated pond in the upstream sub-Montoussé catchment and is surrounded by an area of grass buffer strip (approximately 1.7 ha), whereas MON2 (one order of magnitude larger than MON1), surrounded by wheat, is located in two-thirds of the sub-Montoussé stream. In Mican, ponds did not always connect to the stream, unlike the three first Nuguet ponds. MIC1, MIC4 and NUG3 are the largest ponds and have the longest hydraulic retention time (HRT, Table 1), while MIC3 is a shallow pond with a significant sediment accumulation as it has not been dredged since the 1960s. NUG2 with emergent and submerged aquatic plants, and NUG3, with a lot of organic debris (mainly decayed fallen leaves) covering the pond bed, are closely linked. A fourth pond exists in Nuguet (NUG4, Fig. 1), but the outlet of the Marestaing village wastewater treatment plant (WWTP) downstream of NUG3 is discharged into the Nuguet stream close to NUG4. Regarding the objectives of the study, we thus decided not to sample the inner NUG4 pond in the sampling strategy. The construction data and data from the last dredging together with field observations made it possible to evaluate a greater accumulation of sediments for some ponds (ex. MIC 3) compared to others.

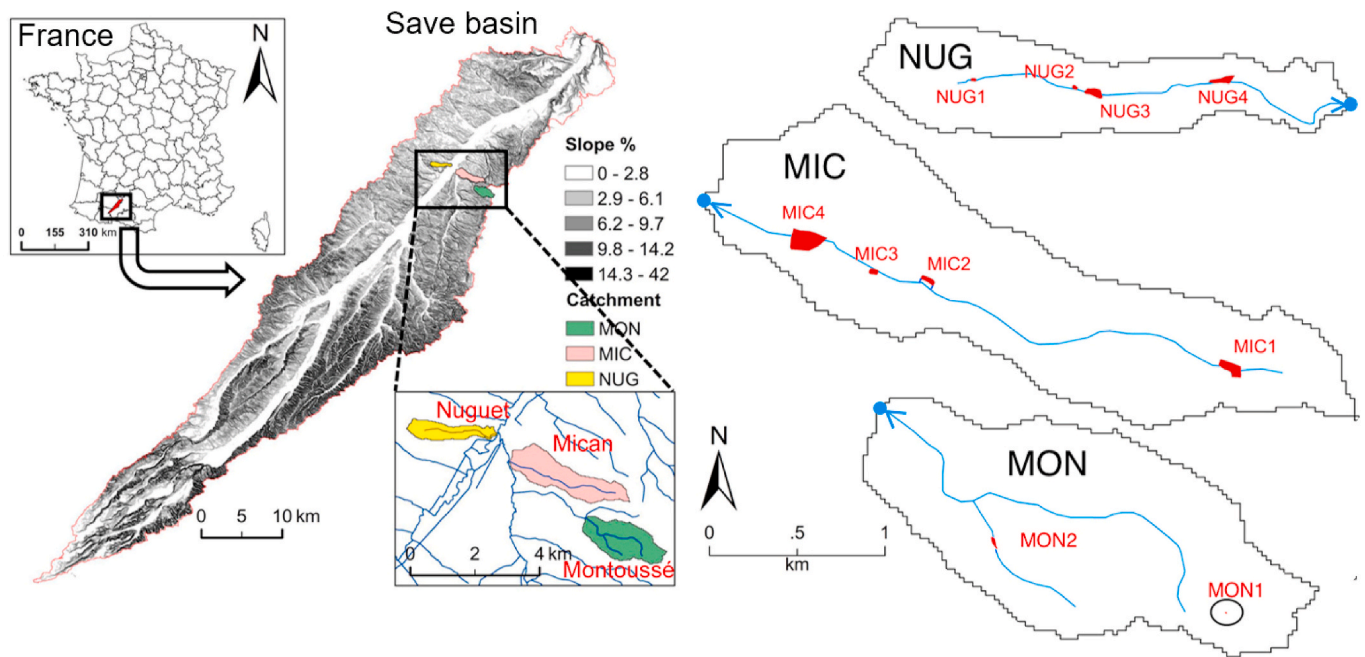


Fig. 1. Location of the studied ponds and of the three related catchments [Montoussé (MON), Mican (MIC), Nuguet (NUG)] within the Save basin, south-western France.

Table 1

Characteristics of sampling sites (catchments and ponds). YC = year of construction, YD = year of last dredging;  $HRT$  (Hydraulic Retention Time) =  $(Pond \cdot volume) / (Q_{outlet} - Q_{inlet})$  where  $Q$  is the discharge. L/W = Length/Width. Note that the inside of pond NUG4 was not sampled (see text); distance = the distance between the pond and the stream source. Connection indicates if the pond is connected or not to the stream. NA = Not Applicable.

Catchment	Montoussé (MON)		Mican (MIC)				Nuguet (NUG)			
Area (ha)	325		277				116			
Slope (%)	6.59 ± 3.10		4.44 ± 2.49				4.01 ± 2.56			
Stream length (m)	900		3595				2550			
Stream elevation (m)	226.4–192.7		220.6–149.0				190.4–146.0			
Pond	MON1	MON2	MIC1	MIC2	MIC3	MIC4	NUG1	NUG2	NUG3	NUG4
Longitude	1.0936	1.0773	1.0762	1.0547	1.0509	1.0460	1.0073	1.0145	1.0157	1.0246
Latitude	43.5480	43.5513	43.5656	43.5696	43.5699	43.5715	43.5815	43.5813	43.5810	43.5818
Distance	NA	0.644	0.068	0.598	0.691	0.789	0.075	0.306	0.329	0.627
Slope (%)	2.72	4.49	1.81	3.94	1.03	1.46	1.46	1.62	3.15	1.63
Area (m <sup>2</sup> )	80	1050	4200	2500	1566	18000	480	550	3800	3641
Depth (m)	0.526	1.602	1.570	1.228	0.844	3.567	0.478	0.657	2.863	2.934
Volume (m <sup>3</sup> )	23.0	1520.7	8515.7	2183.7	1123.3	62213.3	169.3	293.3	10144.7	5481.3
Shape (L/W)	1.525	1.697	1.201	1.293	1.584	1.657	1.831	1.213	1.772	3.183
HRT (day, 2016)	NA	41.8	1202.0	186.8	13.5	1956.7	26.8	41.4	1151.1	379.9
YC	1970	1970	1977	1960s	late 1970s	1965	before 1970s	1970s	early 1980s	1995
YD	Never	2015	Never	1999	Never	1985	1980s	1990s	(c)	Never
Connection	Isolated	Connected	Connected	(a)	Connected	(b)	Connected	Connected	Connected	Connected

<sup>a</sup> This pond is disconnected during high floods periods with large loads of suspended matters and connected the rest of the time. It was connected to the Mican stream while sampling.

<sup>b</sup> It was disconnected unless the owner opens its valve linked to the channel very scarcely, not in flood conditions. It was disconnected while sampling.

<sup>c</sup> Only the border was dredged a little.

## 2.2. Sampling strategies and in situ measurements

Water and sediment samples were collected in 2016 on 15 March (MON1 and MON2), 18 March (MIC1 and MIC2), 23 March (MIC3 and MIC4), and 4 April (three ponds in NUG: NUG1 to NUG3), during the late wet season in the region. No significant flood events occurred either before or during sampling, and thus the water discharge during the time corresponded to a recession flow period, which was representative at that period of the year (Perrin et al., 2008; Paul et al., 2015; Ponnou-Delaffon et al., 2020).

### 2.2.1. Water

Stream water was sampled at the stream inlet (IN) and outlet (OUT) of each pond. Samples were collected from open-channel surface water (<5 cm) with great attention paid to avoiding both the stagnant water zone and contact with the stream sediment or floating debris. Pond water was sampled using a boat in the centre of each pond (CP), which was supposed to be the most representative mixing point, and within the first 30 cm of surface water in the same way for all the ponds, since several ponds are very shallow with a water depth of less than 1 m. Temperature (T, °C), pH, dissolved oxygen (DO, mg L<sup>-1</sup>), and

conductivity (EC,  $\mu\text{S cm}^{-1}$ ) were measured at each sampling point using a WTW Multi 3420 multi-parameter portable meter, which was calibrated in the laboratory before measurement. Water was collected in HDPE bottles and stored and transported on ice in a cooler to the laboratory. After arrival in the laboratory, water samples were filtered through 0.22  $\mu\text{m}$  cellulose acetate membranes (Sartorius, Germany). All the filtered and unfiltered samples were stored in a cool room at 4 °C until analysis. The discharge of the stream at the inlet and outlet ( $Q$ ,  $\text{L s}^{-1}$ ) was measured using a simple bucket method or a flow meter depending on the stream situation.

### 2.2.2. Sediment

Stream sediments at the inlet/outlet of the ponds were grabbed from the surface layer of sediments (<4 cm, beneath the water-sediment interface) by hands in sterilized non-powder gloves and then sealed in straight containers (180 mL). Along the flow direction, the number of sediment sampling points depended on the surface area of each pond: three for the largest ones [upper part (UP), central part (CP) and lower part (LP)] and two (UP and LP) or one (CP) for the smallest ones. A straight core sampler, used from a boat, was inserted vertically into the benthic sediment and then sealed by a rubber stopper at the tail end to obtain a core of sediment. Using a ceramic knife, the surface layer (S) of the sediment (0–4 cm) was extracted from each core and the rest was sliced into one or two layers [called deeper (D) and bottom (B) layers] according to the sedimentation and sediment colours, which were believed to represent different oxic and organic conditions. Three replicate cores were collected at each sampling point. The storage method was the same as for water samples during transportation to the laboratory. Once in the laboratory, some subsamples were dried at 40 °C for texture analysis. Other sets of wet subsamples were preserved at 4 °C for denitrification assay experiments and other physico-chemical analyses, which were performed immediately after arrival at the laboratory, taking into consideration the degradation of nitrogenous ions in sediments caused by bacterial activities. Finally, another set of subsamples were kept at –20 °C prior to DNA extraction and microbiological analysis.

The dry sediments were gently homogenized in an agate mortar and then were sieved into three fractions [a fine fraction (<63  $\mu\text{m}$ ), a coarse fraction (63  $\mu\text{m}$ –2 mm) and a coarser fraction (>2 mm)] using a nylon screen. Each fraction was weighed and kept in a dry place for further analyses.

### 2.3. Physicochemical analysis of waters and sediments

Bicarbonate alkalinity ( $\text{mg L}^{-1}$ ) was analysed by the acid titration method on unfiltered water samples. Nitrate concentration ( $\text{NO}_3^-$ ,  $\text{mg L}^{-1}$ ) was determined by ion-chromatography (Dionex ICS-5000). A Shimadzu TOC-5000 analyser was used to measure dissolved organic carbon (DOC,  $\text{mg L}^{-1}$ ).

Sediment texture was determined by a laser diffraction particle distribution analyser (LA-960, HORIBA). Two fractions were used in the texture analysis, the fine fraction (<63  $\mu\text{m}$ ) and the coarse fraction (63  $\mu\text{m}$ –2 mm). The sediment remaining in the serum bottle after the denitrification assay was dried at 105 °C to a constant weight. The dried material was used to measure the sediment dry bulk density (DBD,  $\text{g cm}^{-3}$ ) and water content (WC, %). Wet sediment subsamples were used to determine the oxidation - reduction potential (ORP, mV) with an ORP probe coupled with the WTW Multi 3420 multi-parameter portable meter. The organic carbon content ( $C_{\text{org}}$ , %) and organic nitrogen ( $N_{\text{org}}$ , %) were determined using an element analyser (Flash2000, ThermoFisher) after decarbonation with 1 mol  $\text{l}^{-1}$  HCl on a heat block (40 °C) until the complete disappearance of effervescence. The concentration of added HCl has been proven to be reliable for obtaining accurate organic carbon measurements in calcareous sediments (Van Iperen and Helder, 1985) like in the study area (Bur et al., 2009).

### 2.4. Potential denitrification rate (PDR) analysis

We applied the commonly used acetylene ( $\text{C}_2\text{H}_2$ ) block technique (Hynes and Knowles, 1978), as described in Iribar et al. (2015).

First, 20 ml of sediment was put into a 125 ml serum bottle, and then the incubation solutions (100  $\text{mg N L}^{-1}$  of  $\text{KNO}_3$  and 50  $\text{mg C L}^{-1}$  of  $\text{CH}_3\text{COONa}$ ) were added into the bottle. After sealing with a septum cap, the headspace of the bottle was purged using nitrogen gas at a speed of 4  $\text{L min}^{-1}$  in 15 min. The serum bottle was then stored in a thermostatic incubator at 13 °C (value representing the average annual air temperature in the field) for 3 h. Meanwhile, it was shaken every hour, firstly to make the reaction homogenous and secondly to guarantee gas-liquid equilibrium. After 3 h, 0.2 mL of gas was extracted from the headspace of the serum bottle and then transferred into a gas chromatograph (GC) equipped with an electron capture detector for the determination of  $\text{N}_2\text{O}$ .

After the injection into the GC, a further 7 mL of gas was extracted from the headspace of the serum bottle and 15 mL of  $\text{C}_2\text{H}_2$  was injected into the bottle. The purpose of the extraction of 7 mL gas was to keep the balance of atmosphere in the bottle. The bottle was stored in a thermostat incubator at the same temperature for 3 h. After the incubation period, 0.1 or 0.2 mL of gas from the headspace, depending on the concentration of  $\text{N}_2\text{O}$ , was injected into the GC. Before injection of the gas into the GC, the serum bottle was vigorously shaken for 2 min to ensure the equilibrium of the gas and water phases. The  $\text{N}_2\text{O}$  emission rate and PDR were expressed as the same unit, microgram of N by gram of dry sediment by hour.

### 2.5. Molecular analysis

In order to identify a potential link between the presence of genes and the denitrification rate, twelve sediments from five ponds (MON2, MIC1, NUG1, NUG2, and NUG3) were selected to perform the qPCR assay according to the great spatial variability of their PDR values (see section 3.2.2), especially for the highest PDR found in MON2 and the relatively low PDR in MIC1. Eleven samples were from surface layers, and one (NUG3-CP-D) was from the deeper layer. The selection proceeded after the denitrification rate measures and considered a large range of PDR conditions (low and high values from upstream ponds, MON2 and MIC1, respectively) and the potential influence of the succession of ponds from upstream to downstream (NUG1 to NUG3, respectively).

According to the method set up by several authors (see references listed in SM Table 1), duplicate DNA samples of each sediment were extracted from an approximately 0.5 g aliquot of sediment using a NucleoSpin® Soil kit (Macherey-Nagel GmbH & Co. KG), according to the manufacturer's instructions. A final elution volume of 60  $\mu\text{L}$  was used. The quantity and quality of DNA isolated were determined using a NanoDrop spectrophotometer (ThermoFisher Scientific). The abundance of total and denitrifier communities was expressed by the gene copies per gram of sediments for 16S rRNA, and *nirS*, *nirK*, and *nosZ* denitrification genes, respectively.

During the denitrification process, first  $\text{NO}_3^-$  is reduced to nitrite ( $\text{NO}_2^-$ ) and then the reduction of nitrite to nitric oxide (NO) is catalysed by two distinguishing nitrite reductases, a cytochrome  $cd_1$  encoded by *nirS* or a Cu-containing enzyme encoded by *nirK* (Zumft, 1997). The last step ( $\text{N}_2\text{O} \rightarrow \text{N}_2$ ) is catalysed by nitrous oxide reductase encoded by *nosZ* (Iribar et al., 2015).

SM Table 1 provides the list of primers and thermocycling programs used for each gene. In a preliminary test, DNA dilution series of 10 were made to detect inhibitory effects and find the optimum concentration for analysis by qPCR. For each gene studied, a standard was used as reference. The standard is a manufactured product (obtained from Eurofins) produced by ligation of a specific version of the gene of concern into the synthetic plasmid pEX-A128 (2450 base pairs, Eurofins). The reaction mixture (10  $\mu\text{L}$ ) consisted of 5  $\mu\text{L}$  of SsoAdvanced Universal SYBR Green

Supermix (BioRad, containing the DNA polymerase, appropriate buffer, dNTPs and the SybrGreen chemical which stains double-stranded DNA), the appropriate primers, and DNA templates (sample or standard). Each reaction was run in triplicate in 96-well plates using a CFX96 Touch™ Real-Time PCR Detection System (BioRad). Gene quantification was based on SybrGreen fluorescence emitted when associated with double-stranded DNA. After the qPCR run for all gene analyses, the efficiency of the amplification was assessed using the standard curve slope, and the specificity of amplification was assessed through the melting curve.

## 2.6. Statistical analysis

The statistical analysis was conducted in R (version 3.4) for the Kruskal-Wallis test and the post hoc Dunn's test for multiple-variable analysis and difference comparisons between various samples. Pearson correlation coefficients were used to evaluate the correlations between measured parameters, which included denaturation enzyme activity.

Principle component analysis (PCA) was carried out based on z-scored data to investigate the relationships between denitrification rate and other physicochemical and environmental variables for the datasets taken together and for ponds and streams separately. Multiple linear regressions were performed to identify the main variables that could explain PDR in stream and pond sediments, respectively. To reach the normal distribution of PDR, a log-transformation was applied. Other variables were not transformed since they met the normality. Independencies (sediment, water, and environmental variables) were initially selected to avoid overfitting and multicollinearity based on the correlation matrix and the all-subsets regression method (Miller, 1984). This method performs an exhaustive search for the best subsets of the various variables for predicting the target dependent variable through an efficient branch-and-bound algorithm. The algorithm returns a best model of each size; thus, the results avoid a penalty model considering the model size. Finally, the analyser can select the proper set of variables with the best fitness according to a number of returned models of each size and the specialist knowledge. The all-subsets regression was conducted by the function of "regsubsets" from the "leaps" package in R.

The statistical figures proposed in this article were mainly generated via the ggplot2 package in R.

## 3. Results

### 3.1. Water characteristics

Waters from the catchments MON, MIC, and NUG had similar cation and anion compositions (Table 2 and SM Table 2) and distributions according to the Piper diagram (SM Fig. 1). Those waters draining carbonate molassic bedrock were well oxygenated (with a lower value for the NUG4 stream outlet), neutral, and dominated by  $\text{Ca}^{2+}$  and  $\text{HCO}_3^-$  (Perrin et al., 2008; Ponnou-Delaffon et al., 2020). DOC concentrations were in the range of values registered in the area (Paul et al., 2015), as was the water temperature (Table 1 and SM Table 2; Ponnou-Delaffon et al., 2020), which showed a higher mean value in NUG due to the lower stream discharge compared to MIC. The average  $\text{NO}_3^-$

**Table 2**

Physicochemical characteristics of waters (ponds and streams) in Mican, Nuguet and Montoussé catchments. n is the number of samples. T: water temperature; DO: dissolved oxygen; Cond: conductivity; DOC: dissolved organic carbon;  $\text{NO}_3^-$ : nitrate concentration.

	Mican (n = 12)	Mean	Nuguet (n = 11)	Mean	MON1 (n = 1)	MON2 (n = 3)	Mean
	Range		Range			Range	
T °C	8.7–12	10.63 ± 0.98	12.5–15.2	13.43 ± 0.86	9.70	10.3–12.3	11.53 ± 1.08
pH	7.76–8.29	8.06 ± 0.16	7.65–8.17	7.89 ± 0.15	8.07	7.88–8.2	8.08 ± 0.18
DO, mg L <sup>-1</sup>	8.83–16.14	11.1 ± 1.87	5.09–12.75	9.39 ± 2.68	13.87	9.88–13.8	11.66 ± 1.99
Cond, $\mu\text{S cm}^{-1}$	507–858	766.42 ± 97.13	701–879	800.73 ± 59.55	452	586–632	604 ± 24.58
DOC, mg L <sup>-1</sup>	2.16–4.32	2.71 ± 0.72	1.65–5.97	3.13 ± 1.37	5.60	1.69–3.77	2.86 ± 1.06
$\text{NO}_3^-$ , mg L <sup>-1</sup>	3.07–44.92	29.39 ± 13.96	11.4–60.56	34.02 ± 16.13	22.87	59.84–61.46	60.63 ± 0.81

concentrations in waters from the two catchments MIC and NUG were close, with the highest values for the upstream ponds (MON2, NUG1, MIC1; Table 2), except for MON1 (an upstream impluvium unconnected to the stream).

### 3.2. Sediment characteristics

#### 3.2.1. Physicochemical characteristics

Sediment physicochemical characteristics are summarized in Table 3. On average, sediment was anoxic, unlike waters, but with a large ORP range (minimum value in NUG1-IN to maximum value in MIC4-OUT), while DBD was variable with an average of  $0.67 \pm 0.19 \text{ g cm}^{-3}$ . No difference was observed between catchments for both parameters but ORP was significantly higher in ponds than in streams ( $p < 0.05$ ) and higher in surface layers than in deep layers ( $p < 0.01$ ), and the reverse was true for DBD. Pond sediments contained more water in surface layers than in deeper ones and more than stream sediments ( $p < 0.01$ ).  $\text{C}_{\text{org}}$  and  $\text{N}_{\text{org}}$  were in the range of what was found in sediments and soils from the same region (Bur et al., 2009). A detectable difference between pond sediment depths (deeper > surface,  $p < 0.05$ ) was observed only for  $\text{N}_{\text{org}}$ , while  $\text{C}_{\text{org}}$  only differed significantly between sediment types (streams > ponds,  $p < 0.01$ ). Finally, the silty-clayey texture of the sediments did not differ between catchments or between pond layers (Table 3). Clay and sand contents were significantly higher and lower in pond and stream sediments ( $p < 0.01$ ), respectively, while silt did not differ.

#### 3.2.2. Potential denitrification rate (PDR)

PDR was always detected in all sediments from the three catchments (Fig. 2) in the order  $\text{MON} > \text{NUG} > \text{MIC}$ , with an average of  $0.218 \pm 0.328 \mu\text{g N g}^{-1} \text{ dry sediment h}^{-1}$ . It also showed a great heterogeneity extending from 0.00126 (MIC2-IN) to 2.19 (MON2-OUT) (Fig. 2), with the highest PDR values in upper catchment ponds (MON2, especially at the stream outlet, Fig. 2B and NUG1, stream inlet and outlet), with the exception of the stream outlet of NUG4. In general, sediments from MIC exhibited a poor PDR performance (significantly different from the other catchments; Kruskal-Wallis test with post hoc Dunn's test,  $p < 0.0001$ ), with the highest values in MIC1 and MIC3 ponds. Even PDR in MON1 (the isolated impluvium) exceeded these values.

The mean and the standard deviation of PDR in sediments from streams were higher than in ponds (except for MIC3 and MIC1,  $p = 0.427$ , Fig. 2Aii and NUG1, Fig. 2C). In the ponds, PDR in surface layers ( $0.305 \pm 0.337$ ) was greater ( $p < 0.001$ ) than in deeper sediments ( $0.118 \pm 0.063$ ) (Fig. 2Aiii). It should be noticed that as NUG2 and NUG3 were very closely connected, the PDR value in NUG3-IN was considered the same as in NUG2-OUT (Fig. 2C). Moreover, due to the analytical limitation regarding the coarse nature of the sediment, PDR could not be analysed in NUG3-OUT. As already stated, only input and output stream sediments were sampled in NUG4.

### 3.3. Relationships between variables

As a synthesis of investigations done using the Pearson correlation

**Table 3**

Physicochemical characteristics of sediments from ponds and streams taken together or separately, taken by catchment and by depth (surface and deep layers) of the sediment cores of the ponds. The asterisk (\*) highlights the significant difference between different kinds of sediments ( $p < 0.05$ ). n is the number of samples; ORP: Oxidation - Reduction Potential; DBD: Dry Bulk Density; WC: Water content; Norg: Organic nitrogen content; Corg: Carbon Organic content.

	Units	All sediments (n = 49)			Catchment								
					Montoussé (n = 9)			Mican (n = 22)			Nuguet (n = 18)		
		min	max	mean	min	max	mean	min	max	mean	min	max	mean
ORP	mV	-228.6	123.1	-71.2 ± 82.0	-175.5	75.7	-47.3 ± 75.2	-191.9	123.1	-67.2 ± 76.5	-228.6	94.7	-87.9 ± 89.4
DBD	g cm <sup>-3</sup>	0.36	1.32	0.67 ± 0.19	0.36	1.32	0.68 ± 0.26	0.39	0.98	0.65 ± 0.17	0.43	1.06	0.70 ± 0.16
WC	%	38.6	221	112.9 ± 39.1	38.6	221	122.0 ± 49.9	56	206.7	115.8 ± 37.8	51.7	181.6	104.6 ± 32.8
Norg	%	0.11	0.36	0.19 ± 0.05	0.13	0.36	0.22 ± 0.06	0.11	0.31	0.18 ± 0.05	0.14	0.33	0.18 ± 0.05
Corg	%	1.02	4.21	1.71 ± 0.74	1.19	3.6	1.86 ± 0.67	1.02	3.92	1.58 ± 0.67	1.08	4.21	1.81 ± 0.83
Clay	%	11.03	51.06	32.2 ± 11.38	22.47	39.52	31.35 ± 5.14	17.88	50.13	34.64 ± 10.61	11.03	51.06	29.51 ± 13.95
Silt	%	37.6	73.51	55.58 ± 6.65	55.95	69.44	60.05 ± 4.52	44.99	73.51	55.64 ± 6.78	37.6	67.02	53.13 ± 6.27
Sand	%	0	51.37	12.22 ± 13.37	3.93	17	8.60 ± 4.26	0.57	35.76	9.72 ± 12.63	0	51.37	17.36 ± 15.87
	units	Type Pond (n = 31)			Stream (n = 18)			Layer Surface (n = 15)			Deeper (n = 14)		
		min	max	mean	min	max	mean	min	max	mean	min	max	mean
ORP	mV	-103.3	94.7	-46.1 ± 54.2*	-228.6	123.1	-114.41 ± 102.06*	-103.3	94.7	-13.13 ± 57.89*	-102.6	-20.4	-74.76 ± 25.35*
DBD	g cm <sup>-3</sup>	0.39	0.94	0.62 ± 0.12*	0.36	1.32	0.77 ± 0.23*	0.39	0.9	0.56 ± 0.11*	0.53	0.94	0.67 ± 0.11*
WC	%	63.9	206.7	122.11 ± 32.43*	38.6	221	96.71 ± 44.48*	66.2	206.7	140.32 ± 31.34*	63.9	135.6	104.93 ± 21.37*
Norg	%	0.14	0.23	0.18 ± 0.03	0.11	0.36	0.21 ± 0.08	0.15	0.23	0.19 ± 0.03*	0.14	0.21	0.17 ± 0.03*
Corg	%	1.02	2.09	1.41 ± 0.26*	1.18	4.21	2.27 ± 0.97*	1.2	2.09	1.51 ± 0.25	1.03	1.82	1.34 ± 0.24
Clay	%	15.19	51.06	38.64 ± 8.28*	11.03	30.28	20.47 ± 5.17*	17.36	48.15	37.61 ± 7.37	15.19	51.06	39.21 ± 9.5
Silt	%	48.94	67.02	56.89 ± 4.6	37.6	73.51	53.18 ± 8.86	51.03	67.02	57.64 ± 4.38	48.94	64.45	56.16 ± 4.94
Sand	%	0	21.98	4.47 ± 4.98*	5.35	51.37	26.35 ± 12.24*	0.82	15.61	4.75 ± 4.02	0	21.98	4.63 ± 6.05

matrix (SM Table 3), three PCAs were performed on water and sediment parameters considering all samples (n = 31), streams (n = 16), and ponds (n = 14, MON1 was not included since it is an isolated impluvium) considered separately (Fig. 3A, B, C, respectively). The input variables in the PCA included (1) PDR and N<sub>2</sub>O as the denitrification rates; (2) pH, ORP, water content, organic carbon, organic nitrogen, clay, and silt content as the sediment physicochemical characteristics; (3) water temperature, pH, DOC, and NO<sub>3</sub><sup>-</sup> concentration as water properties. Additionally, stream discharge was introduced to the PCA for stream sediments. Pond slope, hydraulic retention time, pond length/width ratio, and pond depth were taken into consideration for PCA based on pond sediments. Both Bartlett's test of sphericity and the determinant test showed that the datasets were suitable statistically to perform the further PCAs.

### 3.3.1. Principal component analysis (PCA)

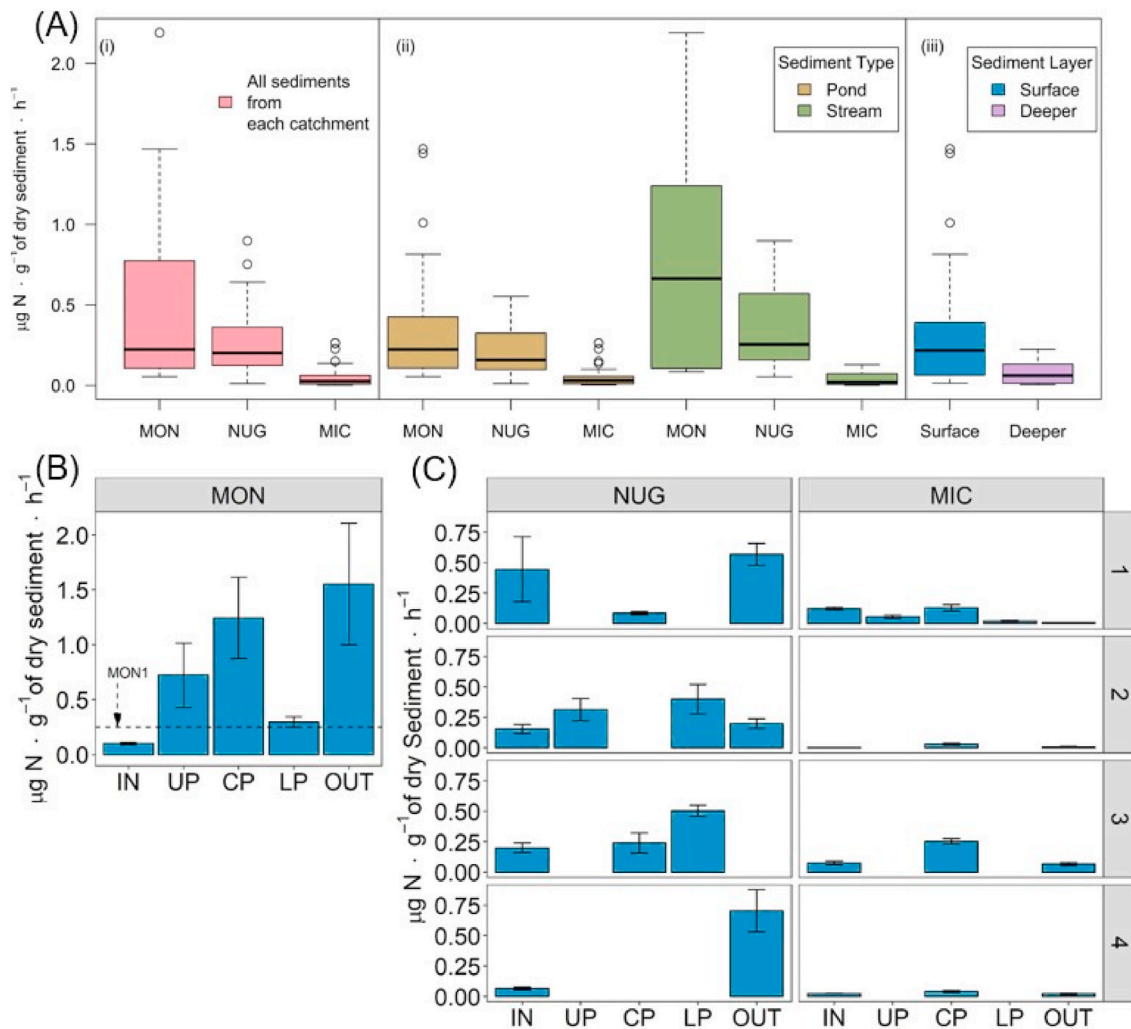
**3.3.1.1. All sediments.** All sediments included both stream and pond sediments considered together. For all sediments, four principal components (PC) explained the majority of the total variance (71.1%). The first two components (PC1 and PC2) were the more explicative ones for the whole data set (25.0% and 20.8% of the total variance, respectively, Fig. 3A and SM Table 4).

The component 1 was mainly driven by biochemical variables including Norg, Corg and PDR (in decreasing order), although to a lesser extent WC also contributed to PC1 (SM Table 5). The majority of samples scored positively, while only seven samples scored negatively (the lowest and highest values for MON2-OUT and MIC4-OUT, respectively). The component 2 mostly gathered physicochemical variables such as clay content, water pH, sediment ORP, sediment pH, silt content and WC, in decreasing order. Although N<sub>2</sub>O was associated to this component, the significance of the correlation was much lower compared to the physicochemical variables listed above. Most pond sediments scored positively and stream sediments negatively (except MON2-OUT). MON2-CP-S and NUG1-IN scored the highest and the lowest, respectively. The majority of pond sediments were clustered in Quadrant I, to

which water content (-), Norg (-), and Corg (-) made the most contribution. In addition, N<sub>2</sub>O was rather strongly associated with PC3 along with NO<sub>3</sub><sup>-</sup> concentration (+) and DOC (-), even if the explained variance (13.5%, SM Table 4) for this component was lower than the first two components. MIC1 and MIC4 sediments scored the highest values, while MON2 sediments scored the lowest negative ones (SM Fig. 2). PC4 (11% of the total variance) represented mainly DOC and water temperature.

**3.3.1.2. Stream sediments.** In the PCA for stream sediments (Fig. 3B), four principal components (PCs) explained the majority of the total variance (77.9%, SM Table 4), with PC1 and PC2 as the most explicative ones (35.2 and 17.3%, respectively, Fig. 3B). As for all sediments, the same variables including N<sub>2</sub>O mostly contributed positively to PC1 (Fig. 3A) and to PC2 (except N<sub>2</sub>O, pH and WC). They were negatively related to PC2, while T<sub>w</sub> was positively related (Fig. 3B and SM Table 5). The majority of samples were clustered negatively in PC1 (Fig. 3B right), whereas four samples were positively scattered (MIC1-OUT, NUG1-OUT, NUG4-OUT, and particularly MON2-OUT). Except for six samples, most individuals were in the positive sector of axis 2, with the highest scores for NUG1-IN, NUG4-IN and particularly NUG4-OUT, and the lowest ones for MON2-IN, MON2-OUT and MIC1-IN. Components 3 and 4 (13.5 and 11.9% of the variance, respectively) were mainly explained by discharge, NO<sub>3</sub><sup>-</sup>, and pH of water and sediment. PDR was partially associated with PC4, in an opposite position to discharge (SM Table 4 and SM Table 5), and it mainly discriminated the sediments in MIC and NUG (highest value in MIC1-OUT and lowest negative value in NUG1-IN, SM Fig. 2).

**3.3.1.3. Pond sediments.** The first four components reached a high score of 80.6% of the total variance (Fig. 3C and SM Table 4), with 29.5 and 22.8% explained by PC1 and PC2, respectively. PC1 was mostly contributed by water NO<sub>3</sub><sup>-</sup>, C<sub>org</sub>, HRT, N<sub>org</sub> and DOC and secondarily by PDR and N<sub>2</sub>O in a decreasing order (SM Table 5), with only HRT and DOC negatively related (Fig. 3C and SM Table 5). PC2 was mainly positively composed of clay content and, negatively related, to water



**Fig. 2.** Potential denitrification rates (PDR) in stream and pond sediments from the three catchments: (A) PDR values (i) in all stream and pond sediments for each catchment, (ii) separately in stream and pond sediments of each catchment, (iii) in two different depths of pond sediment cores. (B) and (C) indicate individual PDR values in each sampling site in the three catchments: (B) Montoussé [two ponds MON1 (dashed line) and MON2], (C) Nuguet (NUG) and Mican (MIC): vertically from upstream (No. 1) to downstream (No. 4). IN: stream pond inlet, UP: upper part of a pond, CP: pond centre part, LP: lower part of a pond, OUT: stream pond outlet.

temperature, silt content, and L/W (length/width of a pond) in decreasing order (SM Table 5). Sediments from three high-HRT ponds (MIC1, MIC4, and NUG3) scored negatively along PC1, with the highest score for MON2-CP-S. Among them, the highest and the lowest scores were for MIC1 and NUG3 in PC2, respectively. Other sediments were clustered in Quadrant I and close to the centre. One can observe that except for MIC4, surface layers of pond sediments were more linked to the axis PC1 or PC2 than to deeper ones.

Despite PC3 explained a lower variance score than the two first components (17%), PDR,  $N_2O$ , and pH of sediment and water, as well as the pond slope and to a lesser extent DOC, were well represented by this axis. Finally, ORP was linked to axis 4 (11% of the variance) with sediment pH, water content, and pond slope.

### 3.3.2. Relationship between PDR and $N_2O$

$N_2O$  emission rate was positively related to  $\log_{10}$  PDR ( $N_2O = (\log_{10} \text{PDR} + 1.98)/482$ ,  $R^2 = 0.64$ ,  $p < 0.0001$ ,  $n = 36$ ; Fig. 4), considering all sediments together if the nine labelled sediments with high  $N_2O$  were not taken into consideration. These nine samples were out of the linear relationship with higher  $N_2O$  emission rate compared to the dotted linear regression line, particularly for MON2. If NUG is separated from MON + MIC (the two closest catchments with the highest slope), the relationships are different: a linear type (dashed line) for the former and

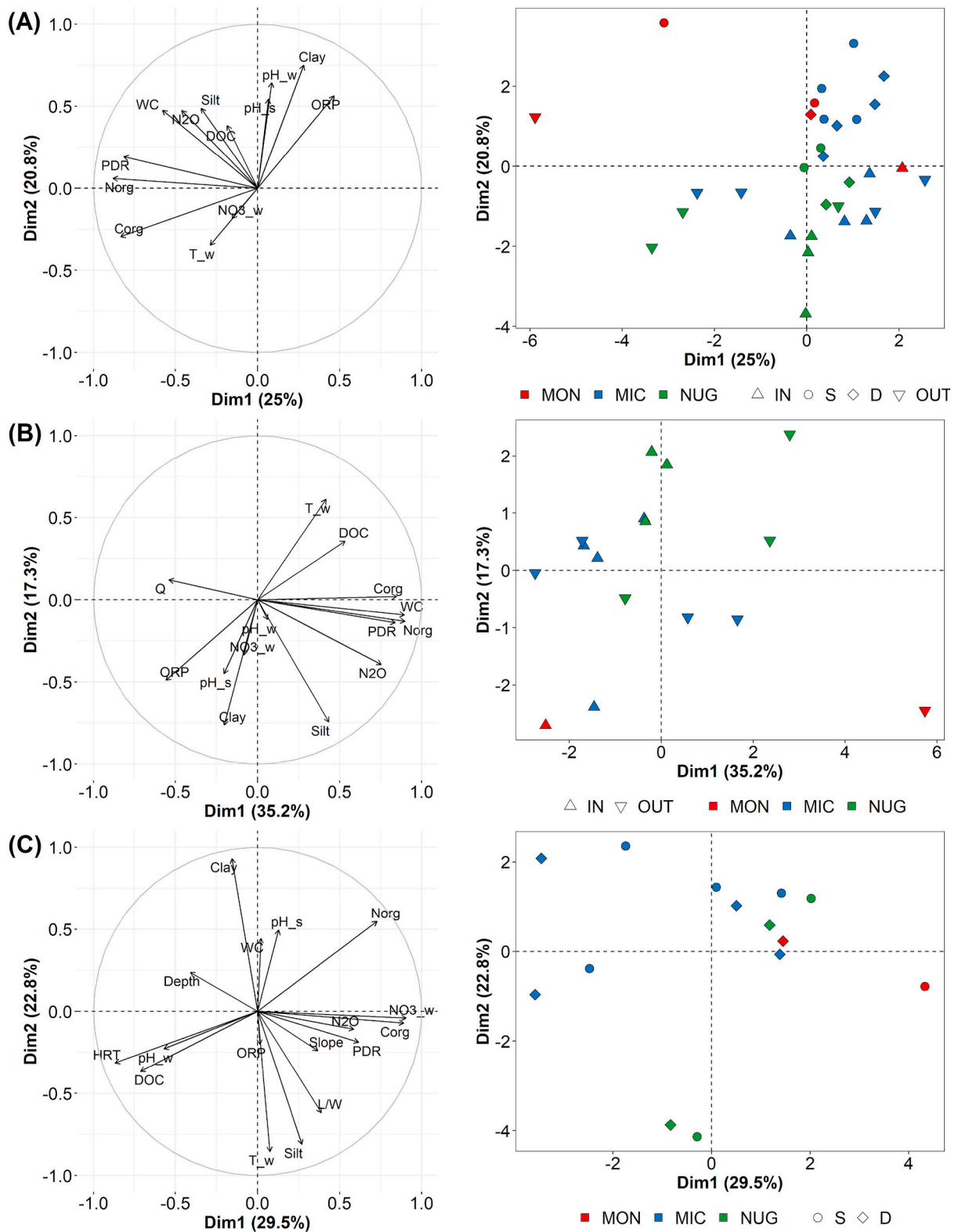
an exponential type for the latter group (solid line) (Fig. 4; see the respective equations in the legend).

### 3.4. qPCR assay for denitrification genes in pond sediments

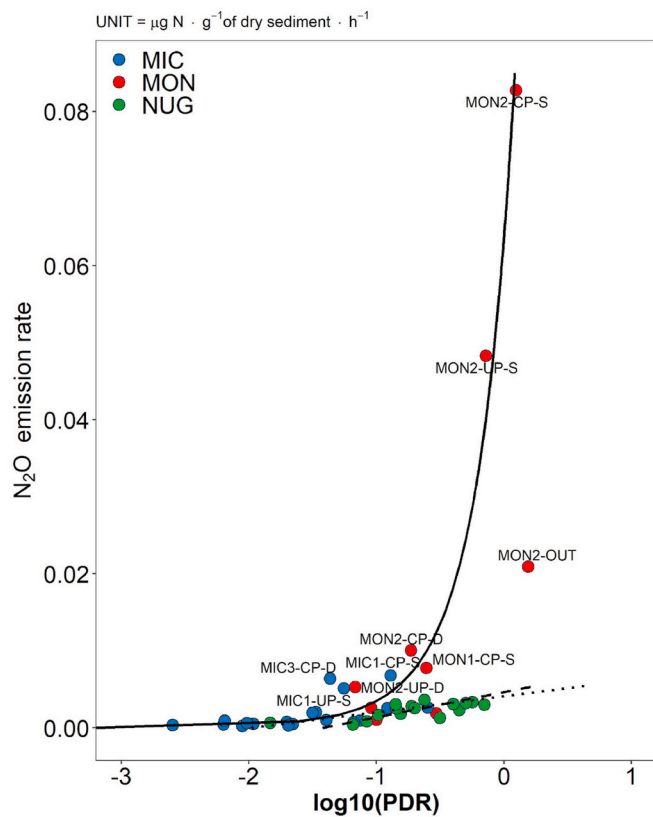
Among the 12 sediments selected from the five ponds, the copy numbers of the 16S rRNA gene had a mean value of  $5.83 \times 10^{10} \pm 2.46 \times 10^{10}$  copies  $g^{-1}$  dry sediment, from  $2.63 \times 10^{10}$  (MIC1-UP-S) to  $1.14 \times 10^{11}$  (NUG1-CP-S). Three denitrifier genes (*nirK*, *nirS*, and *nosZ*) were detected in all analysed samples (Table 4; SM Fig. 3A), but with significantly different abundances from each other ( $p < 0.01$ , SM Fig. 3B). Generally, *nosZ* was the most abundant gene and represented a ratio of *nosZ* to 16S rRNA of  $21.5 \pm 8.8\%$  followed by *nirS* ( $11.5 \pm 3.4\%$ ) and *nirK* ( $2.4 \pm 0.5\%$ ) (Table 4). In the deeper layer of NUG3 (the only deeper layer of pond sediment analysed for genes), the denitrifier abundances were lower in absolute values as well as relative to the whole gene abundances (SM Fig. 3C).

A two-by-two Pearson's correlation matrix was performed (SM Fig. 3) since the limited number of samples investigated for microbiological analysis did not allow a strong multivariate analysis, as done above with PCA. PDR was related to  $N_2O$ ,  $C_{org}$ , and  $NO_3^-$  in waters (SM Fig. 4) consistently with the results above, but considering this dataset, no significant relationship with the abundance of each denitrifier (SM





**Fig. 3.** Principal component analysis (PCA) combining the first two principal components (PC1 and PC2; variable loadings (left) and individual scores (right)). (A) All sediments; (B) Stream sediments; (C) Pond sediments. For variable names, refer to the legend in Table 3 and for sites refer to Section 2.2. For the individual scores (right column), the colour palette distinguishes the catchments, and the sediment types are highlighted by shapes. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)



**Fig. 4.** Relationship between  $N_2O$  emission rate and  $\log_{10}$  potential denitrification rate (PDR) in stream and pond sediments from the three catchments. (1) the dotted line: the general pattern without the nine labelled outliers ( $N_2O = (\log_{10} PDR + 1.98)/482$ ,  $R^2 = 0.64$ ,  $p < 0.0001$ ,  $n = 36$ ); (2) the dashed line: NUG sediments ( $N_2O = (\log_{10} PDR + 1.42)/303.3$ ,  $R^2 = 0.53$ ,  $p < 0.001$ ,  $n = 16$ ); (3) the solid line: MON + MIC ( $N_2O = \sqrt[0.0954]{(3.14 - \log_{10} PDR)/2.41}$ ,  $R^2 = 0.71$ ,  $p < 0.0001$ ,  $n = 29$ ).

**Fig. 4).**

The four gene abundances were positively related to each other with the exception of *nosZ* and *16S rRNA* ( $p < 0.05$ , SM Fig. 4). Only *16S rRNA* was significantly related to water DOC ( $r = -0.62$ ,  $p < 0.05$ , SM Fig. 4). The ratio of *nirK* to *16S rRNA* was positively related to *nirS/16S rRNA* and sediment ORP and was negatively associated with water  $NO_3^-$  concentration ( $p < 0.05$ , SM Fig. 4).

**Table 4**

Gene abundances and their percentages in the selected ponds. Values from 12 samples and the descriptive statistics are listed.

Sample	Abundance (copy numbers per gram of dry sediment)				Ratio			
	<i>16S rRNA</i>	<i>nirK</i>	<i>nirS</i>	<i>nosZ</i>	<i>nirK/16S rRNA</i>	<i>nirS/16S rRNA</i>	<i>nosZ/16S rRNA</i>	<i>nirK/nirS</i>
MON2-UP-S	5.11E+10	1.18E+09	5.49E+09	6.16E+09	2.4	10.7	12.0	22.5
MON2-CP-S	4.26E+10	1.01E+09	7.26E+09	5.04E+09	2.4	17.0	11.8	13.9
MON2-LP-S	7.84E+10	1.48E+09	7.58E+09	7.40E+09	1.9	9.8	11.0	18.8
MIC1-UP-S	2.63E+10	9.10E+08	3.58E+09	6.40E+09	3.1	12.2	23.6	25.3
MIC1-CP-S	7.20E+10	2.00E+09	7.12E+09	1.23E+10	2.8	10.2	17.8	28.2
MIC1-LP-S	5.74E+10	1.26E+09	9.19E+09	1.75E+10	2.6	16.6	29.8	13.2
NUG1-CP-S	1.14E+11	1.82E+09	6.63E+09	1.58E+10	1.6	6.0	13.7	26.8
NUG2-UP-S	7.22E+10	1.74E+09	1.04E+10	1.60E+10	2.8	15.4	25.6	17.7
NUG2-LP-S	5.60E+10	1.34E+09	5.53E+09	1.26E+10	2.4	10.1	22.7	24.4
NUG3-CP-S	6.61E+10	1.92E+09	8.56E+09	2.71E+10	3.0	12.4	40.4	24.9
NUG3-CP-D	3.54E+10	7.10E+08	3.02E+09	7.05E+09	2.2	9.5	23.3	23.6
NUG3-LP-S	2.85E+10	5.72E+08	2.34E+09	7.50E+09	2.1	8.0	26.8	22.6
Min	2.63E+10	5.72E+08	2.34E+09	5.04E+09	1.6	6.0	11.0	13.2
Max	1.14E+11	2.00E+09	1.04E+10	2.71E+10	3.1	17.0	40.4	28.2
mean	5.83E+10	1.33E+09	6.39E+09	1.17E+10	2.4	11.5	21.5	21.8
Sd	2.46E+10	4.76E+08	2.50E+09	6.52E+09	0.5	3.4	8.8	4.9

### 3.5. Multilinear regression model for PDR

To ensure a potential prediction of the spatial variability of PDR in response to physicochemical and environmental variables, multiple linear regressions (MLR) were applied to the potential denitrification rate (PDR) as the dependent variable in stream sediments and ponds considered separately. Only physicochemical and environmental variables were considered in the multilinear regressions since water, sediment, and geomorphological characteristics could be better predictors of the denitrification rate than the denitrifier's properties (SM Fig. 4). Indeed, the smaller number of data for the denitrifiers would weaken the model prediction. We also considered empirical models for easy-to-measure variables that are applicable for pond management and  $NO_3^-$  water quality control by stakeholders or local pond managers.

Preliminary models including all variables were able to explain 89% of the variance of PDR in stream sediments and 91% of the variance of PDR in pond sediments, respectively (Table 5). Although these preliminary models could explain a high percentage of variance of PDR, they could not be adopted since a few independent variables were unrelated or weakly related to  $\log(PDR)$ , and multicollinearity may still exist, which means the information provided by one variable may be explained by other collinear variables that make the model redundant. Hence, the all-subsets regression method was applied to construct the best model with non-redundant variables and a high percentage of the variance of dependent variables. The results of all subset's regressions are shown in SM Fig. 5.

- For stream sediments, the predictive model was:

$$(PDR) = -2.819 \cdot \rho Hs + 0.148 \cdot Clay + 0.487 \cdot Tw + 0.809 \cdot DOC + 0.060 \cdot NO3\_w - 0.001 \cdot Dist + 4.887 \quad (1)$$

with  $p = 0.002$ ,  $R^2 = 0.86$ , adjusted  $R^2 = 0.76$  (see Table 5 for units);  $n = 16$ .

- For pond sediments, two predictive models were finally set up:

$$(1) (PDR) = 0.005 \cdot ORP - 0.064 \cdot Depth + 0.010 \cdot WC + 0.417 \cdot Tw + 1.224 \cdot DOC - 0.002 \cdot HRT - 10.637 \quad (2)$$

with  $p < 0.001$ ,  $R^2 = 0.86$ , adjusted  $R^2 = 0.83$  (see Table 5 for units);  $n = 28$

$$(2) (PDR) = 0.007 \cdot ORP - 0.090 \cdot Depth + 0.026 \cdot Clay + 0.210 \cdot Tw + 0.518 \cdot DOC - 0.049 \cdot NO3\_w - 8.373 \quad (3)$$

with  $p < 0.01$ ,  $R^2 = 0.79$ , adjusted  $R^2 = 0.73$  (see Table 5 for units);  $n$

**Table 5**

Multiple linear regression results for PDR. “Preliminary” indicates the consideration of all variables and “Updated” indicates the variables selected by the all-subsets regression method; ORP: Oxidation-Reduction Potential; Depth: Depth of the pond; Tw: Temperature of the water; DOC: Dissolved Organic Carbon; HRT: Hydraulic Retention Time (days); Dist: Distance to stream source; with \*  $p < 0.1$ ; \*\*  $p < 0.05$ ; \*\*\*  $p < 0.01$ .

Variable	Stream Sediment		Pond Sediment		
	log (PDR)		log (PDR)		
	Preliminary	Updated	Preliminary	Updated 1	Updated 2
pH	-2.28	-2.819**	1.092		
ORP (mV)	0.002		0.003	0.005**	0.007**
Depth (cm)			-0.083**	-0.064**	-0.090***
Water content (%)	-0.001		0.006	0.010*	
Clay content (%)	0.195	0.148**	0.065		0.026
Silt content (%)	-0.065		0.099		
Organic carbon content (%)	0.451		-0.222		
Tw (°C)	0.342	0.487**	0.727**	0.417***	0.210**
DOC (mg L <sup>-1</sup> )	1.161	0.809**	1.816**	1.224***	0.518*
NO <sub>3</sub> <sup>-</sup> (mg L <sup>-1</sup> )	0.06	0.060***	-0.008		0.049***
Discharge (L s <sup>-1</sup> )	0.441				
Slope (%)			-0.219		
HRT (d)			-0.003***	-0.002***	
Dist (m)	-0.002	-0.001***	0.001*		
Constant	4.034	4.887*	-30.291**	-10.637***	-8.373***
Number of observations	16	16	28	28	28
R <sup>2</sup>	0.89	0.86	0.91	0.86	0.79
Adjusted R <sup>2</sup>	0.58	0.76	0.83	0.83	0.73
p value	0.16	0.002		<0.001	<0.001

= 28.

The agreement between measured and predicted values shown in SM Fig. 6 indicated that the proposed predictive models had the ability to calculate the actual PDR values in sediment using a few sets of physicochemical and environmental variables. For ponds, the first model (Eq. (2)) showed better regression results (R<sup>2</sup> and adjusted R<sup>2</sup>) than the second one (Eq. (3)). However, HRT and WC (Eq. (2)) were less accessible data for investigations and applied procedures, and thus the second one (Eq. (3)), involving more commonly measured data, was also proposed.

## 4. Discussion

### 4.1. PDR magnitude and spatial variability

PDR values exhibited a wide range and were one order of magnitude greater than the values of groundwater sediments in the Garonne river, which is the large drainage basin of the area considered (Bernard-Jannin et al., 2017). The observed spatial heterogeneity (Fig. 2A) between and within the three closely adjacent catchments is consistent with the non-uniformity observed in the groundwater sediment in the Garonne River and stream sediments in the Seine River (Bernard-Jannin et al., 2017; Garnier et al., 2010, respectively). Higher PDR values were observed for upper ponds in two catchments (MON and NUG), which behaved similarly, while PDR was very low in the third one (MIC), the ponds having lower PDR than draining streams and a higher PDR at the pond surface than at depth. This heterogeneity could be attributed to the influence of some key factors related to the physicochemical characteristics of waters and sediments, such as the organic matter content (Grebliunas and Perry, 2016) and other physicochemical factors discussed below (see 4.1.2, Burgin et al., 2010; Saggari et al., 2013).

Indeed, this was observed in a period (March) where PDR was supposed to be at lower values compared to the hot season (Birgand et al., 2007) since PDR could vary in time. The results are thus supposed to represent lowered denitrification conditions (Song et al., 2012). One must notice that various methods have been developed to measure the denitrification rate, which may contribute to the difficulty in comparing denitrification rates between different studies (Garnier et al., 2010; Groffman et al., 2006), and currently Almaraz et al. (2020) recommended a standardization of protocols by creating the Global

Denitrification Research Network (GDRN). The present study evaluated PDR considering non-limited NO<sub>3</sub><sup>-</sup> and carbon supply to sediment core samples that could reflect the potential capacity of the denitrification process under optimized conditions (Iribar et al., 2008). However, according to some authors (Seitzinger et al., 1993), the acetylene technique may only measure approximately 50% of the denitrification due to NO<sub>3</sub><sup>-</sup> from the overlying water. Our data could thus be underestimated. Yet, Well et al. (2003) mentioned that the denitrification rates obtained using <sup>15</sup>N *in situ* and using the C<sub>2</sub>H<sub>2</sub> technique in the laboratory were in the same range. These authors thus recommended measuring denitrification in the lab for large-scale investigations because of the easier application of the protocol. This gave us confidence in the PDR estimation as a comparative point of view at our studied scale and in its ability to be related to the environmental factors. Nevertheless, we only compared our data with the literature using a similar protocol.

### 4.2. Controlling factors of PDR

Considering all sediments from the three catchments, each factor taken alone was not able to explain PDR with a strong power (as expressed by the Pearson correlation matrix, SM Table 3). Nevertheless, some key factors such as the sediment water content (WC), the organic carbon content (C<sub>org</sub>), and the NO<sub>3</sub><sup>-</sup> concentration in overlying water columns were highlighted, as indicated by the positive significant correlation coefficients with PDR (SM Table 3). Indeed, sediment C<sub>org</sub> was considered as the “fuel” for denitrification (Birgand et al., 2007) and was not only the carbon source for denitrifiers (García-ruiz et al., 1998) but also a proxy for denitrifier biomass (Iribar et al., 2008). Meanwhile, PDR was associated with WC and ORP positively and negatively, respectively, which indicated that high WC could inhibit the O<sub>2</sub> diffusion in sediment particle pores, and thus providing a desirable anaerobic environment for denitrification. The similar observation for WC and PDR has been reported by other studies (García-Ruiz et al., 1998; Attard et al., 2011; Saggari et al., 2013). Moreover, hotspots of denitrification were observed in upstream ponds with high water NO<sub>3</sub><sup>-</sup> and sediment C<sub>org</sub> (MON2-OUT and NUG1-OUT; Fig. 3A). This was also consistent with the lower PDR values associated with lower sediment C<sub>org</sub> and NO<sub>3</sub><sup>-</sup> concentration in waters (as observed in the MIC catchment). The weak positive relationship between water NO<sub>3</sub><sup>-</sup> and PDR might indicate that in stream sediments, water NO<sub>3</sub><sup>-</sup> was not a very limiting factor controlling

denitrification since  $\text{NO}_3^-$  could be supplied continuously by the stream flow (SM Table 2 and SM Table 5B) in this agricultural context. This observation was similar to that of Oehler et al. (2007) in an agricultural catchment in Britany with heavy nitrogen loads. Meanwhile, the discharge could also play an important role by controlling the sediment texture and consequently  $C_{\text{org}}$  (Luo et al., 2012). A fast discharge might have washed away fine fractions in sediments, contributing to a decrease of the  $C_{\text{org}}$  content (SM Table 2 and Fig. 3B) and leading to a stronger dilution and a reduced PDR (see Mican catchment with higher discharge, PC1, SM Table 5B and Fig. 3B). This was more particularly evidenced in MIC4-OUT, which showed a relatively low clay content and  $C_{\text{org}}$  with the highest discharge (SM Table 2). Indeed, the texture in stream sediments was coarser and more heterogeneous than in pond sediments (Table 3).

A specific discussion is needed for ponds. Similar explanatory variables as for stream sediments were involved in the PDR explanation for PC 1 and 2, but some specific parameters like HRT and pond slope directly influence the PDR of pond sediments. The highest PDR was related with the lowest HRT and the highest pond slope (PC1 and PC3, Fig. 3 and SM Table 5). Indeed, these variables could indirectly affect  $C_{\text{org}}$  and  $\text{NO}_3^-$ . A long HRT meant a more stagnant hydrologic condition that could inhibit the supplementation of  $\text{NO}_3^-$ . The denitrifiers could only utilize the limited  $\text{NO}_3^-$  and  $C_{\text{org}}$  to run the denitrification process, as also indicated by the positive link between DOC and *16S rRNA* gene. Because the nutrients continued to be consumed, the denitrification process in a long-HRT pond would be less active. Nevertheless, the PDR rate in NUG3 (one of the ponds with the longest HRT) was still higher than in other long-HRT ponds (MIC1 and MIC4). Contrary to other ponds, the large amount of leaf debris in the bottom of pond NUG3 could act as an additional carbon source for the denitrification process (Hang et al., 2016). The denitrifiers in MIC1-UP and NUG3-LP (two long-HRT ponds) were the least abundant compared to other analysed short-HRT ponds (Table 4), which also reflected the importance of HRT in the denitrification process of pond sediments. However, the absence of a significant relationship between the abundance of denitrifiers and PDR did mean that they might be inactive during the rather low-temperature conditions of the study (Shrewsbury et al., 2016). In addition, the sediment dredging managed by the farmers might have influenced the denitrification process. The denitrification rate would be decreased after the dredging activity due to the removal of surface sediments enriched with denitrifiers and organic carbon (Smith and Pappas, 2007). However, MON2, which was dredged recently in 2015, still possessed a high denitrification rate. Its upper position in the catchment, the high erosion rate in the surrounding cultivated parcels, and its small scale and elongated shape along the stream connection have led to a quick sediment accumulation (Wu and Probst, 2021). With the supply of water  $\text{NO}_3^-$  due to close connection to soil water drainage (Table 2 and SM Table 2) and low HRT, a preferable denitrification environment could thus be generated soon for the growth of denitrifiers. In MIC3, which has not been dredged in the last three decades, the sediment has accumulated, leading to a shallow water depth, low HRT, and a favorable environment for denitrifiers. Therefore, the denitrification rate in MIC3 was the highest in the Mican catchment. As a result, the dredging activity should be well considered in relation to other environmental conditions.

The higher PDR rates observed in surface layers of pond sediments compared to the deeper layers, were in agreement with previous studies (Li et al., 2010). Although the deeper layer sediment had a more reduced and anoxic condition than surface layer sediment, which should favour the denitrification process (Burgin et al., 2010), the lower  $C_{\text{org}}$  and available  $\text{NO}_3^-$  limited the denitrification process in the deeper layers of sediments. The qPCR results (Table 4) consistently showed that the denitrifier abundances were lower in the deeper layer of NUG3. Although no direct relationships were found between the denitrification rates and the denitrifier abundances, the strong association between water  $\text{NO}_3^-$  concentration and *nirK/16S rRNA* potentially highlighted the

function of denitrifiers, especially for *nirK*, in mitigating the water  $\text{NO}_3^-$  as part of the denitrifying activity.

#### 4.3. Predictive models and interest from a management perspective

In such agricultural contexts, the evaluation of key explanatory factors is important to quantify and allow a good prediction of PDR on the basis of accessible parameters.

The empirical models concerning stream (Eq. (1)) and pond (Eqs. (2) and (3)) sediments have shown the importance of nutrients and some physicochemical characteristics to predict PDR in a convergent way. These parameters (*i.e.*, water  $\text{NO}_3^-$ , water temperature, water DOC, *etc.*) are known in the literature to explain PDR (García-ruiz et al., 1998; Song et al., 2012), but few studies have considered the complexity of streams and ponds taken together (Attard et al., 2011; Tuttle et al., 2014). In flowing water conditions (stream sediments, Eq. (1)), the model highlighted the importance of environmental factors such as the discharge (*i.e.*, regulated by the distance to stream source) as a key parameter influencing sediment pH and the clay component. On the contrary, in pond sediments where water was more stagnant, suitable anaerobic sediment conditions and HRT were prevalent factors (Eqs. (2) and (3)), Li and Irvin, 2007; Vymazal, 2017). The predictive models could thus identify the denitrification hotspots in both stream and pond sediments.

In a context of climate and land cover changes, the water demand in cultivated areas will probably be more and more important. Stakeholders and farmers had in mind that water storage in ponds in upstream catchments might be an interesting local solution. However, they might have potential negative influences on the water cycle (increased evaporation for example), water transfer downstream, and other ecological impacts. One major environmental issue was related to the greenhouse gas emissions such as  $\text{N}_2\text{O}$  when the denitrification process was not complete (Beaulieu et al., 2011). Indeed, the  $\text{N}_2\text{O}$  versus PDR pattern (Fig. 4) depended on the catchment characteristics (linear or exponential), with some PDR saturation limit (MON) and outlier ponds having high  $\text{N}_2\text{O}$  emission (MON and MIC). The upstream position of ponds with high  $\text{NO}_3^-$  loads might thus contribute to greenhouse gas emissions in some cases.

The interest of our detailed area investigations was that a set of field conditions in the same region was considered at a given time. Even if some previous studies found beneficial to consider only one short-period sampling in an extensive area rather than a small site under long-term monitoring as well (García-ruiz et al., 1998), one must be cautious of the study limitations.

Moreover, since denitrification might vary temporally (Chen et al., 2014), samples could thus be representative only for lower values in seasonal spring conditions. Additional campaigns in contrasting seasonal conditions would thus be advantageous at least regarding the importance of the  $\text{N}_2\text{O}$  emissions (which are more erratic to evaluate, Well et al., 2003), which could be constrained using additional *in situ* measurements of atmospheric  $\text{N}_2\text{O}$  at the pond surface. Finally, these management tools must also be combined with better agricultural practices in the drainage catchments. Among them, the limitation of fertilizer inputs and/or the choice of periods of spreading, the location of strip bands along the streams and a landsharing/landscaping approach were shown to reduce nitrogen inputs and/or improve surface water quality in the area regarding  $\text{NO}_3^-$  (Ferrant et al., 2011; Casal et al., 2019; Ponnou-Delaffon et al., 2020).

## 5. Conclusion

Our study brought new insights about the spatial variation of denitrification rate in ponds and streams at a catchment scale. A significant spatial variability of the potential denitrification rate (PDR) was observed in stream and pond sediments collected from three adjacent agricultural catchments (the Save basin, southwestern France). It was affected by several distal and proximal factors related to water and

sediment characteristics and the pond management. Regardless of the sediment type, PDR was generally controlled by sediment organic carbon content ( $C_{org}$ ). The stream sediment was additionally controlled by  $NO_3^-$  concentration in overlying water, while the pond sediment was not. Therefore, PDR was more active in the stream sediment than in the pond sediment due to the sustainable supply of “fuels” ( $NO_3^-$  and  $C_{org}$ ) brought by the stream discharge compared to the continuously consumed “fuels” in the pond sediment. Higher PDR in the stream sediment could contribute to  $NO_3^-$  mitigation, but the high discharge diluted  $NO_3^-$  and could indirectly lower the denitrification activity downstream.

Low PDR was observed with a flat pond slope and/or large ponds with long HRT, which limited the supply of “fuels” and contributed to their consumption and reduced the denitrifier abundance. The availability of  $NO_3^-$  and  $C_{org}$  also led to higher PDR in the surface pond sediment compared to the deeper pond sediment. Some ponds located in the upper stream catchments exhibited a higher PDR, while a high  $N_2O$  emission rate was also observed due to the incomplete denitrification process, which should be further estimated to get a complete nitrogen budget and perform the best management practice (dredging, position and size of pond etc.).

Meanwhile, this study presented efficient empirical models based on some key and easily measured factors to predict PDR. Therefore, the pond management can be optimized further through the combination of long-term hydrological surveys, measurements of key factors, and empirical models to limit the  $NO_3^-$  contamination in surface waters from agricultural catchments.

#### Author contribution

Conceptualization, X.W. and A.P.; methodology, X.W., A.P. and M.B.; validation, X.W., A.P. and M.B.; formal analysis, X.W.; investigation, X.W., A.P. and M.B.; resources, X.W., V.P.-S., T.C., and F.G.; data curation, X.W.; writing—original draft preparation, X.W. and A.P.; reviewing, MB; visualization, X.W; supervision, A.P; project administration, and funding acquisition, A.P. All authors have read and agreed to the published version of the manuscript.

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#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Appendix A. Supplementary data

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