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Denitrification responses to increasing cadmium exposure in Baltic Sea sediments

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ABSTRACT

Benthic ecosystems have come under intense pressure, due to eutrophication-driven oxygen decline and industrial metal contamination. One of the most toxic metals is Cadmium (Cd), which is lethal to many aquatic organisms already at low concentrations. Denitrification by facultative anaerobic microorganisms is an essential process to transform, but also to remove, excess nitrate in eutrophied systems. Cd has been shown to decrease denitrification and sequester free sulfide, which is available when oxygen is scarce and generally inhibits complete denitrification (i.e. N\textsubscript{2}O to N\textsubscript{2}). In polluted sediments, an interaction between oxygen and Cd may influence denitrification and this relationship has not been studied. For example, in the Baltic Sea some sediments are double exposed to both Cd and hypoxia. In this study, we examined how the double exposure of Cd and fluctuations in oxygen affects denitrification in Baltic Sea sediment. Results show that oxygen largely regulated N\textsubscript{2}O and N\textsubscript{2} production after 21 days of exposure to Cd (ranging from 0 to 500μg/L, 5 different treatments, measured by the isotope pairing technique (IPT)). In the high Cd treatment (500μg/L) the variation in N\textsubscript{2} production increased compared to the other treatments. Increases in N\textsubscript{2} production are suggested to be an effect of 1) enhanced nitrification that increases NO\textsubscript{3}\textsuperscript{−} availability thus stimulating denitrification, and 2) Cd successfully sequestrating sulfide (yielding CdS), which allows for full denitrification to N\textsubscript{2}. The \textit{in situ} field sediment contained initially high Cd concentrations in the pore water (~10μg/L) and microbial communities might already have been adapted to metal stress, making the effect of low Cd levels negligible. Here we show that high levels of cadmium pollution might increase N\textsubscript{2} production and influence nitrogen cycling in marine sediments.

1. Introduction

Benthic habitats are highly diverse ecosystems important for the ecosystem functions of nutrient cycling between the pelagic waters and the sediment (Griffiths et al., 2017). A significant part of biogeochemical transformation is conducted in sediments by bacteria and archaea (Burdige, 2006). These microbial communities are essential in, e.g., the reduction and oxidation of nitrogen and carbon compounds (Burdige, 2006). Denitrification is one of the most important microbially driven biogeochemical processes in transforming and removing NO\textsubscript{3}\textsuperscript{−} (Galloway et al., 2004). The process becomes particularly important in eutrophied environments, where overload of nutrients from industrial and agricultural production cascade into phytoplankton blooms (Conley, 2012). As a large portion of this phytoplankton bloom is sedimented (Conley, 2012; Emeis et al., 2000; Tallberg and Heiskanen, 1998) and heterotrophically respired by microorganisms, oxygen levels may decline (Middelburg and Meysman, 2007). Once dissolved oxygen becomes scarce, bacterial and archaeal populations start to respire a variety of other electron acceptors depending on energy gain, with NO\textsubscript{2}\textsuperscript{−} being the most favorable after oxygen (Burdige, 2006). As a result, the reduction of NO\textsubscript{3}\textsuperscript{−} by bacteria becomes prevalent when previously oxygen-rich sediment turns hypoxic (< 2 mg/L O\textsubscript{2}) (Marchant et al., 2017). Denitrification is carried out in several enzymatic steps, reduction of 1) NO\textsubscript{3}\textsuperscript{−} to nitrite (NO\textsubscript{2}\textsuperscript{−}), 2) NO\textsubscript{2}\textsuperscript{−} to nitric oxide (NO), 3) NO to nitrous oxide (N\textsubscript{2}O), and finally 4) N\textsubscript{2}O to nitrogen gas (N\textsubscript{2}) (Devol, 2015). The enzymes nitrite reductase (nit i.e. NO\textsubscript{2} to NO) and
nitrous oxide reductase (nos i.e. N₂O to N₂) catalyze key steps and their respective genes (nirS/K and nosZ) have been used as functional genetic markers of denitrification (e.g. Braker et al., 2000; Throbäck et al., 2004). In addition to studying gene abundance, transcript abundance of genes in general is commonly used as indicator of functional activity of corresponding enzymes (Aguiar-Pulido et al., 2016). Geochemical measurements and molecular tools are therefore widely used when studying nitrogen cycling. However, many polluted sites are also affected by other contaminants that influence denitrification rates and gene activity.

It is well documented that Cadmium (Cd), originating from industrial production or wastewater, combustion and agricultural activities (Carolin et al., 2017; Sigel et al., 2013), is one of the most toxic metals (Tchounwou et al., 2012). Cd is lethal to many aquatic organisms already at low concentrations (Strydom et al., 2006) by affecting aerobic respiration through oxidative stress with subsequent cell damage (Cherkasov et al., 2007; Kurochkin et al., 2009; Stohs and Bagchi, 1995). Moreover, Cd is known to adsorb to organic material, such as algae (Chu et al., 1997), which might transfer Cd to higher trophic levels through ingestion by, e.g., zooplankton (Reinfelder and Fisher, 1991) and macrofauna (Wang and Fisher, 1996), or deposit it onto the benthos. On the other hand, addition of organic matter is known to stimulate benthic nitrogen cycling (Devol, 2015). Under hypoxic conditions, Cd forms complexes with sulfide and precipitates as cadmium sulfide (CdS) (Banks et al., 2012). The solubility of Cd from the sediment to the overlying water is therefore indirectly dependent on oxygen, and high concentrations of Cd may accumulate in anoxic sediments (Banks et al., 2012). Cd might therefore have a key role in the nitrogen cycle, as sulfide has been shown to inhibit the reduction of N₂O to N₂ (Bonaglia et al., 2016; Sørensen et al., 1980). A couple of studies have shown that high concentrations of Cd decrease denitrification rates in sediment and soil (Liu et al., 2016; Sakadevan et al., 1999), as well as the copy numbers of denitrification genes in soil (Liu et al., 2016). However, it remains to be investigated how oxygen fluctuations affect the intensity of Cd exposure on denitrification.

The Baltic Sea, located in northern Europe, is one of the most contaminated seas with high concentrations of both nutrients and toxic metals (Andersen et al., 2010). In addition, hypoxic bottom zones are widespread, as well as hypoxia in coastal areas (Conley et al., 2011). In the Baltic Sea, Cd pollution in coastal sediments is therefore likely to co-occur with hypoxia (Andersen et al., 2010, 2009). Especially in coastal areas adjacent to factories and industrial harbors the Cd concentrations in the sediment can be high, exceeding levels of 5 mg kg⁻¹ dry weight (dw) (Tobiasson and Andersson, 2013), relative to the Swedish national threshold values for ‘good environmental status’ at 0.5 mg kg⁻¹ dw (Andersen et al., 2010). The Baltic Sea is thus a good study system to investigate the effect of Cd on coastal ecosystem processes.

In this study, we analyzed the combined effect of Cd and varying oxygen levels on denitrification using mesocosms with coastal Baltic Sea sediments. Sediments were exposed to different concentrations of Cd (0–500 μg/L, 5 different treatments), and one Cd treatment was also exposed to algal organic matter (to simulate dead algal biomass adsorbed with Cd sinking to the sediment). Denitrification rates (N₂O and N₂) and the abundance of transcripts of denitrification genes (nirS and nosZ) were analyzed after 21 days of exposure to Cd under fluctuating oxygen conditions. We hypothesized that Cd amendment would reduce sediment denitrification rates, drawing from previous studies on the effect of cadmium on denitrification (as described above). Differences in oxygen would either increase (nitrification) or decrease available NO₃⁻ (denitrification). However, high loads of cadmium would eventually have a stronger effect than that of oxygen. We also hypothesized that Cd added with OM, as another possible route to reach the sediment, could reduce the negative effects of Cd on denitrification.

2. Materials and methods

2.1. Field sampling

Soft bottom sediment was collected during October 12 2018 on the east coast of Sweden in the Stockholm archipelago, Baltic Sea (Hållsviken, WGS84 coordinates: Lat 58.832177, Long 17.534229). The water depth was 30 m and conductivity, temperature, pH, and dissolved oxygen were measured in the water surface (~50 cm) and bottom water (~29 m) using multi-probe field sensors (WTW Multi431). Bottom water was collected using a 5 L Niskin bottle and transferred into rinsed 25 L carboys. Sediment with intact overlying bottom water was collected using a box corer (Jonasson and Olaison, 1966). Transparent acrylic cores (length: 30 cm, inner diameter: 6.4 cm, surface area: 17 cm²) were inserted into the box corer, collecting sediment and bottom water, and sealed underneath and at the top using silicon plugs. From three cores the sediment surface (0–1 cm) was sliced directly on the boat to measure Cd concentrations in the field sediment. The sliced sediment was transferred into sterile 15 mL centrifuge tubes (Sarstedt) for chemical measurements. For the experimental studies, an additional 34 cores were collected. All samples were immediately stored in a cooling box and during transport back to the laboratory. Samples for chemical measurements were then stored at ~20 °C, and the experimental sediment cores were stored in darkness at in situ temperature (~9 °C).

2.2. Cd exposure experiment

After three days of sediment acclimation, the water overlying the sediment was replaced with collected in situ bottom water without disturbing the sediment. Sediment height was measured and all cores were topped up. This was to ensure all sediment cores had the same water volume and a similar background interface. This procedure added fully oxygenated bottom water to all cores and was conducted carefully by spraying bottom water using a syringe directly on top of a floating, flat disk placed above the sediment surface. The cores were placed in a circular pattern and magnets, attached to a tube with the same size as the inner side of the cores, were inserted and kept stable in the overlying bottom water. Using an external rotating magnet, the bottom water was gently mixed without disturbing the sediment surface. At the start of the experiment four cores were sacrificed and the 0–1 cm sediment surface was sliced and kept to provide sediment zero-time points. The sliced sediment was collected, homogenized and handled as mentioned above for the field samples. In addition, from each core 2 ml of homogenized sediment for RNA extraction was rapidly transferred into cryogenic tubes (2 ml, VWR), instantly frozen, and stored at ~80 °C.

The remaining 30 cores were divided into five treatments of different concentrations of Cd (n = 6 for each treatment): 1) 0 μg/L Cd, 2) 2 μg/L Cd, 3) 2 μg/L Cd + organic matter (OM), 4) 100 μg/L Cd, and 5) 500 μg/L Cd. Treatments are referred to as 0 Cd, 2 Cd, 2 Cd + OM, 100 Cd, and 500 Cd. Concentrations represent the Cd in the bottom water plus the sediment surface (top 1 cm) inside the sediment cores. These concentrations can be compared to the European Union threshold of 5 μg/L Cd for drinking water (European Commission, 1998). From a stock solution of CdCl₂ (anhydrous ≥ 99.0%, Fluka) the cores were carefully amended with calculated amounts (in relation to volumes of bottom water and the 0–1 cm sediment surface) of Cd by pipetting directly into the bottom water of each core. Cores amended with organic matter were prepared from a sampled algal spring bloom. The spring bloom was sampled during April 2017 (Hållsviken, WGS coordinates Lat 58.833333, Long 17.533333) using a 90 μm-mesh plankton net and zooplankton removed using a light trap. The spring bloom material was transferred into 50 ml centrifuge tubes and stored at ~20 °C until further analysis. The carbon concentration of the spring bloom was determined, after thawing at 4 °C overnight, from sub-samples (2 ml on
pre-combusted GF/F filters, \( n = 10 \) with a Thermo Scientific Flash 2000 Elemental 189 Analyzer. Wet spring bloom biomass representing \( \sim 2 \) g carbon was thoroughly mixed with Cd, and added together into the respective cores. All treatments were left open at the top, to oxygenate cores through air-water diffusion, in darkness at in situ temperature and exposed for 21 days.

2.3. Chemistry measurements and \(^{15}\)N isotope experiment

Dissolved oxygen was measured in the 0 Cd treatment cores on day 7, 11, 14 (only control cores were measured to not cause unnecessary damage from Cd to the sensor), and in all cores on day 21 (LD0101 sensor with HQ40d meter, Hach). After 21 days exposure time, a \(^{15}\)N isotope pairing analyses technique (IPT) experiment was carried out to determine rates of sediment denitrification as N\(_2\) and N\(_2\)O production (Nielsen, 1992). Briefly, each sediment core’s overlying water was spiked with 40–60 \( \mu \)L of a 100 mM \( ^{15}\)NO\(_3^-\) solution, to a final concentration of ca. 25 \( \mu \)M \( ^{15}\)NO\(_3^-\) in the water phase of each core. The \( ^{15}\)NO\(_3^-\) solution was made by dissolving 430 mg Na\(^{15}\)NO\(_3\) (99.4 \( ^{15}\)N atom %; Sigma-Aldrich, DE) into 50 mL Milli-Q water. Incubation started after a pre-incubation time of ca. 1 h, which was necessary to homogeneously mix the added \( ^{15}\)N nitrate with the \( ^{14}\)N nitrate and to establish a linear production of labeled N\(_2\) and N\(_2\)O over time (De Brabandere et al., 2015). At the start of the incubation cores were capped with rubber stoppers so that no air bubbles formed, and the water was continuously mixed by externally driven magnetic bars. Cores were kept closed and incubated for 4–5 h. The incubation was terminated by uncapping each core and withdrawing 20 mL sample without oxygen using a syringe equipped with a rubber tubing (Bonaglia et al., 2017). The water sample was immediately transferred into a 12 mL Exetainer\textsuperscript{®} (Labco, UK) to which 250 \( \mu \)L of a 7 M ZnCl\(_2\) solution was added to stop biological activity. The experimenters were stored refrigerated and upside down until later analysis by UC Davis facilities (USA). Concentrations of \( ^{29}\)N\(_2\), \( ^{30}\)N\(_2\), \( ^{45}\)N\(_2\)O, and \( ^{46}\)N\(_2\)O were determined injecting headspace samples into a gas chromatograph-isotope ratio mass spectrometer (GC-IRMS, UC Davis, USA). Rates of denitrification were calculated from \( ^{29}\)N\(_2\), \( ^{30}\)N\(_2\), \( ^{45}\)N\(_2\)O and \( ^{46}\)N\(_2\)O as determined by GC-IRMS, and were split into N\(_2\) production over time (N\(_2\)-denitrification) and N\(_2\)O production over time (N\(_2\)O-de-nitrification) (Bonaglia et al., 2017; De Brabandere et al., 2015; Nielsen, 1992).

The experiment was ended by slicing the sediment surface (0–2 cm) from each core. The sediment was rapidly homogenized and portioned both for chemistry measurements (15 mL centrifuge tubes) and for RNA extraction (2 mL cryotubes that were directly flash frozen in liquid nitrogen). Samples for chemistry measurements were stored at \( \sim 20 \) °C, while the flash frozen samples for RNA extraction were stored at \( -80 \) °C until further processing. Pore water was collected from thawed sediment samples by centifying at 2200 \( \times \) g and filtering the supernatant through a sterile 0.2 \( \mu \)m pore-size syringe filter (polyethersulfone 26 mm filter; Filterpur S, Sarstedt). Cd concentration analyses, of bulk sediment (dried, and extracted 30 min at 120 °C in 7 M HNO\(_3\)) and pore water, respectively, were done by atomic absorption spectrometry (Spectra A 55B, Agilent, USA) equipped with a flame atomizer and graphite furnace (GTA100, Agilent) according to Svensk Standard: ISO 15586:2004 (2004) Svensk Standard: ISO 15586:2004 (2004).

2.4. RNA extraction and Real time-qPCR assays

RNA was extracted from 1 g of sediment sample using the FastRNA\textsuperscript{®} Pro soil direct Kit (MP Biomedicals\textsuperscript{®}) according to manufacturer’s instructions, with the following modifications: after processing the sample in the FastPrep\textsuperscript{®} (MP Biomedicals), the 3/4 of the liquid phase was transferred to a new eppendorf tube and the remaining liquid phase was homogenized a second time. The samples were incubated for 90 min at \( -20 \) °C after the addition of 660 \( \mu \)L of cold 100% isopropanol. RNA concentration and quality were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). Reverse transcription reactions were performed immediately after extracting the RNA, with Quantitect\textsuperscript{®} Reverse Transcription kit (Qiagen), following the manufacturer’s directions. DNA traces were digested using gDNA wipout buffer provided in the kit.

Real-time qPCR was used to quantify transcript abundance through cDNA conversion of transcripts for key functional genes coding for nitrite- (\( nitrS \)) and nitrous oxide- (\( nosZ \)) reductases involved in denitrification pathways. The transcript copy numbers of \( nitrS \) were determined by primer pair c33aF and R33c (Throbäck et al., 2004). The reaction mixture (15 \( \mu \)L) contained 7.5 \( \mu \)L of SsoAdvanced SYBR Green supermix (Bio-Rad laboratories), 0.2 pmol/\( \mu \)L primers, 1 \( \mu \)L template and RNase-free water (Qiagen) and qPCR was performed on Bio-Rad C1000 Touch™ Thermal cycler, CFX96™ Real-Time System. The cycling conditions consisted of an initial denaturation step for 3 min at 98 °C followed by 40 cycles of 15 s at 95 °C, 20 s at 53 °C, and 20 s at 72 °C. For \( nosZ \) analyses, primers used were \( nosZ\) F (Henry et al., 2006) and \( nosZ\) R789TR (Rösch et al., 2002), and each 20 \( \mu \)L reaction contained 10 \( \mu \)L of SsoAdvanced SYBR Green supermix (Bio-Rad laboratories), 1 \( \mu \)L of cDNA template and 0.25 \( \mu \)M of each primer. qPCR cycling conditions were as follows: 95 °C 5 min, 40 cycles of 15 s 95 °C, 15 s 55 °C, and 40 s at 72 °C and melt curve analysis. All qPCR amplifications were run in triplicates and melt curves were used to test the stringency of the reaction. Standard curves were obtained with five-step serial dilutions (10\(^{-2}\)–10\(^{6}\)) of linearized plasmids harboring synthetic gene fragment inserts of the targeted genes (Motwani and Gorokhova, 2013). The \( nitrS \) gene from Paracoccus denitrificans and \( nosZ \) gene from Marinobacter sp BC38 were synthesized at Eurofins MWG Operon (Ebersberg, Germany). The standard curves had a \( r^2 \) > 0.99 in all assays and efficiency of 98% and 96% for \( nitrS \) and \( nosZ \) gene, respectively.

2.5. Statistics

The data were tested with Shapiro-Wilk and variables not following a normal distribution were transformed (N\(_2\)O boxcox transformation for ANCOVA test, and square root transformation of all variables for Pearson correlations). Statistics were conducted with Analysis of covariance (ANCOVA) using N\(_2\) and N\(_2\)O as dependent variables and O\(_2\) as a covariate, and One-Way ANOVA (with post hoc Tukey tests) to detect differences between variables and treatments. Pearson correlations of all data points from the experiment were used to detect trends. Statistical tests were conducted in the software SPSS 25 (IBM) and the R 3.5.1 (R Core Team, 2013) package “car” (Fox and Weisberg, 2018).

3. Results

Field data indicated that the water column was mixed at the time of sampling. Surface water had a temperature of 8.5 °C, pH 8.5, conductivity 12.57 (mS/cm), and a dissolved oxygen concentration of 10.0 mg/L. Bottom water temperature was 8.7 °C, pH 7.7, conductivity 12.69 (mS/cm), and the oxygen concentration was 8.5 mg/L.

3.1. Cd concentrations in the sediment and pore water

After 21 days of exposure to Cd in the different treatments, Cd concentrations in the bulk sediment and pore water showed that the majority of the amended Cd had been adsorbed to the sediment particles (Fig. 1). Sediment sliced in the field (0–1 cm sediment surface) and at the start of the exposure experiment (0–1 cm sediment surface) had similar Cd concentrations in pore water (11.3 ± 0.7 \( \mu \)g/L, \( n = 4 \)) and bulk sediment (19.3 ± 1.7 \( \mu \)g/Kg dw, \( n = 4 \)) (compared to field data: 10.6 ± 1.8, and 19.8 ± 5.1, respectively; \( n = 3 \), one standard deviation shown; Fig. 1). Concentrations were also similar to that of the 0 Cd controls (0–2 cm sediment surface sliced at end of experiment) at the
end of the exposure experiment for pore water (11.2 ± 0.6 μg/L, \( n = 6 \)) and bulk sediment (16.4 ± 0.4 μg/Kg dw, \( n = 6 \)) (Fig. 1). All treatments had significantly different concentrations of Cd (One-Way ANOVA, post hoc Tukey tests; \( P < 0.05 \)) in both the pore water and bulk sediment, except for the 2 Cd and 2 Cd + OM. For example, compared to the 0 Cd controls (< 16.5 μg/L Cd measured) the 500 Cd treatments had 29.4 ± 0.8 μg/L Cd in the pore water (\( n = 6, P < 0.01; \) Fig. 1A) and 204.2 ± 3.8 μg/Kg dw in the bulk sediment (\( n = 6, P < 0.01; \) Fig. 1B). Water content in the sediment samples was ~85%, and
Cd concentrations as mg/Kg wet weight (ww) in the different treatments therefore approximately correspond to: 0 Cd (0.07 mg/Kg ww), 2 Cd (0.09 mg/Kg ww), 2 Cd + OM (0.10 mg/Kg ww); 100 Cd (0.37 mg/Kg ww); and 500 Cd (0.82 mg/Kg ww).

3.2. Oxygen concentrations and N2O/N2 production rates

Oxygen concentration, in the bottom water overlying the sediment, fluctuated inside the cores throughout the experiment (see Data S1 for concentrations measured in the control cores on day 7, 11, and 14), and on day 21 at the end of the experiment oxygen was: 3.1–6.9 mg/L in the 0 Cd treatment; 1.9–7.2 mg/L in the 2 Cd; 2.9–6.8 mg/L in the 2 Cd + OM; 0.4–6.2 mg/L in the 100 Cd; and 0.8–7.5 mg/L in the 500 Cd treatment (n = 6 for all treatments with no statistical significance between treatments; Data S1 and Data S3). N2O had a significant negative correlation with oxygen at the end of the experiment when testing all data from the experiment (Pearson correlation, r = -0.801, P < 0.001, n = 30; Fig. 2A). However, there was no correlation between N2O and Cd concentrations in the sediment or pore water (Fig. S1 and Data S2). The N2O data was tested for significant differences (as a dependable variable) between the treatments with ANCOVA, using the treatment groups as an independent factor, and oxygen as a covariate. Oxygen had a statistical significance with the N2O production rates in the different Cd treatments (F = 26.489, P = 0.00003; Data S3), as well as the Cd treatment groups (F = 8.911, P = 0.000147; Data S3).

N2 had a significant positive correlation with oxygen when tested with all data from the experiment (Pearson correlation, r = 0.382, P = 0.041, n = 29; Fig. 2B). N2 production rates also correlated positively with the Cd concentration in the sediment (r = 0.428, P = 0.021; Fig. 2C), and pore water (r = 0.418, P = 0.024; Data S2). ANCOVA analysis with N2 as a dependent variable, using the treatment groups as an independent factor, and oxygen as a covariate, showed that the N2 production rates varied statistically significantly with the oxygen and the different Cd treatment groups (F = 9.192, P = 0.00593; and F = 3.099, P = 0.03534, respectively; Data S3). Finally, there was no statistical difference between the 2 Cd and 2 Cd + OM (One-Way ANOVA tests) for the N2O and N2 production rates, and Cd concentrations in the sediment did not correlate with oxygen concentrations in the bottom water overlying the sediment surface (Fig. 2D).

3.3. Nitrite reductase and nitrous oxide reductase transcript abundance

The amount of nirS (coding for nitrite reductase, which produces N2O) and nosZ (coding for nitrous oxide reductase, which produces N2) RNA transcript copy numbers were 2 577 500 ± 203 039 and 2 055 000 ± 234 023 (copies g⁻¹ ww) in the sediment surface at the start of the exposure experiment, respectively (i.e. in zero-time point cores sliced at the start; n = 4, Data S1). Compared to the start of the experiment there was a significant lower amount of nirS and nosZ transcripts at the end of the experiment in all treatments (< 800 000 gene copies, P < 0.01, One-Way ANOVA post hoc Tukey tests; Fig. 3A). Results from both nirS and nosZ showed significantly lower RNA transcript copy numbers in the 100 Cd and 500 Cd treatments, with nirS in the 100 Cd (7 600 ± 1 020 copies g⁻¹ ww) and 500 Cd (1 512 ± 199) treatments (Fig. 3A); and nosZ in the 100 Cd (6 645 ± 731) and 500 Cd (1 296 ± 170) treatments (Fig. 3B) (compared to 100 942 - 7 711 000 nirS and nosZ gene copies in the 0 Cd, 2 Cd, and 2 Cd + OM treatments; P < 0.01; Fig. 3). Looking closer at the nirS and nosZ copy numbers, there was a significant negative correlation with Cd concentrations in the sediment (nirS, r = -0.873, P < 0.001; and nosZ, r = -0.866, P < 0.001) and pore water (nirS, r = -0.906, P < 0.001; and nosZ, r = -0.900, P < 0.001; Data S2). Both nirS and nosZ transcript abundance also correlated with oxygen and N2O production rates (r = -0.37 (for N2O), and r = 0.37 (for O2), P < 0.05, same for both nirS and nosZ; Data S2). Finally, there was a significant difference between nirS and nosZ with both the Cd treatment groups and oxygen (P < 0.05; Data S3) as shown by ANCOVA tests, using the treatment groups as an independent factor, nirS and nosZ as dependable variables, with oxygen as a covariate.

4. Discussion

4.1. Production of N2 increased in the high Cd treatment

This study shows that N2 production was affected by Cd concentrations in the sediment, indicating that the addition of Cd had an influence on microbial denitrification. This was in contrast to our hypothesis that Cd would reduce denitrification rates. N2 production occurred in the majority of the cores in the 500 Cd treatment with high oxygen (Fig. 2B and C). This might have been due to an effect of CdS formation sequestering free sulfide (known to inhibit N2O reduction to N2) which would allow for full denitrification to N2 (Bonaglia et al., 2016; Sørensen et al., 1980), and nitrification in the sediment surface (Stenstrom and Poduska, 1980) that has been shown to increase NO3⁻ availability and denitrification in coastal sediments (Seitzinger, 1988). Even though we did not measure CdS concentrations, a large part of the added Cd was measured to be in the sediment surface where it would have been available for CdS formation. Nitrifying microbial populations have been shown to be initially inhibited by Cd (1.5 mg/L) but adapt over time to the metal stress (Ganguly and Jana, 2002). Cd in the sediment did not correlate with oxygen (Fig. 2D). However, Cd correlated with N2 production rates although with a low r-value of ~0.4 (likely...
due to \( N_2 \) production rates being higher only in the highest Cd treatment). Despite the weak correlation the Cd treatments and \( N_2 \) were significantly different in the ANCOVA analysis with \( O_2 \) as a covariate. This indicates that Cd had an effect on \( N_2 \) production in the sediment.

Rates of nitrate and nitrite reduction have been shown to increase with decreased oxygen concentrations and to be especially high at hypoxic/anoxic conditions (Bonin and Raymond, 1999; Marchant et al., 2017). Oxygen correlated negatively with \( N_2O \) production rates (Fig. 2A) and these results are in accordance with previous findings that \( N_2O \) production is sensitive to oxygen presence (Kock et al., 2016). This further indicates that cores amended with high concentrations of Cd (500 Cd treatment) resulted in a different \( N_2 \) production pathway when compared to \( N_2O \). A discrepancy between \( N_2 \) and \( N_2O \) production rates have previously been reported and it was suggested that Cd acts differently on the various denitrification enzymes (Magalhães et al., 2007). Results from this study further strengthen the argument that Cd acts differently on specific enzymes involved in the denitrification pathway.

Previous studies have observed decreased denitrification rates in wetland sediment amended with glucose and Cd (incubated 120 ml vials with 500 mg/Kg Cd ww) (Sakadevan et al., 1999), and sandy intertidal sediments amended with glucose, \( NO_3^- \), and Cd (incubated 6–8 g sediment in septum bottles with 70 mg/Kg Cd ww) (Magalhães et al., 2007). In contrast, our results showed an increased variation in \( N_2 \) production rates in the 500 Cd treatment (Fig. 2C). In this treatment, Cd likely formed CdS below the oxic-anoxic interface, which would sequester dissolved sulfide in the sediment pore water (Banks et al., 2012). The sediment in the sampled bay has previously been shown to have an oxygen penetration depth of \( \approx 5 \) mm (sampled during July 2012; Bonaglia et al., 2014b). The sliced sediment (0–2 cm) in this study would therefore include a redox cascade, including sulfide production from sulfate reduction (Burdige, 2006). This could explain why the variation of \( N_2 \) production was higher in the 500 Cd treatment, as Cd has a higher affinity than iron to sequester sulfide (Bostick et al., 2000), and sulfide has been shown to inhibit the reduction of \( NO_3^- \) to \( N_2 \) and thus promote accumulation of \( N_2O \) (Bonaglia et al., 2016; Sørensen et al., 1980). Furthermore, distinctive from previous studies using the acetylene inhibition technique (Sørensen, 1978) with amended slurries (e.g. Magalhães et al., 2007; Sakadevan et al., 1999), this study used the whole-core IPT method, which increases accuracy due to traceable \( ^{15}NO_3^- \) and allows for all ecosystem processes in the sediment core to influence denitrification rates (Nielsen, 1992). It is therefore possible that previous experiments conducted with homogenized slurries did not account for interactions over, across, and below the oxic-anoxic sediment interface, such as stimulated denitrification (due to nitrification) and CdS formation.

4.2. Field samples contained high amounts of Cd in the pore water

The field samples had \( \approx 10 \mu g/L \) Cd in the pore water (Fig. 1); compared to 5 \( \mu g/L \) threshold for drinking water (European Commission, 1998) and it is therefore possible that some of the members of the microbial community were already adapted to the presence of heavy metals. Metal polluted sediments (containing \( \approx 5 \) mg/Kg Cd ww, and other metals) have been shown to contain different microbial community structures with more metal resistance genes, compared to unpolluted sediments (Chen et al., 2018). Furthermore, Baltic Sea open water sediment has been shown to be overrepresented in Cd-resistance genes when compared with that of other sediment datasets globally (Thureborn et al., 2013). It is therefore possible that the high Cd treatment (500 Cd) in our study contained a different microbial community compared to the other treatments, and this could partly explain why more variation in \( N_2 \) production was observed in high Cd concentrations. We are aware of that the experiment was conducted during October when microbial activity might have been low compared to the summer. For example, comparing our \( N_2 \) production rates in the control cores (47–121 \( \mu mol N_2/m^2 d^{-1} \)) with \( \approx 400 \mu mol N_2/m^2 d^{-1} \) from Bonaglia et al. (2014a), who sampled at a nearby bay during August 2011–2012. However, low microbial activity and reduced \( N_2 \) production rates would have affected both the control and treatment cores, and it is therefore unlikely that this explained the higher \( N_2 \) production rates in the 500 Cd treatment.

Cadmium has beneficial effects for algae at low concentrations, but is toxic at high concentrations with species-specific sensitivities (Xu and Morel, 2013). Cd is adsorbed to algal biomass (Chu et al., 1997) that might sink down to the seafloor and this is therefore an alternative route for Cd to reach the sediment surface. In addition, considering that Cd is known to stimulate denitrification (Devol, 2015) we therefore added Cd with algal OM in the 2 \( \mu L/Cd \) treatment to test for any potential effect on denitrification. However, there was no significant difference in \( N_2O \) and \( N_2 \) production rates after 21 days of Cd exposure. Considering the high concentrations of Cd in the field, an addition of 2 \( \mu L/Cd \) might have been negligible for the microbial communities.

4.3. Cd decreased nirS and nosZ transcripts in the sediment surface

At the end of the experiment abundances of nirS and nosZ RNA transcripts were lower in the sediment of the high Cd treatments (100 and 500 Cd; Fig. 3), suggesting an inhibitory effect of Cd on the expression of these denitrification genes. In addition, gene expression or nirS and nosZ transcription might have been generally low throughout the experiment as suggested by comparing the start samples with the end samples (Data S1). Here we analyzed RNA transcripts, and results showed a similar pattern when compared to studies looking at DNA gene abundances in relation to metal pollution. For example, in metal-polluted soil (6–10 mg/Kg Cd ww, and containing other metals also) the gene abundance of nirK (functionally equivalent with nirS; Zumft, 1997) and nosZ were lower when compared to unpolluted soil (Liu et al., 2016), as well as in a laboratory bioreactor feed with increasing amounts of Cd (Miao et al., 2018). Previous DNA-based studies have shown a collinearity between nirS gene copies and \( N_2O \) production (e.g. Ji et al., 2015, 2018), and we could not observe such a link in our RNA transcript dataset. Potentially, the time passing between the isolate pairing experiment and actual slicing of the sediment surface may have influenced the abundance of RNA transcripts of these genes. Another possible explanation is that denitrifying bacteria carrying the nirK gene were more active or prevalent in the Cd-polluted sediment during the exposure experiment, as such microbial populations have been shown to largely be taxonomically different from nirS denitrifies (Lee and Francis, 2017; Sanford et al., 2012; Shi et al., 2019). In addition, \( N_2 \) production rates did not correlate with the amount of nosZ transcripts in the sediment. Atypical nosZ genes belonging to nondenitrifying populations have previously been described, and such prokaryotic organisms would reduce \( N_2O \) undetected from conventional nosZ primers (Sanford et al., 2012). However, it was surprising that the amount of nosZ transcripts were unrelated to the measured \( N_2 \) production rates as this has previously been shown (although not in the combination with Cd) (Chen et al., 2015). Results suggest that steps in, or directly connected to, transcription of denitrification enzyme genes are more sensitive to Cd than the protein catalytic activity of nitrous oxide reductase or nitrite reductase. Further studies are needed to understand transcription of denitrification genes during Cd exposure.

5. Conclusions

Coastal sediment was incubated with different Cd concentrations for 21 days, and results showed that bottom water oxygen largely regulated the \( N_2O \) and \( N_2 \) production in the sediment. In the high Cd treatment (500 \( \mu L/Cd \)) \( N_2 \) production increased more than the other treatments. It is suggested that increases in \( N_2 \) production was an effect of 1) enhanced nitrification resulting in higher \( NO_3^- \) availability in oxygenated sediments, which stimulated denitrification and 2) Cd successfully
sequestering sulfide (yielding CdS) in the sediment, which would allow for full denitrification to N₂. Sediment from the field contained initially high Cd concentrations in the pore water (∼10 μg/L Cd) and microbial communities might already have been adapted to metal stress. To conclude, oxygen is known to regulate denitrification in sediment (hence decreasing the flux of N₂O and N₂ to the water column) and here we show that cadmium pollution might increase N₂ production. This suggests that coastal ecosystems exposed to high concentrations of Cd can alter nitrogen cycling in marine sediments.

Author contributions
EB sampled in the field, set up the experiment, conducted chemistry laboratory work, data analysis, and drafted the manuscript. NHM sampled in the field, set up the experiment, conducted chemistry plus molecular laboratory work, and contributed to writing the manuscript. SB designed the study, sampled in the field, helped in the laboratory, and contributed in writing the manuscript. TL conducted cadmium analyses laboratory work and gave feedback on the manuscript. FJAN designed the study, helped with data analysis, and gave feedback on the manuscript. SS designed the study, helped in the laboratory, and gave feedback on the manuscript. All authors read and approved the final manuscript.

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
Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.aquatox.2019.105328.

References