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RESEARCH ARTICLE

Environmental factors determining distribution and activity of anammox bacteria in minerotrophic fen soils

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One sentence summary: Anammox was found to be diverse but poorly active in water-saturated and nitrate-bearing soil layers along a catena from an amended pasture to an ombrotrophic bog.

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ABSTRACT

In contrast to the pervasive occurrence of denitrification in soils, anammox (anaerobic ammonium oxidation) is a spatially restricted process that depends on specific ecological conditions. To identify the factors that constrain the distribution and activity of anammox bacteria in terrestrial environments, we investigated four different soil types along a catena with opposing ecological gradients of nitrogen and water content, from an amended pasture to an ombrotrophic bog. Anammox was detected by polymerase chain reaction (PCR) and quantitative PCR (qPCR) only in the nitrophilic wet meadow and the minerotrophic fen, in soil sections remaining water-saturated for most of the year and whose interstitial water contained inorganic nitrogen. Contrastingly, aerobic ammonia oxidizing microorganisms were present in all examined samples and outnumbered anammox bacteria usually by at least one order of magnitude. 16S rRNA gene sequencing revealed a relatively high diversity of anammox bacteria with one *Ca*. Brocadia cluster. Three additional clusters could not be affiliated to known anammox genera, but have been previously detected in other soil systems. Soil incubations using ^{15}N-labeled substrates revealed that anammox processes contributed about <2% to total N_2 formation, leaving nitrification and denitrification as the dominant N-removal mechanism in these soils that represent important buffer zones between agricultural land and ombrotrophic peat bogs.

Keywords: nitrogen cycling; ammonia oxidation; wetland; ecotone; generalized linear model

INTRODUCTION

Microbial nitrogen (N) transformations in soils have a major influence on agricultural production (Hofstra and Bouwman 2005), water quality (Vitousek *et al*. 1997) and the emission of greenhouse gases such as nitrous oxide (Butterbach-Bahl *et al*. 2013). Our notion, however, of microbial N-cycling and its interactions with other element cycles has changed greatly in recent

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years. Novel processes have been discovered such as denitrification by eukaryotes (Finlay, Span and Harman 1983), denitrification coupled to methane oxidation (Raghoebarsing *et al.* 2006), archaeal ammonium oxidation (Könneke *et al.* 2005), complete nitrification by a single organism (Daims *et al.* 2015; van Kessel *et al.* 2015), as well as anaerobic ammonium oxidation with nitrite (anammox) or iron (Feammox) as oxidants (Javanaud *et al.* 2011; Yang, Weber and Silver 2012).

The significance of anammox in natural environments was first recognized in marine sediments (Thamdrup and Dalsgaard 2002), oceanic oxygen minimum zones (Kuypers *et al.* 2003) and later in chemoclines of permanently stratified freshwater lakes (Schubert *et al.* 2006; Wenk *et al.* 2013). Sequences of anammox bacteria were also detected in a wide range of terrestrial ecosystems including water-saturated soils of wetlands and riparian zones, aquifers (e.g. Humbert *et al.* 2010a; Humbert, Zopfi and Tarnawski 2012; Kumar *et al.* 2017), fertilized paddies (e.g. Zhu *et al.* 2011) and agricultural soils (Long *et al.* 2013).

The quantitative significance of anammox in terrestrial systems has been investigated by comparatively few studies (Humbert et al. 2010a, 2012; Zhu *et al.* 2011; Sato *et al.* 2012; Long *et al.* 2013; Naeher *et al.* 2015; Yang *et al.* 2015; Shan *et al.* 2016; Shen *et al.* 2016 2016; Xi *et al.* 2016). They revealed that in terrestrial systems, anammox bacteria are usually less active than in marine systems, despite the greater phylogenetic diversity. Anammox activity and/or abundance appear to increase (Humbert, Zopfi and Tarnawski 2012; Meng *et al.* 2016) or to decrease with soil depth (Sher *et al.* 2012; Hui *et al.* 2017), possibly depending on anoxic conditions (Humbert *et al.* 2010b; Long *et al.* 2013) and the availability of the required substrates. For instance, the importance of anammox seems to increase with the availability of ammonium (e.g. Sher *et al.* 2012; Shen, Xu and He 2014; Shen *et al.* 2015, 2016) or nitrate (Humbert *et al.* 2010b; Yang *et al.* 2015; Shan *et al.* 2016). Other factors such as soil organic matter content, C:N-ratio and soil pH have also been found to correlate with anammox activity (e.g. Yang *et al.* 2015; Zhou *et al.* 2017). To date, however, identification of general environmental factors that constrain anammox abundance and/or activity in terrestrial ecosystems is still difficult. Possible reasons include the facts that (i) studies often focus on one type of soil, (ii) they consider only few environmental variables, and (iii) some soil types are over-represented, including agricultural soil, rice paddies and wetlands (Table 1).

In order to address this, we investigated anammox bacteria (together with other ammonia oxidizers) along a catena ranging from an amended pasture to a peat bog. This system represents an ecotone (Attrill and Rundle 2002) and acts as an important buffer zone, mitigating N-loadings from agriculture or other human activities to the environment. We investigated four different soil types that characterize this catena and related the occurrence of anammox to soil characteristics and a wide range of environmental parameters, monitored during an annual cycle. We used exploratory and predictive statistics to determine the environmental factors that explain best the occurrence and the abundance of anammox. Furthermore, anammox was compared with other N-cycling microbial metabolisms, such as aerobic ammonia oxidation (through qPCR analyses) and denitrification (through ^{15}N incubation analyses).

MATERIAL AND METHODS

Study site

The investigated catena is located close to Bellefontaine in the French part of the Jura Mountains (Fig. 1). Four different stations were selected based on the vegetation map of Gallandat (1982): an amended pasture (Soil 1; N 46°34'12.30", E 006°04'50.94", WGS84), a nitrophilic wet meadow (Soil 2; N 46°34'11.40, E 006°04'51.00"), a minerotrophic fen (Soil 3; N 46°34'8.76", E 006°04'53.28") and an ombrotrophic peat bog (Soil 4; N 46°34'6.36", E 006°04'55.20"). The underlying soils were identified according to the French soil classification system (AFES, Baize and Girard 2009). Soil 1 is a SATURATED BRUNISOL, a carbonate-rich soil with a clayey structure due to the parental material issued from Oxfordian marls (Guillaume and Guillaume 1963). Soil 2 is similar but was classified as a TYPIC REDUCTISOL (i.e. gley soil) because groundwater level fluctuations and associated reduction/oxidation processes led to the redistribution of iron and the formation of visible Fe(III)-oxide patches. Soil 3, located in the minerotrophic fen, was identified as a SAPRIC HISTOSOL (i.e. muck soil). This soil is seasonally water saturated up to the surface, leading to the accumulation of organic matter with a doughy texture. Soil 4, described as FIBRIC HISTOSOL (i.e. peat soil) is permanently water-saturated, acidic and oligotrophic. The soil organic matter has a fibrous texture and consists essentially of dead *Sphagnum* biomass. Schematic drawings of the profiles of Soils 1–4 are presented in Fig. 1C. Table 2 summarizes the general bulk soils characteristics. Soils 2, 3 and 4 are water-saturated at least part of the year. Self-constructed multilevel-piezometers (Fig. S1, see online supplementary material) were installed in these locations to sample repeatedly the interstitial water from various depths and determine the physical/chemical characteristics during an annual cycle.

Physico-chemical analysis of interstitial soil water

In Soils 2, 3 and 4, *in situ* temperature, pH and pO_2 of the free-water were measured down to 1 m below the surface every 10 cm by lowering a multi-meter (HQ40d, Hach Lange GmbH, Germany) in a piezometer tube, seven times during a complete annual cycle starting in November 2007. To determine concentrations of dissolved compounds (NH_4^+, NO_2^-, NO_3^-, Fe(II), colloidal Fe(III), S(-II) and SO_4^{2-}), soil water was sampled down to 1 m below the surface every 6.25 or 12.5 cm using self-constructed multilevel piezometers five times between February and November 2008. More details about the design of the multi-level piezometers can be found in Fig. S1, see online supplementary material. Interstitial water was withdrawn from each piezometer level using a peristaltic pump and gas-tight tubings (PharMed®, Saint-Gobain Performance Plastics, USA). The outlet of the tube was connected directly to a 60 mL syringe, allowing sample collection without any contact with atmospheric oxygen. Then, water samples were directly filtered through 0.45 μm nylon membranes and treated as follows. For nitrite determination, a sample of 6.6 mL was fixed with 3.3 mL of sulfanilamide on site and analyzed within 24 h in the laboratory using the colorimetric method of Griess (1879), with a detection limit of 0.5 μmol L^{-1}. Nitrate was quantified by a modified cadmium reduction protocol (Wood, Armstrong and Richards 1967): 100 μL of Na-borate buffer (pH 11) and one third of a NitraVer®6 Nitrate Reagent bag (Hach Lange GmbH, Germany) were added to 1 mL of water sample. The sample was agitated for 1 h and the produced nitrite was quantified using the Griess assay. Ammonium was determined by the hypochlorite–phenol reaction (Chaney and Marbach 1962), with a detection limit of 0.5 μmol L^{-1}. Fe(II) and Fe(III) were measured with the ferrozine assay (Stookey 1970) on samples fixed with HCl (1 M final concentration). Dissolved S(-II), i.e. the sum of H_2S, HS^- and S^{2-} species, was determined using the methylene-blue assay (Cline 1969) on Zn-acetate

Table 1. Literature review of anammox diversity, abundance and activity in soils where this metabolism has been detected.

Soil type	Sample site	Number of soils / samples	Brocadiaceae diversity									Abundance		Activity	References
			Proportion of Brocadiaceae population (%)†						Proportion of non-annamox sequences	Target gene	NCBI accession number (for 16S rRNA gene sequences only)	Anammox abundance (range of copies g^{-1})	Target gene	Anammox N_2 contribution (range)	
			Anammo-xoglobus	Brocadia	Jettenia	Kuenenia	Scalindua	Unkown Broca-diaceae							
Agricultural soil	Jiangsu province, China	2 / 2	-	-	-	-	-	-	-	-	-	4.5×10^6 - 1.2×10^7 *	*hzsB*		Zhou et al., 2017
												1.3 - 4.0×10^6 *	*hzo*	-	
Paddy soil		2 / 2	-	-	-	-	-	-	-	-	-	3.1 - 3.2×10^7 *	*hzsB*		
												1.6×10^7 *	*hzo*	-	
Forest soil		1 / 1	-	-	-	-	-	-	-	-	-	5.2×10^6 *	*hzsB*		
												1.0×10^6 *	*hzo*	-	
Agricultural soil	Jiangsu province, China	4 / 12	0	>0	>0	0	0	0	-	*hzo*	-	3.2×10^3 - 2.7×10^4	*hzsB*	-	Hui et al., 2017
Wetland soil	Seine Estuary, France	1 / 3	0	100	0	0	0	0	0	16S rRNA and *hzo*	KJ701283 - 9	3.5×10^6 - 1.4×10^7	16S rRNA	2.9 - 6.4% *	Naeher et al., 2015
Agricultural soil	6 sites accross USA	6 / 6	0	0	100	0	0	0	-	*hzo*	-	5.5×10^3 - 1.1×10^7	*hzo*	39.1 - 77.9%	Long et al., 2013
Paddy soil	Jiaxing, China	1 / 10	0	8	0	12	0	80	0	16S rRNA	GU083863 - 952	8.6×10^5 - 1.1×10^7	16S rRNA	4 - 37%	Zhu et al., 2011
Peat soil	Hierdense Beek, Netherland	1 / 3	0	0	0	50	0	50	0	16S rRNA	HQ637487 - 9	-	-	-	Hu et al., 2011
Permafrost	Creux-du-Van, Switzerland	1 / 1	0	0	100	0	0	0	50	16S rRNA		-	-	-	Humbert et al., 2010a
Wetland soil	Cadagno, Switzerland	5 / 5	0	0	0	100	0	0	33	16S rRNA		2.02×10^6	16S rRNA	-	Humbert et al., 2010a, and 2012
Lake shore soil	Shore Lake Neuchâtel, Switzerland	2 / 2	0	0	0	100	0	0	80	16S rRNA		6.48×10^4	16S rRNA	-	
Wetland soil	Grande Cariçaie, Switzerland	3 / 5	0	100	0	0	0	0	88	16S rRNA	FM174251 - 320, FN908027 - 44	2.14×10^5	16S rRNA	-	
Minerotrophic fen	Bellefontaine, France	1 / 2	0	0	0	0	0	100	0	16S rRNA		1.8 - 2.3×10^6	16S rRNA	-	
Wetland soil	Camargue, France	6/9	0	0	0	67	33	0	86	16S rRNA		4.01×10^5	16S rRNA	-	
Porous aquifer	Wallis, Switzerland	1 / 1	0	100	0	0	0	0	0	16S rRNA		1.83×10^5	16S rRNA	-	
Lake shore soil	Shore Lake Loclat, Switzerland	5 /10	0	13	0	33	0	54	42	16S rRNA		$4.0\ 10^4$ - 6.7×10^6	16S rRNA	0.5 - 9.2% *	Humbert et al., 2010a, 2010b, and 2012
Wetland soil	Greensboro, NC, USA	3 / 3	0	100	0	0	0	0	0	16S rRNA	GQ424188	-	-	-	Dong & Reddy, 2010
Paddy soil	Honghe State Farm, China	3 / 11	0	0	0	0	100	0	23	16S rRNA and *hzo*	JF965466 - 88	-	-	-	Wang & Gu, 2013
Paddy soil	Zhejiang Province, China	1 / 3	16	14	1	49	0	21	0	16S rRNA	JN176677 - 804	2.3×10^6 - 2.9×10^6	*hzsA*	-	Hu et al., 2013
Paddy soil	Ibaraki prefecture, Kanto plains, Japan	1 / 5	0	57	0	4	0	39	0	16S rRNA	AB602629 - 748	-	-	1-5%	Sato et al., 2012
Paddy soil	Subtropical China	1 / 10	0	86	14	0	0	0	-	*hzsB*	-	0 - 2.7×10^6	*hzsB*	-	Wang et al., 2012
Paddy soil	11 paddy soils accross China	11 / 11	-	-	-	-	-	-	-	-	-	5.1×10^4 - 7.6×10^5	*hzsB*	4.5–9.2%	Shan et al., 2016
Temperate forest soil	Qingyuan Forest CERN, China	2 / 6	0	42	58	0	0	0	-	*hzsB*	-	-	*hzsB*	0.5 - 14.4%	Xi et al., 2016
Forest soils	Nanling National Nature Reserve, China	5 / 10	0	9	0	91	0	0	-	*pmoA*	-	2.2×10^5 - 1.20×10^6	16S rRNA	-	Meng et al., 2016
Mangrove soils	Sai Keng, China	1 / 1	-	-	-	-	-	-	-	-	-	1.2×10^5 *	16S rRNA	-	Chen et al., 2016
Wetland soil	Baguazhou wetland, China	6 / 6	0	38	0	55	0	7	3	16S rRNA	KT162107 - 40	2.3×10^5 - 2.2×10^6	*hzsA*	1.5 - 20.1%	Shen et al., 2016
Agricultural soil	Nanjing City, China	5 / 5	7	53	4	29	0	7	0	16S rRNA	KM887957 - 87	2.8×10^5 - 3.0×10^6	*hzsA*	5.9 - 20.5%	Shen et al., 2015
Paddy soil	12 sites accross southern China	12 / 12	0	91	0	0	0	9	40	16S rRNA	KJ508414 - 750	1.2×10^4 - 9.7×10^4	*hzsB*	1.4 - 12.2%	Yang et al., 2015
Paddy soil	Zhejiang Province, China	1 / 10	0	8	0	17	0	75	8	16S rRNA	KF754815 - 36	1.0×10^5 - 2.0×10^6	*hzsA*	-	Shen et al., 2014
Paddy soil	Baijiang, China	1 / 1	0	0	0	12	88	0	7	16S rRNA	KF286660 - 82, KF286780 - 818	2.6×10^5 - 7.7×10^5 *	16S rRNA	-	Han et al., 2013
Agricultural soil	32 sites across China	32 / 32	0	78	0	20	0	2	0	16S rRNA	JQ918892 - 9160	6.4×10^4 - 3.7×10^6	*hzsA*	-	Shen et al., 2013
Clay soil	Shfelat Yehuda, Israel	1 / 11	0	0	100	0	0	0	59	16S rRNA	HQ652082 - 103	2.0×10^3 - 2.0×10^7 *	16S rRNA	-	Sher et al., 2012
Typic Reductisol	Bellefontaine, France	1 / 11	0	0	0	0	0	100	0	16S rRNA	KT873861 - 88	0 - 7.0×10^5	16S rRNA	<2.1%	This study
Minerotrophic fen	Bellefontaine, France	1 / 10	0	35	0	0	0	65	26	16S rRNA		0 - 2.7×10^7	16S rRNA	<1.4%	

* data extracted with WebPlotDigitizer from article's figures

† 16S rRNA gene-based anammox population were re-analysed from reads in this study

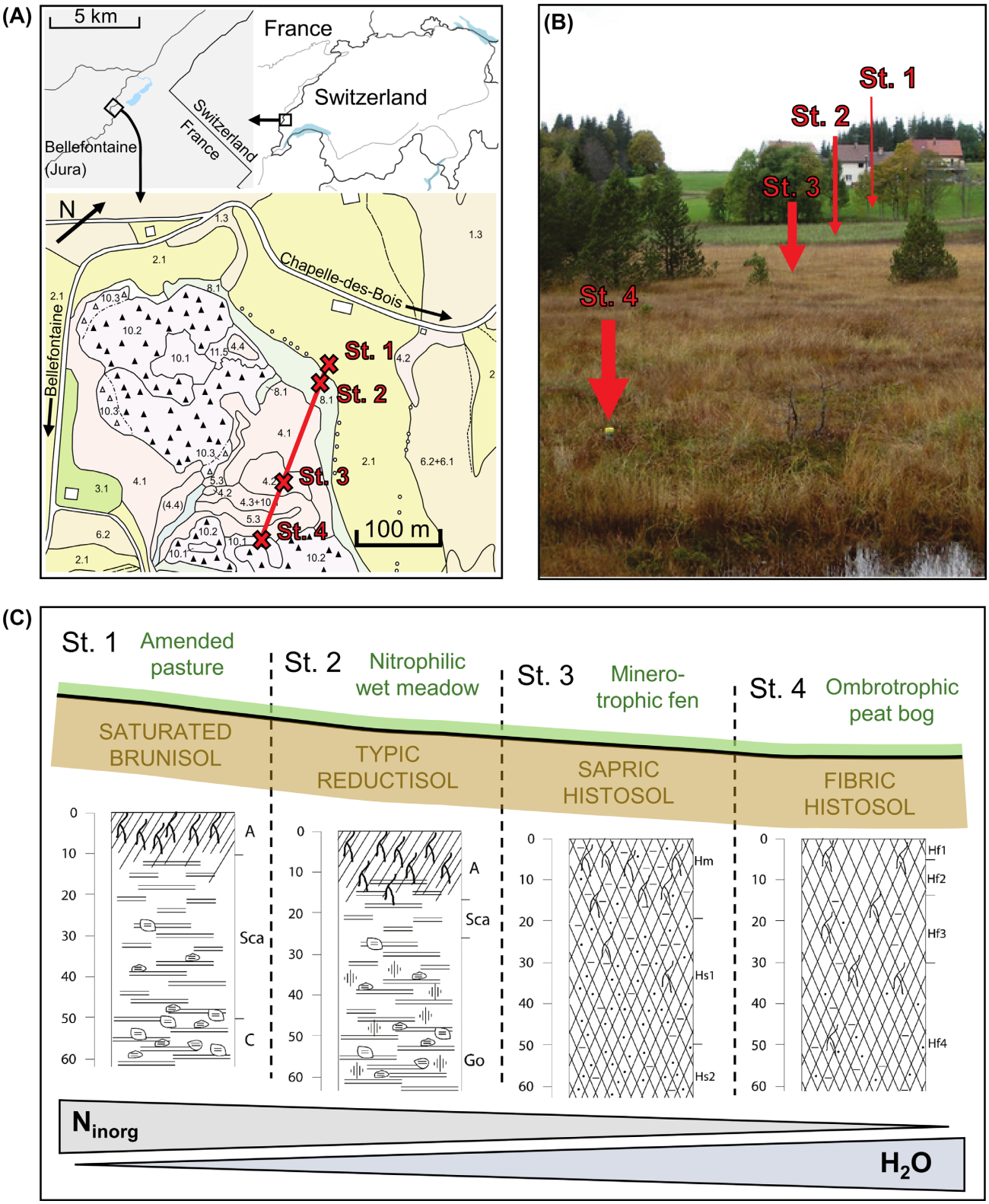

Figure 1. (**A**) Location of the study area in the French Jura mountains close to the border with Switzerland. Four stations representing a sequence of different soil types along a hill slope have been selected based on vegetation type (colored map modified from Gallandat 1982). Each station (St.) is indicated by a red cross. (**B**) Red arrows indicate the approximate positions of the four stations (St.). (**C**) Schematic representation of the studied catena, including the vegetation type, the soil type and the soil profile. Soils from stations 1 and 2 consist of an organo-mineral horizon 'A' (0–15 cm) with some living plant roots. Below, horizon 'Sca', is a carbonate-rich layer with a clayey structure, containing some carbonate stones, and consisting of weathered bed rock. The last horizon of Soil 1, 'C', consists of fragmented bed rock, whereas the last horizon of Soil 2, 'Go', is characterized by rusty stains, indicating fluctuating ground water level. Soils 3 and 4 are composed of a succession of holorganic horizons formed under water-saturated anoxic conditions. 'Hf' designates horizons with a fiber content >40%, 'Hs' stands for horizons with a fiber content between 10 and 40%, and 'Hm' for horizons with a fiber content <10%.

Table 2. Summary of the general soil characteristics: average (±standard deviation) percentage of organic carbon content determined as weight loss on ignition, organic carbon:nitrogen ratio, pH (H_2O); KCl-extractable NH_4^+, NO_2^- and NO_3^-, and HCl-extractable Fe(II) and Fe(III). 'n. d.' stands for 'not determined'.

	Loss on ignition [%]	C_{org}/N_{org}	pH H_2O	NH_4^+ [μmol·g^{-1} dry soil]	NO_2^- [μmol·g^{-1} dry soil]	NO_3^- [μmol·g^{-1} dry soil]	Fe(II) [μmol·g^{-1} dry soil]	Fe(III) [μmol·g^{-1} dry soil]
Soil 1 Amended pasture	11.6 ± 5	11 ± 1.3	6.6 ± 0.2	0.1 ± 0	0 ± 0	0.01 ± 0.01	1.7 ± 0.6	30.4 ± 7.4
Soil 2 Wet meadow	13.8 ± 6.7	12.9 ± 0.9	7 ± 0.3	0.2 ± 0.1	0 ± 0	0.03 ± 0.02	4.8 ± 3	15.7 ± 9.6
Soil 3 Fen	53.8 ± 9	14 ± 0.6	6.9 ± 0.1	0.5 ± 0	0 ± 0	0.02 ± 0.01	78.5 ± 43.9	31.1 ± 32.8
Soil 4 Peat bog	94.9 ± 1.4	66.2 ± 24	5.2 ± 0.1	n. d.	n. d.	n. d.	12.2 ± 3.5	0 ± 0

fixed water samples (0.1% w/v final concentration). Interstitial SO_4^{2-} was quantified by ion chromatography (Dionex DX-120, USA).

Pedological and geochemical characterization of the soils

An Edelman auger (for Soils 1 and 2) and a Wardenaar peat profile sampler (for Soils 3 and 4; Eijkelkamp, The Netherlands) were used to collect soil material from each station along a depth profile (10 cm depth resolution) in September 2007. Soil cores sampled in triplicate from the same depth were manually homogenized in the field and sub-sampled for the different analyses. Three grams of soil were placed immediately in 30 mL of HCl 1 M for the later determination of Fe(II) and Fe(III). For determining inorganic N-forms, 15 g of soil sample were placed in 30 mL of 2 M KCl. Samples for DNA extraction were filled into two sterile 2 mL cryotubes and immediately frozen in liquid nitrogen. Soil material for all remaining analyses was stored in sealed plastic bags at 4°C.

Soil water content was determined by weight loss after drying 10 g of fresh soil at 105°C for 2 days. The same samples were muffled at 450°C for 2 h (for samples of Soils 1 and 2) and at 600°C for 4 h (for samples of Soils 3 and 4). Soil organic matter content was measured by the weight loss on ignition (LOI; Ball 1964).

For pH and organic matter analysis, samples were dried at 40°C and sieved at 2 mm. Soil pH (H_2O) was determined with a pH-electrode (Mettler, Switzerland) in a suspension of 50 g of dried soil in 30 mL of deionized water that had been equilibrated on a rotary shaker for 1 h. Organic carbon to organic nitrogen ratios (C_{org}:N_{org}), and total organic carbon (TOC) were determined on a Carlo Erba CHN analyzer (Disnar *et al.* 2003).

For quantification of adsorbed inorganic nitrogen compounds (NH_4^+, NO_2^-, NO_3^-) and Fe(II) and Fe(III), the soil samples were agitated for 1 h at 120 rpm in their sampling solution. The supernatant was then analyzed as described for interstitial water within 24 h. Adsorbed inorganic N-forms are not presented for samples from Soil 4 because of erratic measurements due to problems with the KCl extraction protocol applied to the peat samples.

Biological oxygen demand after 5 days (BOD_5) was used as a proxy for organic matter degradability in Soil 3 and was determined using manometric Warburg respirometers (Chase and Gray 1957).

^{15}N incubation experiments for denitrification, anammox and Feammox

Potential rates of anammox and denitrification were determined by ^{15}N-label incubation experiments under anoxic condition (Thamdrup and Dalsgaard 2002; Naeher *et al.* 2015). The experimental set-up consisted of three treatments: (i) a control with addition of $^{15}NH_4^+$; (ii) addition of $^{15}NH_4^+$ and $^{14}NO_3^-$, where production of $^{29}N_2$ is indicative for anammox; (iii) addition of $^{15}NO_3^-$, where production of $^{30}N_2$ is caused by denitrification and $^{29}N_2$ by anammox.

As the formation of labeled N_2 was observed in the controlled samples amended with $^{15}NH_4^+$ only, this control incubation was repeated with slurries from Soil 3 in order to determine if this was due to an oxygen contamination or to another microbial process, such as Feammox (Yang, Weber and Silver 2012). This time, absolute care was taken regarding oxygen contamination, and *in vitro* oxygen contamination was closely monitored using high-sensitivity oxygen sensors (PSt6-NAU from Presens, Germany) with a detection limit of 0.5 ppb. Soil slurries for five specific depths, as well as a soil-free extract with no amendment, amended with hydrous ferric oxides (HFO) or amended with MnO_2, were used for this second incubation experiments. Methodological details concerning these incubations are presented in the supplementary material available online.

Potential nitrification rates

Potential aerobic nitrification rates were measured in a subset of samples from Soils 1–4 (Prosser and Nicol 2012). Ten grams of fresh soil were placed in an Erlenmeyer flask and amended with 50 mL of a solution containing 500 μM NH_4Cl, 50 μM of KH_2PO_4 and 10 mM of $NaClO_3$, to inhibit biological NO_2^- oxidation to NO_3^-. The flasks were continuously agitated (100 rpm) at room temperature in the dark for 24 h. The linear increase in NO_2^- concentrations in the supernatant was followed using the Griess method and used to calculate potential nitrification rates.

Molecular analyses

DNA was extracted using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Santa Ana, USA) following the manufacturer's protocol. A nested-PCR approach was used to determine the diversity of anammox bacteria in the soil samples (Humbert *et al.*

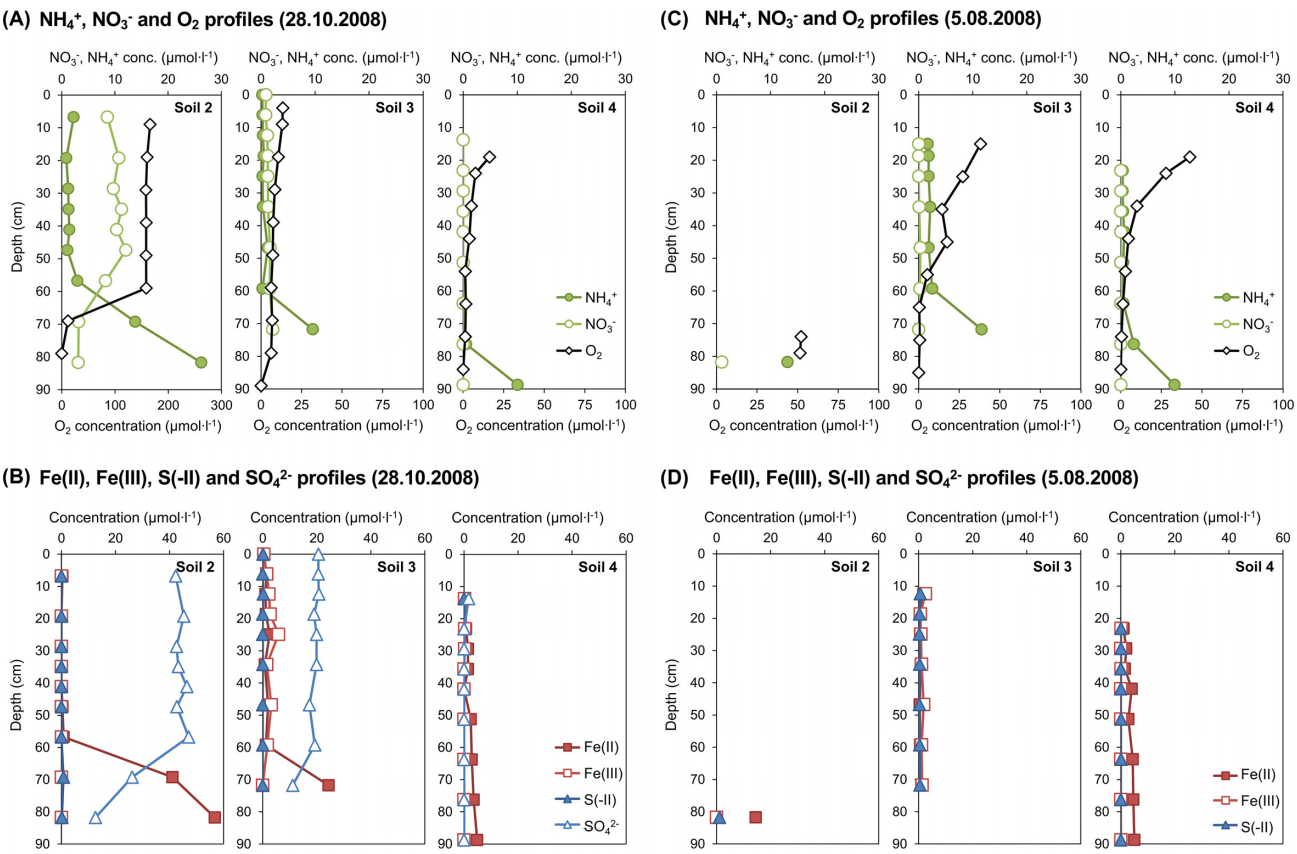

Figure 2. Depth profiles of NH_4^+, NO_3^- and O_2 concentrations (**A** and **C**), colloidal Fe(III), dissolved Fe(II), S(-II) and SO_4^{2-} concentrations (**B** and **D**) in the interstitial water of Soils 2, 3 and 4, measured in fall (A and B) and in summer (C and D). No interstitial water could be extracted from Soil 1. NO_2^- concentrations are not shown as they were usually below the detection limit (0.5 μM).

2010a), using GoTaq DNA polymerase (Promega, Madison, Wisconsin, USA). Planctomycetes 16S rRNA genes were first amplified by PCR using Pla46f (Neef *et al.* 1998) and Univ1390r (Zheng *et al.* 1996) primers. DNA concentration in the reaction mix was between 0.04 and 2.5 ng μL^{-1} (0.45 ng μL^{-1} on average). The anammox 16S rRNA genes were then amplified in a second step using primers Amx368f and Amx890r (Schmid *et al.* 2005), by diluting PCR products of the previous PCR assay 100-fold in the new PCR mix. Weak or multiple bands of PCR products were considered as anammox negative. Purified PCR products of the correct size were ligated into pGEM-T vectors (Promega) and cloned in electrocompetent *Escherichai coli* XL1 cells. For sequencing, 27 clones were picked randomly from sample 3.4 (i.e. Soil 3, 22–28 cm depth), 50 clones from sample 3.7 (i.e. Soil 3, 41–48 cm depth), 24 clones from sample 3.10 (i.e. Soil 3, 60–75 cm depth) and 16 clones from sample 2.10 (i.e. Soil 2, 56.5–62 cm depth). Amplicons from each clone were digested by two restriction enzymes (AluI and MspI). Clones with similar restriction profiles were considered as identical and grouped in the same operational taxonomic unit (OTU). Eight OTUs from sample 3.4, 13 from sample 3.7, 3 from sample 3.10 and 4 from sample 2.10 were sequenced by Sanger sequencing (Eurofins Genomics, Germany). Sequences were uploaded to the NCBI database and can be retrieved with the accession numbers KT873861–KT873888.

DNA extracts were analyzed by three qPCR assays in order to quantify anammox bacteria (based on 16S rRNA gene copy numbers), ammonia oxidizing archaea (AOA; based on archaeal *amoA* copy numbers) and ammonia oxidizing bacteria (AOB; based on bacterial *amoA* copy numbers). For all qPCR assays, each sample was run in triplicate and with three different DNA dilutions (4x, 8x and 16x) in order to limit inhibitory effects from soil contaminants co-extracted with DNA. Dilutions yielding higher copy numbers (per g of dry soil) were selected.

Copy numbers of anammox 16S rRNA genes were determined using the SensiMixPlus SYBR® qPCR master mix (Quantace, Biolabo, Châtel-St-Denis, Switzerland) and the primers A438f/A684r (Humbert, Zopfi and Tarnawski 2012). Amplification efficiencies were between 71 and 73%, with $R^2 > 0.99$. A detailed description of the qPCR amplification conditions for anammox bacteria is described elsewhere (Humbert, Zopfi and Tarnawski 2012). Genes encoding subunit A of the ammonium monooxygenase (*amoA*) of AOA and AOB (Prosser and Nicol 2012) were quantified using the Power SYBR® Green PCR Master Mix (Applied Biosystems™, Darmstadt, Germany), as well as the primer set amoA-1f/amoA-2r (Rotthauwe *et al.*, 1997) and 19f/CrenamoA616r48x (Nicol *et al.* 2008), respectively. qPCR efficiencies ranged between 64 and 71% with $R^2 > 0.98$ for the AOB qPCR assay, and between 72 and 78% with $R^2 > 0.99$ for the AOA qPCR assay. Detailed amplification conditions for AOA/AOB are described in Hai *et al.* (2009).

Bioinformatic analyses

A reference alignment was constructed by aligning reference 16S rRNA genes from known anammox organisms (see Fig. 6 for Silva accessions numbers) and two outliers: *Gemmata obscuriglobus* (ABGO01000192) and *Pseudomonas aeruginosa* (AAQW01000001)

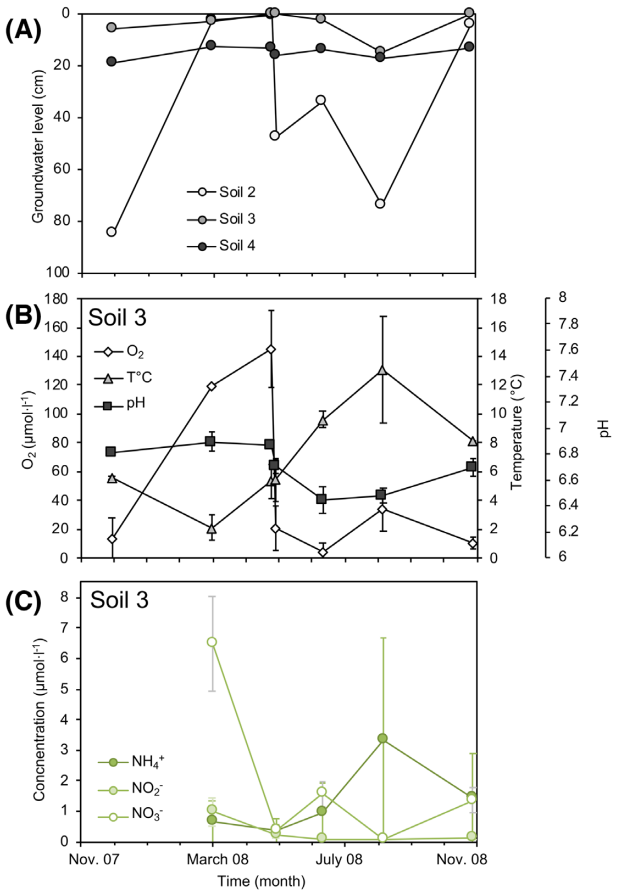

Figure 3. (**A**) Seasonal groundwater level variations in the nitrophilic wet meadow (Soil 2), the minerotrophic fen (Soil 3) and the peat bog (Soil 4). Seasonal changes of the mean values of the physical and chemical parameters of the interstitial water in Soil 3: (**B**) depth-weighted averages of temperature, pH and dissolved O_2; (**C**) depth-weighted average concentrations of ammonium, nitrite and nitrate. In B) and C), error bars represent standard deviations along the soil profile.

with default parameters of MAFFT v.7 (http://mafft.cbrc.jp/alignment/server/; Katoh and Standley 2013). Shorter 16S rRNA anammox sequences retrieved in this study were added to the reference alignment using the '–addfragment' option of MAFFT. The alignment was cleaned using the default parameters of BMGE v.1.12 (https://galaxy.pasteur.fr/; Criscuolo and Gribaldo 2010) and the alignment edges were manually trimmed off using AliView v.1.18 (Larsson 2014). Finally, a maximum-likelihood tree was constructed using the default parameters of IQ-TREE v.1.5.3 (http://iqtree.cibiv.univie.ac.at/; Minh, Nguyen and von Haeseler 2013; Nguyen *et al.* 2015). The tree was then annotated with iTOL v.3 (http://itol.embl.de/; Letunic and Bork 2016) and Inkscape v.0.91 (https://inkscape.org).

To compare 16S rRNA genes from anammox-related microorganisms detected in different soil surveys, sequences were downloaded via NCBI Batch Entrez (https://www.ncbi.nlm.nih.gov/sites/batchentrez) using accession numbers. To obtain the same directionality, sequences were added to the anammox reference alignment using the '–addfragments' and '–adjustdirection' in MAFFT. UPARSE implemented in USEARCH v10.0.240 (Edgar 2013) was then used for sequence dereplicating, OTU picking (using a similarity threshold of 97% and a minimum size of 1) and for building an OTU table. OTUs were taxonomically annotated using UCLUST (Edgar 2010) implemented in QIIME v1.9.1 (Caporaso *et al.* 2010) and the SILVA v.128 as database (Pruesse *et al.* 2007). OTUs annotated as Brocadiaceae were placed on a reference tree (generated as described above but only containing reference sequences) by RAxML v.8.29 (Stamatakis 2014). The detailed bioinformatic analyses can be found at https://github.com/alex-bagnoud/anammox-soil-tree

Statistical analyses

In order to integrate all measurements (i.e. those from soil samples, the piezometer tubes and from multi-level piezometers) into a single data matrix, the depths of soil samples were defined as the standard depths. Where necessary, values and depths of other measurements (e.g. those measured on water samples) were adjusted by simple interpolation in order to match their depths with the standard depths. Statistical analyses were performed in R version 3.3.1 (R Core Team 2011). Pairwise correlations between variables such as:

- depth,
- anammox presence (as a binary variable), abundance of anammox bacteria, AOB and AOA,
- the number of days below the water table,
- organic carbon (measured as the loss on ignition), carbon and nitrogen content in soil samples,
- the extractable concentrations of NH_4^+ and NO_3^- from soil samples (only for Soils 1, 2 and 3),
- the concentrations of extractable Fe(II), Fe(III), and S(-II) from soil samples,
- and the average pH and concentrations of NH_4^+, NO_2^-, NO_3^-, O_2, Fe(II), Fe(III), S(-II) and SO_4^{2-} in the interstitial water (for Soils 2, 3 and 4),

were computed using Pearson's correlation coefficient and the *Hmisc* package (https://CRAN.R-project.org/package=Hmisc). Presence of anammox bacteria and their abundance was modeled using generalized linear models (GLM), based on binomial and Poisson distribution, respectively, and significantly correlated variables as predictors, also including interactions. When considering interaction between variables as predictors, the Bayesian version of GLM was used to address a 'quasi complete separation problem', using the *arm* package (https://CRAN.R-project.org/package=arm). In order to account for putative dependency of samples, generalized linear mixed models (GLMM), were computed using the *glmm* package (https://CRAN.R-project.org/package=glmm). The detailed statistical analysis, including the data set and the script, can be found at https://github.com/alex-bagnoud/AnammoxBellefontaine

RESULTS

Interstitial water chemistry

Free interstitial water could only be sampled in Soils 2, 3 and 4. Data from August and October are presented in Fig. 2 to illustrate the temporal variability of porewater chemistry in the different soils. August concentration profiles mirror the summer situation with dry weather and active vegetation. In October, vegetation dormancy commenced and fall precipitation led to increased water levels (Fig. 3A). Generally, we observed a decrease in the concentrations of soluble N-compounds from Soil 2 towards Soil 4. Ammonium concentrations in interstitial waters were highest in the deepest soil sections (i.e. below 60 cm) and decrease gradually towards the upper, oxic soil layers. Concentrations of nitrate, when present, were higher in the oxic soil layers and

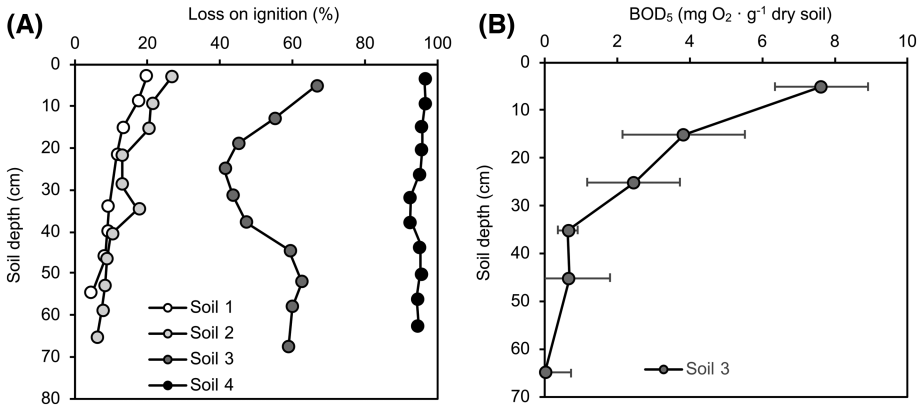

Figure 4. (A) Soil organic matter content in Soils 1–4 expressed as loss on ignition. (B) Depth profile of the biological oxygen demand (determined as BOD_5) in the minerotrophic fen (Soil 3). Error bars represent the standard errors of the mean (SEM).

decreased with depth together with decreasing oxygen concentrations. Particularly in Soils 2 and 3, overlapping zones of ammonium and nitrate were observed, albeit mostly at times of vegetation dormancy (i.e. October, February, April). Highest nitrate concentrations were detected in February (not shown) and reached 30–55 μmol L^{-1} in Soil 2 and 4–8 μmol L^{-1} in Soil 3. No inorganic nitrogen was detected in the interstitial water of Soil 4, with the exception of ammonium below a depth of 62 cm. Nitrite concentrations in all soils were mostly below the detection limit of 0.5 μmol L^{-1}.

Dissolved Fe(II) and Fe(III) as well as SO_4^{2-} (sulfate) and S(-II) (sulfide) provide additional information on the redox conditions in the soils (Fig. 2C and D). For all soils and both seasons (except Soil 3 during summer), Fe(II) concentration increased and/or SO_4^{2-} concentration decreased with depth, mirroring the development of anoxic conditions in the deepest part of the soils. This is consistent with generally decreasing O_2 concentrations with depth (Fig. 2A and C).

The annual variation of the water level in Soils 2, 3 and 4 is presented in Fig. 3A. Even though the water table of Soil 3 was stable throughout the year (Fig. 3A), this soil showed a pronounced seasonal dynamic of the physical and chemical parameters (Fig. 3B and C). Water level, NO_3^- and O_2 concentrations, and pH are higher during winter, while NH_4^+ concentrations and temperature are higher during summer. For Soil 4, no significant seasonal variations occurred in the interstitial water chemistry or the water level (Figs 2 and 3A).

Abiotic soil properties and potential biotic activities

Table 2 summarizes the pedological characterization of Soils 1–4. Average NH_4^+ concentrations in soil extracts increase slightly towards the minerotrophic fen from 0.1 to 0.5 μmol g^{-1} dry soil. Nitrate concentrations of soil extracts were low for all samples (between 0.01 and 0.03 μmol g^{-1} dry soil) and nitrite was always undetectable. Average total organic carbon contents and C_{org}:N_{org} ratios increase towards the peat bog.

Biological oxygen demand (i.e. soil respiration) of Soil 3 was decreasing with depth, reaching values close to zero below 35 cm (Fig. 4). In all soils, except Soil 4, potential nitrification rates decreased with depth (results not shown), from 0.1 μmol NH_4^+ g^{-1} dry soil h^{-1} for Soils 1 and 3, and from 0.2 μmol NH_4^+ g^{-1} dry soil h^{-1} for Soil 2, and reached rates <0.01 μmol NH_4^+ g^{-1} dry soil h^{-1} below a depth of 60 cm. Potential nitrification rates remained low throughout the Soil 4 profile (0 to 0.02 μmol NH_4^+ g^{-1} dry soil h^{-1}).

Abundance of aerobic and anaerobic ammonia oxidizers

The nested-PCR approach for anaerobic ammonia oxidizers (anammox) yielded positive amplification of a 477 bp long fragment for all samples from Soil 2 below 55 cm depth and for all samples of Soil 3 below 20 cm depth. Results of direct qPCR amplification of anaerobic ammonia oxidizers (Fig. 5) were consistent with the nested-PCR data. Anammox bacteria were absent from the upper part of Soils 2 and 3 and increase with depth to reach 7 x 10^5 and 2.7 x 10^7 copies g^{-1} dry soil, respectively.

In contrast to anammox bacteria, both AOA and AOB were detected in all four soils and all depths, with *amoA* gene abundances ranging between 4.3 x 10^6 and 3.1 x 10^9 copies g^{-1} dry soil (Fig. 5). Decreasing AOA and AOB abundances with soil depth were only observed in Soil 2. In all other soil profiles, the copy numbers remained relatively constant. In Soil 3, an increase in the AOA:AOB ratio with depth was observed; archaeal *amoA* was up to 70 times more abundant than bacterial *amoA*. There was a good correlation between the log of the number of AOB copies g^{-1} dry soil and the log of potential nitrification rates (linear regression, $R^2 = 0.86$) but not between the log of the number of AOA copies g^{-1} dry soil and the log of potential nitrification rates (linear regression, $R^2 = 0.12$; Fig. S2, see online supplementary material).

Diversity of anammox bacteria

All anammox-like sequences from three samples from Soil 3 (22–28 cm, 41–48 cm, 60–75 cm) and one sample from Soil 2 (56–62 cm) fall into the *Brocadiaceae* and form four distinct clusters with high ultrafast bootstrap support of ≥88% (Figs 5 and 6). A fifth group of retrieved sequences was not related to anammox bacteria and hence was not examined any further. Ten clones out of a total of 107 (9.3%) represented non-anammox sequences, indicating that this primer pairs can yield false positive amplification in samples with low anammox abundance as reported earlier (Sonthiphand and Neufeld 2013). The relative abundance of these anammox clusters for each of the sequenced samples is summarized in Fig. 5B. With the phylogenetic analysis presented in Fig. 6, Cluster 2 could be assigned to *Ca. Brocadia* and Cluster 4 to *Ca. Anammoxoglobus*, while Clusters 1 and 3 could not be assigned to any known anammox candidate genus. However, when using UCLUST annotation based on the SILVA 128 database, Cluster 4 was not assigned to any

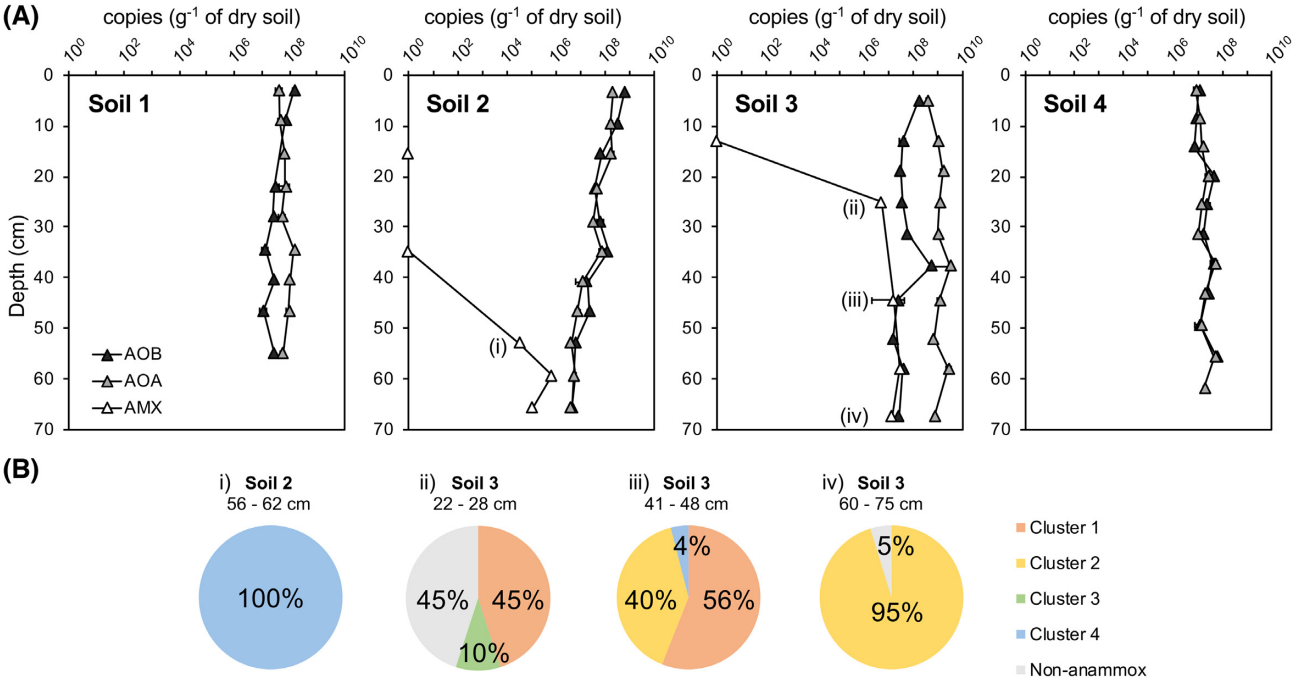

Figure 5. (**A**) Number of amoA gene copies g^{-1} dry soil of ammonia oxidizing bacteria (AOB; black triangles) and archaea (AOA; grey triangles), and number of 16S rRNA gene copies g^{-1} dry of anammox bacteria (AMX; open triangles). Error bars indicate standard deviations of triplicate runs. The roman numbers in parenthesis indicate the samples for which the anammox community composition has been determined by clone sequencing. (**B**) Pie charts showing the phylogenetic repartition of the clones retrieved from one sample of Soil 2 (59 cm, 16 clones) and three samples of Soil 3 (25 cm, 20 clones; 44.5 cm, 50 clones; 67.5 cm, 21 clones). The four anammox clusters and their colors correspond to the ones shown in Fig. 6. Light grey represents non-anammox sequences.

known anammox genus (Cluster 4 falls within OTU67 in Fig. S5, see online supplementary material).

BLASTN analyses revealed that the anammox bacteria from this study are highly similar (≥99% sequence identity) to representatives from other soil environments (Hu *et al.* 2011, 2013; Zhu *et al.* 2011; Humbert, Zopfi and Tarnawski 2012; Sato *et al.* 2012; Yang *et al.* 2015; Shen *et al.* 2016). Sequences from Cluster 1 are highly similar to anammox sequences detected in paddy soils (e.g. AB602695.1, GU083873.1, JN176720.1 and KJ508609.1), in a peat soil (e.g. HQ637487.1), in a wetland soil (e.g. KT162137.1) and in a lake-shore soil (e.g. FM174261.1). Members of Clusters 2 and 4 share high similarity with paddy soil anammox sequences (e.g. AB602629.1 for Cluster 2, and AB602743.1, GU083864.1 and KJ508632.1 for Cluster 4). There are no highly similar hits for sequences from Cluster 3.

USEARCH was also used for defining OTUs at 97% identities in order to compare sequences from various terrestrial surveys, including those from the present study. This clustering analysis allowed 113 OTUs to be defined, but only 26 were annotated as *Brocadiaceae*. Among them, all sequences from Cluster 1 (except the one from clone 3b-1 that was considered as a noisy chimeric by the pipeline) were assigned to OTU4, which was also detected in 8 other studies conducted in wetland soils (Humbert, Zopfi and Tarnawski 2012; Shen *et al.* 2016), paddy soils (Hu *et al.* 2011, 2013; Zhu *et al.* 2011; Sato *et al.* 2012; Yang *et al.* 2015) and lake shore soils (Humbert *et al.* 2010a; Humbert, Zopfi and Tarnawski 2012). All sequences from Cluster 2 were assigned to OTU1, also detected in 10 other studies (Humbert *et al.* 2010a; Humbert, Zopfi and Tarnawski 2012; Sato *et al.* 2012; Han, Li and Gu 2013; Hu *et al.* 2013; Shen *et al.* 2013; Shen, Xu and He 2014; Naeher *et al.* 2015; Yang *et al.* 2015; Shen *et al.* 2016). The two sequences from Cluster 3 were assigned to OTU67, only detected in 1 other study in a paddy soil (Sato *et al.* 2012). Finally, all sequences from Cluster 4 were assigned to OTU5, detected in 6 other studies from wetland soils (Shen *et al.* 2016), paddy soils (Zhu *et al.* 2011; Sato *et al.* 2012; Hu *et al.* 2013; Yang *et al.* 2015) and agricultural soils (Shen *et al.* 2015). *Brocadiaceae* OTUs were placed on the reference tree using an evolutionary placement algorithm (Fig. S5, see online supplementary material). This Figure also summarizes the repartition of OTUs among the studies and among the different soil types.

Anaerobic ammonium oxidation in selected soil layers

Anoxic incubations of soil slurries with ^{15}N-labeled NH_4^+ and/or NO_3^- were carried out to confirm anammox and denitrification activities in Soil 2 (50–60 cm) and Soil 3 (40–50 cm) where molecular data indicated the presence of anammox cells. Incubations amended only with $^{15}NH_4^+$ produced $^{29}N_2$ and $^{30}N_2$, indicating that NH_4^+ was oxidized during the incubations (Fig. S3, see online supplemenatry material). This made the assessments of denitrification and anammox rates more complicated. Therefore, it was decided to use data from the $^{15}NO_3^-$ incubation only, assuming the intrinsic presence of $^{14}NH_4^+$, and the possibility of its oxidation into $^{14}NO_3^-$. In these incubations, rates of $^{29}N_2$ and $^{30}N_2$ production over 12 h are steady (Fig. S3, see online supplemenatry material). Denitrification was the most active process in both soils with NO_3^- reduction rates of 0.99 μmol g^{-1} dry soil d^{-1} and 6.63 μmol g^{-1} dry soil d^{-1} in Soils 2 and 3, respectively. Anammox activity was low, accounting for about 2.1% in Soil 2 and 1.4% in Soil 3 of the N_2 produced, corresponding to anammox potential rates of 0.02 μmol g^{-1} dry soil d^{-1} in Soil 2, and 0.09 μmol g^{-1} dry soil d^{-1} in Soil 3.

Additional incubations were performed with fresh soil material for determining whether alternative anaerobic ammonium oxidation pathways with Fe(III) or Mn(IV) as oxidant were taking

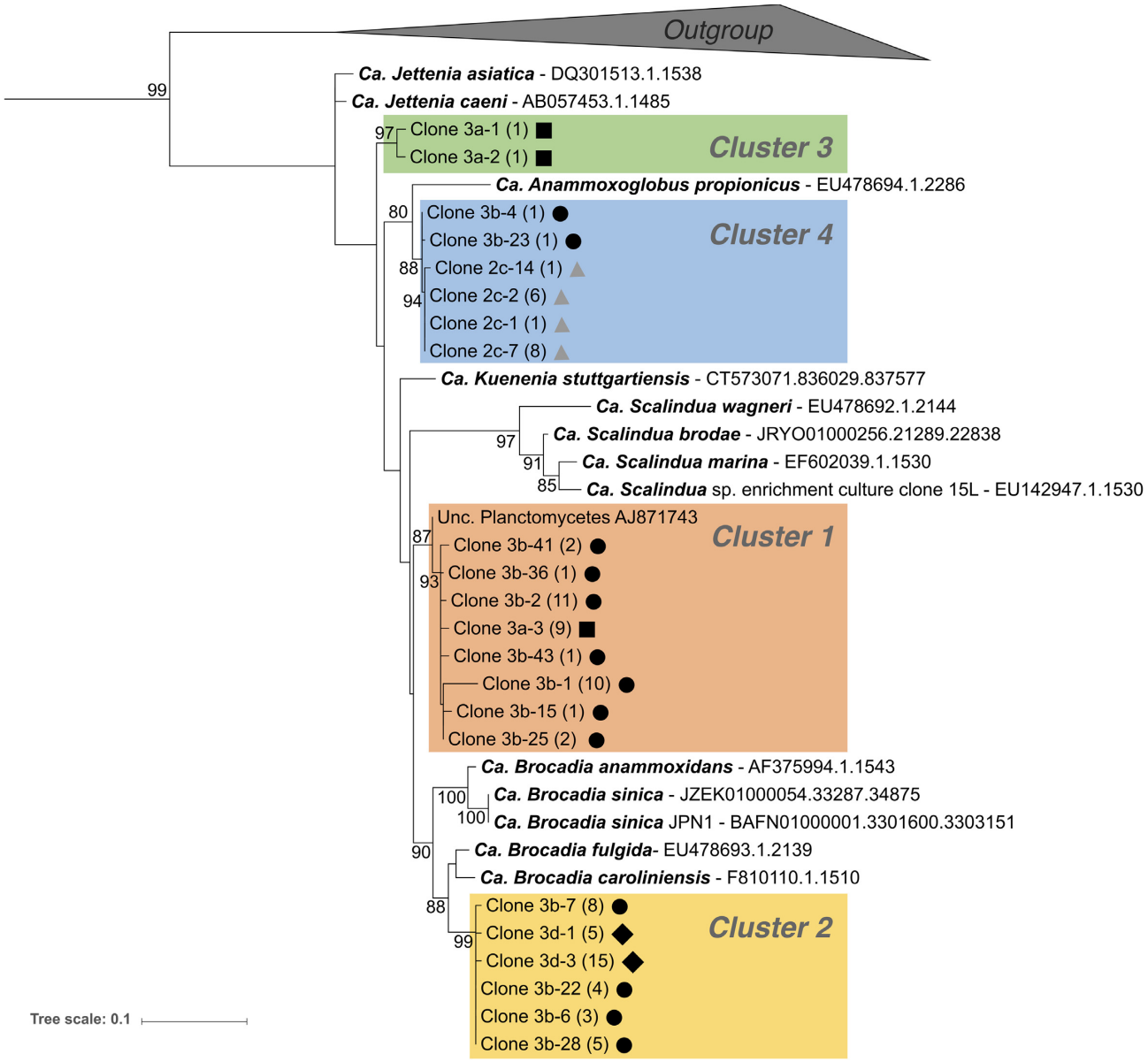

Figure 6. Maximum-likelihood tree showing the phylogenetic relationships between known anammox bacteria and closely related 16S rRNA gene sequences retrieved from the nitrophilic wet meadow (Soil 2) and the minerotrophic fen (Soil 3). Clone names are composed as follows: soil number (2 or 3), sampling depth (a = 22–28 cm, b = 41–48 cm, c = 56–62 cm and d = 60–75 cm), clone number with the number of identical clones retrieved in parenthesis. Clones 2c are labeled with grey triangles, 3a with black squares, 3b with black circles and 3d with black diamonds. The colored boxes highlight the four anammox clusters shown in Fig. 5. Ultrafast bootstrap values >80% are indicated for each node. Sequences from *Gemmata obscuriglobus* (ABGO01000192) and *Pseudomonas aeruginosa* (AAQW01000001) were used as outgroups for rooting the tree.

place, as suggested by the production of labeled N_2 in the control incubations amended only with $^{15}NH_4^+$ (Fig. S3,A, see online supplementary material). During the entire incubation time that lasted 3 months, the concentration of dissolved oxygen in the incubation bottles stayed below the detection limit of the high sensitivity O_2-sensor spot (<0.5 ppb). After subsampling, 1 ppb O_2 was detected transiently in a handful of bottles. After 86 days of incubation, $^{15}N_2$ reached at maximum 0.003 μmol g^{-1} soil for the soil slurry incubations, 0.0004 μmol L^{-1} for the soil-less incubations with no amendment, 0.0005 μmol L^{-1} for the soil-less incubations amended with HFO, and 0.0007 μmol L^{-1} for the soil-less incubations amended with MnO_2 (Fig. S4). This insignificant NH_4^+ oxidation was likely due to very small oxygen contamination through the bottle septa and did not match what was observed in the control experiments of the first set of ^{15}N-incubations, where labeled N_2 reached more than 10 μmol L^{-1} in <10 h when only $^{15}NH_4^+$ was amended (Fig. S3, see online supplemenatry material). There was no significant stimulation of anaerobic NH_4^+ oxidation in the presence of Mn(IV)- or Fe(III)-oxides.

Statistical model for explaining the presence of anammox bacteria

The presence of anammox bacteria was significantly correlated with the number of days a soil sample was below the water table per year (r^2 = 0.50, *P*-value = 0.0009), but also with the organic N content of soil samples (r^2 = 0.45, *P*-value = 0.0035), with the

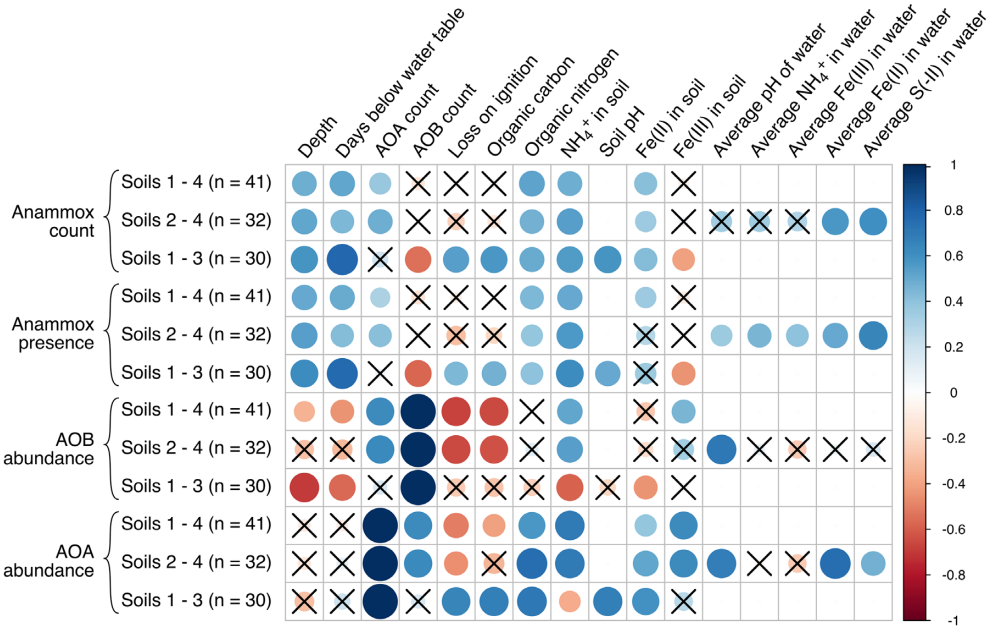

Figure 7. Significant correlations between selected environmental parameters and anammox abundance (16S rRNA gene copies g^{-1} dry soil) and presence (binary variable), and between AOB and AOA abundance (amoA gene copies g^{-1} dry soil). Three groups of samples are considered here: Soils 1–4 ($n = 41$), soils 2–4 ($n = 32$) and soils 1–3 ($n = 30$). Environmental parameters are the following: depth (cm), number of days a soil sample spent under the water table (over a year), AOA and AOB abundance (amoA copies g^{-1} dry soil), loss of ignition, content in organic carbon and nitrogen, soil pH, soil content in NH_4^+, Fe(II), Fe(III) ($\mu g\ g^{-1}$ dry soil), and average concentration of NH_4^+, Fe(II), Fe(III) and S(-II) in the interstitial water (μM). The size of the dots and their color represent the Pearson's correlation coefficient (see scale for more details). Crosses represent non-significant correlations (*P*-value > 0.05) and empty boxes represent incomplete datasets.

pH of soil samples ($r^2 = 0.50$, *P*-value = 0.0008), the concentration of NH_4^+ extracted from soil samples ($r^2 = 0.51$, *P*-value = 0.0042), with the average NH_4^+ concentration in the interstitial water ($r^2 = 0.46$, *P*-value = 0.0087) and with the average Fe(II) concentration in the interstitial water ($r^2 = 0.41$, *P*-value = 0.0212). Fig. 7 summarizes the significant correlations between environmental parameters and the presence/absence of anammox, as well as the abundances of anammox, AOA and AOB. Based on these results, GLMs were fitted to predict the presence/absence of anammox bacteria (using binomial distribution). Three models could accurately describe the presence of anammox bacteria in the soil system. The models use as predictor variables (i) the number of days that soil samples were submerged per year (*P*-value = 0.0044), (ii) the pH of soil samples (*P*-value = 0.0273) and (iii) the average NH_4^+ concentration in interstitial water (*P*-value = 0.0131). The two last-mentioned models were considered redundant because their respective predicting variables, the pH of soil samples and the average NH_4^+ concentration, are correlated ($r^2 = 0.59$, *P*-value = 0.0004). Also, the pH of soil samples correlates with the presence of anammox bacteria because the low-pH values were all measured in Soil 4, where NH_4^+ and NO_3^- concentrations are negligible and thus anammox is absent. All three predictor variables interact significantly with a fourth variable, the average NO_3^- concentration in the interstitial water (*P*-values of interactions = 0.0079, 0.04 and 0.0166, respectively). Akaike information criterion (AIC) scores of GLMs are 13.95, 10.65 and 22.73, respectively. The same set of variables and variable interactions can also significantly model the abundance of anammox bacteria (in a log scale), based on a Poisson distribution.

Additionally, GLMMs were tested in order to account for the fact that the soil samples were not independent, as they originate from four soil depth profiles. However, the random effects of these models (that express how much variability there is between the different soils) were not significant and hence the GLM was sufficient for modeling the presence and abundance of anammox in these soils. The detailed results of the statistical analyses can be found at https://github.com/alex-bagnoud/AnammoxBellefontaine

DISCUSSION

Chemical conditions in the soils and sources of inorganic nitrogen compounds

The physical and chemical conditions vary strongly in the different soils during the annual cycle, particularly in Soils 2 and 3. During the cold season, water levels are higher and the concentrations of oxygen and NO_3^- are elevated (Fig. 2A). This period of low vegetation activity allows the source of inorganic nitrogen compounds in the different soils to be identified. For example, in fall, interstitial water concentrations of inorganic nitrogen compounds were highest in the nitrophilic wet meadow (Soil 2) at the foot of a gentle hill slope adjacent to an amended pasture (Figs 1 and 2A.). Nitrogen brought out by manuring likely reaches this soil by lixiviation (Barakat, Cheviron and Angulo-Jaramillo 2016). The nitrophilic wet meadow (Soil 2) serves thus as a buffer zone between the amended pasture (Soil 1), the fen (Soil 3) and the peat bog (Soil 4). There, concentrations of dissolved nitrogen compounds never exceeded 1 $\mu mol\ L^{-1}$ (except for ammonium in the deeper layer; Fig. 2), because of the ombrotrophic nature of the peat bog and the presence of *Sphagnum* sp. moss, that efficiently scavenge atmospherically deposited nutrients (Fritz *et al.* 2014).

In spring, the induction of biological activity happens within a remarkably short time. Evapotranspiration lowered the

groundwater table in Soils 2 and 3 (Fig. 3A), as was also observed in another bog system (Lafleur *et al.* 2005). Furthermore, respiration of plant roots and microorganisms, stimulated by rising temperature and rhizodeposition, drastically reduced pO_2 in the waterlogged soil zones (Fig. 3B; Revsbech *et al.* 1999; Kuzyakov and Cheng 2001). Nitrate is assimilated by the growing plants (Miller and Cramer 2005) or reduced by microorganisms, leading to the production of nitrogen gas or ammonium (Tiedje 1988; Fig. 3C). Ammonium is also liberated continuously by mineralization of organic matter (Landi *et al.* 2006). The C_{org}:N_{org} ratios in Soils 1, 2 and 3 indicate that organic matter still contains significant amounts of nitrogen, as opposed to Soil 4, which is particularly poor in nitrogen (Table 2).

Soil biological activity and water level are key parameters for the distribution of nitrate and ammonium along the soil profiles. Waterlogged conditions reduce the transport of oxygen into deeper soil sections, facilitating anoxic conditions and anaerobic metabolism. Hence, Soils 2 and 3 are characterized by periods of oxic and anoxic conditions that alternate predictably on a seasonal scale, but may also change on a short-term scale according to meteorological conditions and groundwater table fluctuation.

Environmental factors controlling the distribution of anaerobic ammonia oxidizers

Abundances of anammox bacteria in Soils 2 and 3 are in the same range as found in other soil surveys (Humbert, Zopfi and Tarnawski 2012; Hu *et al.* 2013; Long *et al.* 2013; Naeher *et al.* 2015; Shen et al. 2015, 2016; Yang *et al.* 2015; Meng *et al.* 2016; Shan *et al.* 2016; Hui *et al.* 2017; Zhou *et al.* 2017). We observe, however, a distinct spatial distribution, where anammox bacteria are only present below a certain depth, i.e. 50 cm below the surface in Soil 2 and 20 cm below the surface in Soil 3, respectively. Statistical modeling clearly shows that in this catena anammox bacteria are found in soil sections that remain waterlogged for an extended period of time and contain NO_3^- and NH_4^+ in the interstitial water. Nitrite, however, which is one of the two primary substrates of anammox bacteria, does not explain anammox distribution. Nitrite concentrations are close to or below the detection limit in all soils and at all times. Hence, anammox bacteria in this catena depend on the continuous production of nitrite by nitrate-reducing and/or ammonia-oxidizing microorganisms. Which one of the two processes provides nitrite to anammox bacteria is likely changing during the year along with the water level and degree of oxygenation. The fact that anammox bacteria are significantly correlated with AOA (Fig. 7) suggests that anammox utilize the NO_2^- produced from AOA.

Besides availability of inorganic nitrogen, extended waterlogged conditions seem to be key for the establishment of anammox bacteria. Indeed, anammox was not detected in the part of the soils that are above the groundwater table for longer periods. This is the case for the shallow, well-drained Soil 1 as well as for the upper parts of Soils 2 and 3. In these soils, there is a good correspondence between the groundwater level measured during the summer (Fig. 3A) and the depths where anammox can be detected (Fig. 5). This observation is consistent with Humbert *et al.* (2012) who detected anammox bacteria in the predominantly water-saturated zones of a wetland.

Against initial expectations, soil organic matter content did not have any statistical relation with the distribution of anammox bacteria. High contents of organic matter may favor heterotrophic nitrate-reducing bacteria but do not hinder the presence of anammox bacteria. Anammox bacteria were detected in Soil 3, which consists of more than 60% organic matter (Fig. 4A). The quality, i.e. degradability, of organic matter, rather than the absolute quantity, is likely the determining factor for the distribution and activity of anammox and denitrifying bacteria. For example, in Soil 3, the abundance of anammox bacteria (Fig. 5) is anti-correlated to soil respiration (Fig. 4B). The fact that the organic matter of Soil 3 below 25 cm is poorly degradable by microorganisms may explain the presence of anammox bacteria in this part of the profile, because they do not rely on an organic source of carbon. Conversely, the N content of organic matter shows a positive correlation with anammox and particularly AOA, suggesting that the N (as ammonium) liberated during organic matter mineralization serves as an important substrate for AOA and anammox, particularly in Soil 3 where free inorganic N pools and external N inputs are low.

Distribution and abundance of aerobic ammonia oxidizers

Copy numbers of AOA and AOB *amoA* were generally 1–2 orders of magnitude higher than maximum values of anammox bacterial 16S rRNA gene copy numbers. Assuming a single *rrn* operon per anammox bacterial genome, it means that aerobic ammonia oxidizing microorganisms are clearly more abundant than the anaerobic ones, and thus remain the key players in microbial ammonia oxidation. Only in Soil 3 were AOB distinctly outnumbered by their archaeal counterpart. The observed differences were less pronounced than reported by Leininger *et al.* (2006) who observed up to 3000 times more AOA than AOB. The abundance of AOA in this soil, in the range of 10^9 copies g^{-1} dry soil is higher than what is typically observed in soil (Leininger *et al.* 2006; Erguder *et al.* 2009). Because Soil 3 is holorganic, this observation could signify that AOA can exhibit a mixotrophic mode of growth, and preferably oxidize NH_4^+ derived from organic matter mineralization (Hatzenpichler 2012). Indeed, a positive correlation was observed between soil organic N content and AOA *amoA* copy numbers (Fig. 7). Also, the high density of AOA in Soil 3 is consistent with their ability to grow in environments with low O_2 content (Hatzenpichler 2012), because this soil is less oxygenated than Soil 2 (Fig. 2B). In Soil 1, AOA abundances remain stable with depth, while AOB decrease, which corresponds to findings in other agricultural soils (Erguder *et al.* 2009). However, it is unclear as to why AOA abundances are decreasing with depth in Soil 2, because this latter is similar to Soil 1 in terms of structure, composition and nitrogen content. In Soil 4, AOA and AOB are less numerous than in the other soils of the catena, which is consistent with the very low level of NH_4^+ in the interstitial water and the low N content of the organic matter (Fig. 2; Table 2).

We found a good correlation between potential nitrification rates and the bacterial *amoA* abundances but not the archaeal *amoA* abundances (Fig. S2, see online supplementary material). This lack of correlation has been observed before (Bernhard *et al.* 2010) and may indicate that the classical potential nitrification assay is unsuitable for AOA, which are adapted to low O_2 and low NH_4^+ concentrations. AOA preferably oxidize ammonia at low concentrations derived from organic matter mineralization (Hatzenpichler 2012; Prosser and Nicol 2012).

Diversity of anammox bacteria

The observation of four distinct anammox clusters in Soil 3 is consistent with earlier findings of an increased phylogenetic diversity of anammox bacteria in terrestrial environments

(Table 1) as opposed to aquatic ecosystems (Schmid *et al.* 2007). Similar observations were made in river estuary sediments, where the highest diversity was found in the least saline part of the estuary (Dale, Tobias and Song 2009). A reduced ecological pressure and the heterogeneity of the soil (and sediments), as opposed to the more homogenous water column habitats, may explain this higher diversity. The change in composition of the anammox community along the profile of Soil 3 suggests niche segregation between the different anammox organisms. In Soil 2, however, the anammox diversity is minimal, with only the presence of Cluster 4 representatives. The anammox sequences detected in this study are also present in other terrestrial environments (with similarities >99%), suggesting that they may have a widespread distribution and are not tied to a specific location or soil type (Fig. S5, see online supplementary material).

Activity of anammox

Anammox and denitrification activities were assessed in anoxic soil incubation experiments, where the ^{15}N-labeled substrates were added to levels above natural concentrations. Such incubation experiments do not provide actual *in situ* rates, but provide proof of activity and serve as proxies for the potential metabolic activity of denitrifying and anammox bacteria, respectively, under substrate replete conditions. The actual *in situ* rates will be lower due to substrate limitation, and variable in time because of e.g. changing redox conditions.

The incubation bottles had been flushed with O_2-free gas and pre-incubated for 3 days to ensure anoxic conditions and to deplete the intrinsic NO_3^- pool. The production of $^{30}N_2$ in the $^{15}NH_4^+$ incubation (control) can be explained by oxygen contamination during tracer addition, incubation and/or subsampling. When repeating these control incubations, no consistent oxygen contamination or significant labeled N_2 could be detected. Still, this illustrates that O_2 contamination can occur during manipulations and can significantly influence the outcome of such experiments. Careful experimental procedures (e.g. subsampling under an N_2 atmosphere in an anaerobic chamber) and oxygen monitoring is indispensable to highlight anoxic ammonium oxidation pathways.

Despite this, the $^{15}NO_3^-$ incubations can be used to quantify denitrification and to estimate the maximum contribution of anammox to N_2 formation. Production of $^{30}N_2$ is due to denitrification, whereas $^{29}N_2$ can be formed by denitrifying bacteria from $^{15}NO_3^-$ and $^{14}NO_3^-$ (from re-oxidized $^{14}NH_4^+$) or by anammox bacteria from $^{15}NO_3^-$ and $^{14}NH_4^+$. Our data show that denitrification is dominating anammox in both soil samples and that anammox accounts for <2.1% in Soil 2 and <1.4% in Soil 3 of total N_2 produced. This strong dominance of denitrification may be due to the higher organic matter content in soils than in aquatic environments. Similar observations have been made in marine sediments where anammox was hardly detectable in organic-rich bay and shelf sediments, but became progressively more important with distance from the shore, in concert with decreasing quantity and reactivity of organic matter (Dalsgaard, Thamdrup and Canfield 2005). The potential rates of anammox and its contribution to N_2 production reported here fall within the lower range of what was observed in soils (Table 1). Higher anammox activity in certain soils can be explained by greater availability of NH_4^+ and NO_3^-. For instance, in fertilized paddy soils, where anammox contributes up to 37% of total N_2 formation (Zhu *et al.* 2011), the concentration of inorganic N is one or two orders of magnitude higher than in the nearly pristine wetland soils investigated here.

CONCLUSION

In this soil system, anammox bacteria can be found in deeper soil compartments where the conditions are favorable for their growth. They depend on long-term water-saturated conditions, and nitrate concentrations at least in the μmol L^{-1} range, which is consistent with the GLM analysis. They can be present even if organic carbon contents are elevated. Anammox bacteria are less abundant and active than aerobic ammonia oxidizing microorganisms or denitrifying bacteria. Their activity in this system falls into the lower range of what has been determined for soil environments, which is probably related to the low concentrations of ammonium in this natural wetland system. Nevertheless, the studied soil transect revealed a remarkably high diversity of anammox bacteria, which greatly contrasts with results from marine and lacustrine water columns where typically a single candidate genus dominates. Four distinct anammox clusters could be identified, which are also present in various other soil systems, as shown by pairwise comparison of 16S rRNA sequences. Along the depth profile of Soil 3, the structure of the anammox community changes, which suggests niche segregation between the different species. Even though anammox bacteria are not the main actors in the biological N cycle in the investigated soil system, their distinct distribution along environmental gradients provides important insights about the key environmental factors that shape anammox communities in terrestrial systems.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

Authors' Contributions

J.Z and A.B. designed the study, A.B., S.G.-H. and J.Z. performed the field work, B.S.-H. and M.S. supported the *amoA* qPCR analysis, A.B. and S.G.-H. performed laboratory analyses, all authors analyzed the data, A.B. and J.Z. wrote the manuscript, all authors discussed and revised it.

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