Salinity shifts in marine sediment: Importance of number of fluctuation rather than their intensities on bacterial denitrifying community

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ABSTRACT

The sensitivity of denitrifying community to salinity fluctuations was studied in microcosms filled with marine coastal sediments subjected to different salinity disturbances over time (sediment under frequent salinity changes *vs* sediment with "stable" salinity pattern). Upon short-term salinity shift, denitrification rate and denitrifiers abundance showed high resistance whatever the sediment origin is. Denitrifying community adapted to frequent salinity changes showed high resistance when salinity increases, with a dynamic nosZ relative expression level. Marine sediment denitrifying community, characterized by more stable pattern, was less resistant when salinity decreases. However, after two successive variations of salinity, it shifted toward the characteristic community of fluctuating conditions, with larger proportion of *Pseudomonas*-nosZ, exhibiting an increase of nosZ relative expression level. The impact of long-term salinity variation upon bacterial community was confirmed at ribosomal level with a higher percentage of *Pseudomonas* and lower proportion of nosZII clade genera.

1. Introduction

Coastal lagoons are productive ecosystems with high economic importance, frequently threatened by eutrophication and oxygen depletion due to anthropogenic nitrogen and/or phosphorus enrichment. In aquatic ecosystems, nitrogen cycle regulates the amount of nitrogen available for phytoplankton. The largest source of nitrogen (N_2) is located in Earth's atmosphere, however, it has limited availability for biological use. In contrast, ammonium (NH_4^+), nitrate (NO_3^-) or nitrite (NO_2^-) that are easily absorbed and metabolised, are in much lower concentrations. The scarcity of usable nitrogen molecules and their availability can affect some key processes, such as, the productivity, dynamics and the eutrophication level of many types of environments (Kjerfve, 1985; Kjerfve et al., 1996; Ogilvie et al., 1997; Bonin et al., 1998; Rysgaard et al., 1999; Bonin, 2000; Gardner et al., 2006). Nitrogen cycle is a network of processes by which nitrogen is converted into multiple forms including organic nitrogen, ammonium, nitrite, nitrate, nitrous oxide (N_2O), nitric oxide (NO) or dinitrogen (N_2) gases. In shallow zones, biogeochemistry of the sediments and the overlying water are tightly coupled (Howarth et al., 2011). As a result, the relative percentages of the different forms of nitrogen ex-fluxing from the sediment into the water column have important implications for the status of coastal zones such as lagoons (Eyre and Fergusson, 2002), suggesting the major role of sediment microorganisms in achieving these processes. Among them, denitrifiers, that are able to reduce stepwise nitrate or nitrite through few intermediate gaseous nitrogen compounds to dinitrogen (Zumft, 1997), have a pivotal role in removing nitrogen from ecosystems and in counterbalancing possible excesses. Denitrification is catalysed by diverse facultative anaerobic microorganisms composed almost exclusively of Bacteria and Archaea (Rusch, 2013). Bacterial denitrifiers are affiliated to > 80 genera, spread over different phyla including *Proteaobacteria* (Alpha-, Betat-, Gamma-, Deltat-classes), *Bacteriodetes*, *Firmicutes*, *Verromicrobia*, *Gemmatimonadetes*, *Spirocheates*, *Deferribacters*. In contrast, Archaea denitrifiers are affiliated to a lower number of genera (26) that belong mainly to *Euryarchaeoa* phylum (24 genera) dominated by *Halobacteria* class (23 genera) over *Archaeglobi* class (1 genus, *Ferroglobus*); and to a lower extend of *Crenarchaeota* phylum (1 genus, *Thermoprotei* class) and *Thaumarchaeota* phylum (1 genus, *Nitrososphaeria* class). The nosZ gene, encoding for nitrous oxide reductase enzyme and catalysing the last step of denitrification, is one of the genes used to follow the denitrifying community (Scala and Kerkhof, 1998; Rosch et al., 2002; Rontani et al., 2010). Recently, phylogenetic studies of nosZ genes in soil revealed two distinct clades; nosZI and nosZII (Jones et al., 2013) of which organism would react differently toward environmental parameters such as pH, nitrogen, calcium concentration and cropping system (Domeignoz-Horta et al., 2015).

Microbial community of lagoons and coastal ecosystems are subject to continuous environmental changes, such as those involving salinity or nutrient concentrations. To maintain their ability to function they

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must tolerate rapid and repeated fluctuations (Castel et al., 1996; Baho et al., 2012). Variations in salinity impose considerable osmotic and matric stress on microorganisms and can cause decreases or increases in cytoplasmic volume; damage to membranes, proteins and nucleic acids; and cellular lysis.

In literature, contradictory tendencies have been reported on the effects of salinity changes on denitrification. Salinity has no effect on denitrification for several coastal or estuarine areas (Nielsen et al., 2001; Fear et al., 2005; Magalhaes et al., 2005), whereas other studies have shown an inverse relationship between denitrification rates and salinity (Rysgaard et al., 1999; Giblin et al., 2010). However, it is difficult to assess the direct impact of salinity changes in field studies because many environmental and chemical parameters are correlated or interact at sampling sites (Jackson and Vallaire, 2009). Only a few experimental studies (*e.g.*, (Nowicki, 1994; Rysgaard et al., 1999; Magalhaes et al., 2005; Laverman et al., 2007) address the resistance and resilience of microbial sediment activity in the face of salinity perturbations but without taking into account microbial community.

In the present study, we used a microcosm approach to examine specifically the effects of short-term salinity variations on denitrifying activity and community structure, taking into account the disturbance history of the sediment. The sediments selected for this study originated from the brackish semi-enclosed Berre lagoon subjected to eutrophication (SE France, Fig. S1) that contained extremely low macrofauna, that is a parameter that could impact proportion of nosZ-harbouring bacteria (Bonin et al., 2015). An indirect effect of salinity fluctuation on macrofauna was in consequence excluded in this study.

Different scenarios of salinity variations were performed in this study in order to test the resistance of the communities from both stations that experience different patterns of long-term salinity stress. Several questions were addressed in this study (i) whether the long-term salinity history has an effect on the denitrification rates and community (including nosZI/nosZII taxon repartition), (ii) whether short-term salinity variation has an impact on denitrification rates and community (nosZI/nosZII taxon repartition, (iii) what are the main factors affecting community resistance and which taxa take advantage of fluctuating conditions?

2. Materials and methods

2.1. Sampling sites

The sediments selected for this study originated from the brackish semi-enclosed Berre lagoon subjected to eutrophication, which offers a particular historical record of salinity. The northern part is subjected to frequent salinity changes due to Mediterranean river flows sharp fluctuation (Delpy et al., 2012) and to a hydroelectric power plant that introduces freshwater inputs in a fluctuating mode. The southern part of the lagoon is in front of a channel leading to the Mediterranean Sea. It is, consequently, under marine influence and experiences only few salinity fluctuations of lower intensity. The two selected stations showed, from an annual survey, similar parameters in porous water such as nitrate, nitrite, ammonium concentrations and % of Lost-On-Ignition (LOI, 11 ± 2% and 13 ± 2% for S1 and S3) (Zaghmouri et al., 2013). The two sampling stations (Fig. S1) were distinguished by their contrasting salinity patterns. The first station (S1, ~6 m deep) is located in the northern part of the lagoon in front of the power plant and the Arc and Touloubre River mouths and is subject to freshwater loads. Its annual salinity ranges between 15 and 30. The second station (S3, ~9 m deep) is located in the southern part of the lagoon beyond the Caronte channel, which connects the lagoon to the Mediterranean Sea. Station S3 is under marine influence; its annual salinity ranges from 20 to 35.

2.2. Sample collection

The sediments used in the experiment were collected in December 2010 at station S3 (with an *in situ* salinity of 33 and an *in situ* temperature of 15.9 °C) and in March 2011 at station S1 (with an *in situ* salinity of 24 and an *in situ* temperature of 14.6 °C) (Fig. S1). Both sediments were rich in mud and thus characterized by a low permeability. An annual survey has shown no difference in concentration of O_2 in bottom water, or concentration of nitrate, nitrite and ammonium in the porous water nor in organic matter content between both stations (Zaghmouri et al., 2013). For each station, a set of 5 Plexiglas cores (25 cm length, 10 cm inner diameter) was collected. The bottom half of the cores was filled with sediment (12 cm length). The top half was filled with *in situ* bottom water and aerated to maintain sediment saturation and to prevent the establishment of anoxic conditions during transportation.

2.3. Description of microcosms

The Plexiglas cores were used for microcosms incubations. They consisted of cylinders of 10 cm in diameter and 25 cm in height with a total volume of approximately 2 L (Fig. S2). The microcosms were filled with undisturbed sediment from the cores to a height of 12 cm and were fully re-filled with synthetic seawater (SSW) (Baumann et al., 1971) and NaCl to adjust the desired salinity, then hermetically closed to the atmosphere so that no gaseous headspace remained. In each reservoir, salinity was controlled by refractometry. The salinity of the SSW was adjusted by adding NaCl. The $Na^{15}NO_3^-$ and $^{14}NH_4Cl$ concentrations in the reservoirs were adjusted to 250 μM and ~5 μM, respectively. Each microcosm was equipped with an inflow device (0.3 cm inner diameter), extending into the water column and ending 1 cm above the surface sediment, through which the flowing medium entered the system. The outflow device was located on the opposite side and extended 1 cm above the surface sediment. Cores were connected to a 5L-SSW reservoir providing the experimental treatment. The reservoirs were open and provided with a stream of air bubbles to prevent N_2 accumulation and to maintain air saturation in the water phase. Water was pumped *via* a peristaltic pump from the 5L SSW reservoir to the individual microcosms at a flow rate of $0.09 \, L \cdot h^{-1}$, creating a residence time of approximately 10.5 h in each microcosm. During the incubation, the water column in each microcosm was gently mixed using a magnetic stirrer driven by an external magnet (30 r.p.m.). Mixing tests prior to the experiment showed complete mixing within the water portion of the microcosms. The microcosms were incubated at the *in situ* temperature. The microcosms were stabilised at *in situ* seawater salinity for 72 h to reach a steady state (*i.e.*, constant N-NO$_3^-$ and N-NH$_4^+$ uptake rates) before beginning the experiment.

2.4. Microcosm experiment

To assess the impact of salinity variations on the nitrogen cycle, three set up were developed to simulate salinity fluctuations mimicking those recorded in the lagoon with each type of sediment (S1, S3) (Fig. 1).

The first scenario aimed to maintain the salinity close to the *in situ* values throughout the experiment (Experiment S1M1 and S3M1). The second one was designed to follow the effect of one salinity shift: one increase from 20 to 35 for S1 (Experiment S1M2) or two decrease levels for S3: from 35 to 25 (Experiment S3M2A) or from 35 to 15 (Experiment S3M2B). The third one intended to follow the shift back to the initial *in situ* salinities after the previous disturbance: S1M2 to 20 (Experiment S1M3) and S3M2 A and B to 35 (Experiment S3M3A, S3M3B). The SSW in each microcosm reservoir was renewed at the beginning of each salinity fluctuation phase.

Before starting the experiments, during the 1st phase (3 days), five replicates of each station microcosms were maintained at *in situ* salinity in order to stabilise the system (salinity of S1 = 20, S3 = 35). During the 2nd phase (~0.5 days of transition plus 4.5 days of stability) M1 microcosms were kept at *in situ* salinity, duplicates of microcosms were