Denitrification by benthic foraminifera and their contribution to N-loss from a fjord environment

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Abstract.

Oxygen and nitrate availabilities impact the marine nitrogen cycle at a range of spatial and temporal scales. Here, we demonstrate the impact of denitrifying foraminifera on the nitrogen cycle at two oxygen and nitrate contrasting stations in a fjord environment (Gullmar Fjord, Sweden). The foraminifera contribution to benthic denitrification was estimated by coupling living foraminifera microhabitat, denitrification rate measurement and sedimentary nitrate 2D distribution, combining diffusive equilibrium in thin films (DET) colorimetry and hyperspectral imagery. Oxygenated bottom waters with high nitrate content in sediment porewaters were dominated by the non-indigenous species (NIS) *Nonionella* sp. T1 which could denitrify up to 50-100 % of nitrate porewater. Contrastingly, hypoxic bottom waters where sediment porewaters were nitrate low, denitrifying foraminifera were scarce and did not contribute to nitrogen removal (~ 5 %). Our study showed that benthic foraminifera can be a major contributor of nitrogen mitigation in oxic coastal ecosystems and should be included in ecological and diagenetic models aiming at understanding biogeochemical cycles coupled to nitrogen.

1 Introduction

Hypoxic water occurs frequently in bottom-waters of shallow coastal seas, due to remineralization of organic matter and water stratification. In this study we used the hypoxia threshold of 63 µmol L^{-1} (e.g. Diaz et al., 2008; Breitburg et al., 2018). Hypoxia may have large ecological effects (Levin et al., 2009; Rabalais et al., 2010; Zhang et al., 2010), such as an increase of fauna mortality (Diaz et al., 2001). However, certain microorganisms, e.g. bacteria and foraminifera, can perform denitrification by respiring nitrate (Risgaard-Petersen et al., 2006) and thereby survive in depleted oxygen environments. The effects of decreasing dissolved oxygen availability at spatial and temporal scales will impact biogeochemical cycles such as the nitrogen cycle (Childs et al., 2002; Kemp et al., 2005; Conley et al., 2007; Diaz et al., 2008; Neubacher et al., 2013; Breitburg et al., 2018). The nitrogen cycle in marine sediments is a perpetual balance between nitrogen inputs (e.g. terrestrial runoff, atmospheric precipitations) and outputs (e.g. denitrification from sediment and water column) (Galloway et al., 2004; Sigman et al., 2009). In most semi-enclosed marine environments as the Baltic Sea, the nitrogen loss through benthic denitrification exceeds the inputs of nitrogen through nitrogen fixation. These sink regions of the ocean are mostly associated with anoxic regions (Gruber and Sarmiento 1997). This study focuses on how one important compartment of the marine meiofaunal community - the benthic foraminifera - is coupled to the nitrogen cycle during contrasted dissolved [O_2] conditions at two different stations, focusing on the impact of a non-indigenous species (NIS).

The nitrogen cycle occurring in marine sediments is dependent on the bottom-water oxygenation. In oxic bottom water conditions (Fig. 1a), ammonium (NH_4^+) produced from remineralization of particulate organic nitrogen (PON) in sediments, diffuses toward the oxic sediment-superficial layer and through the water-sediment interface. Nitrification can occur in the oxic sediment and in the oxic water column through the conversion of NH_4^+ to nitrate (NO_3^-) (Rysgaard et al., 1994; Thamdrup and Dalsgaard, 2008). Conversely, denitrification occurs in sediment when oxygen is scarce (below 5 µmol L^{-1}, Devol et al., 2008) and organic carbon and nitrate are available. Denitrification named “canonical denitrification” ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) is an anaerobic process whereby nitrate is used as the terminal electron acceptor in the oxidation of organic matter by facultative anaerobic metabolisms when oxygen is exhausted. Denitrification participates in the loss of the fixed Nitrogen to N_2 gas (Brandes et al., 2007 and references within). Another process can contribute to this loss of N_2 gas: Anammox (anaerobic ammonia oxidation) (Engström et al., 2005; Brandma et al., 2011). According to Brandes et al. (2007 and references within) the “total denitrification” can be defined as the sum of the canonical denitrification plus the anammox. Nitrification and denitrification are thus strongly coupled, and denitrification can be enhanced by adjacent sedimentary nitrification zones or by direct NO_3^- diffusion from the overlying water towards the sediment (Kemp et al., 1990; Cornwell et al., 1999). When bottom water turns hypoxic, the nitrogen cycle occurring in the sediment is strongly affected (Fig. 1 b). Nitrate production is reduced since nitrification cannot process under low oxygen conditions (~ 0 µmol L^{-1}; Rysgaard et al., 1994; Mortimer et al., 2004). However, deeper into reduced sediment, nitrification can occur through secondary reactions with NH_4^+ oxidation by Mn and Fe oxides (Luther et al., 1997; Mortimer et al., 2004). Denitrification is the dominant process of nitrate reduction in coastal marine sediments (Thamdrup and Dalsgaard, 2008; Herbert, 1999). However, dissimilatory nitrate reduction to ammonium (DNRA) can also contribute to nitrate depletion in reduced sediment leading to NO_3^- conversion into NH_4^+ instead of nitrogen (N_2) (Christensen et al., 2000) and compete denitrification.

Benthic foraminifera were the first marine eukaryotes found to perform denitrification (Risgaard-Petersen et al., 2006), but not all foraminifera species can denitrify (Piña-Ochoa et al., 2010). Denitrifying foraminifera species are defined in our study as species able to perform denitrification proved by denitrification rate measurements. These denitrifying species have a facultative anaerobic metabolism and nitrate-storing foraminifera can use either environmental oxygen or nitrate to respire (Piña-Ochoa et al., 2010). *Nonionella* cf. *stella* (Charrieau et al., 2019 and references therein) and *Globobulimina turgida* were identified as the first denitrifying foraminifera species (Risgaard-Petersen et al., 2006). Currently, nineteen denitrifying species are known (Glock et al., 2019). Foraminifera denitrification rates show a large range from 7 ± 1 pmol N indiv.$^{-1}$ d^{-1} to 2241 ± 1825 pmol N indiv.$^{-1}$ d^{-1} (Glock et al., 2019).

Recently, *Nonionella stella* was described as invasive in the North Sea region and reported in the Gullmar Fjord (Sweden) (< 5 %, Polovodova Asteman and Schönfeld, 2015). However, *Nonionella stella* sampled in the Santa Barbara Basin (California USA) differs morphologically (Charrieau et al., 2018) and genetically (Deldicq et al., 2019) from the specimens sampled in Kattegat and Oslofjord (Norway), respectively. Deldicq et al. (2019) describe these specimens as the *Nonionella* sp. T1 morphotype, a non-indigenous and suspected invasive species in the Oslofjord. The genus *Nonionella* is potentially capable to denitrify as demonstrated with *Nonionella* cf. *stella* by Risgaard-Petersen et al. (2006). Denitrification rates of two species from the Gullmar Fjord have been measured: *Globobulimina turgida* (Risgaard-Petersen et al., 2006) and *Globobulimina auriculata* (Woehle et al., 2018). Additionally, *Stainforthia fusiformis* and *Bolivina pseudopunctata* are two dominant species in the deepest part of the fjord (Gustafsson and Nordberg, 2001; Filipsson and Nordberg, 2004). These species are also potential candidates for denitrification. Indeed, the denitrification rates of *Stainforthia fusiformis* from Perú were measured by Piña-Ochoa et al. (2010) and several species of *Bolivina* from Perú, Bay of Biscay and Santa Barbara were measured by Glock et al. (2019); Piña-Ochoa et al. (2010) and Bernhard et al. (2012), respectively. On the other hand, other typical fjord species such as *Bulimina marginata*, *Cassidulina laevigata*, *Hyalinea balthica* are considered as non-denitrifying species by Piña-Ochoa et al. (2010) as their intracellular nitrate reserves are almost absent. The anaerobic metabolism of some other species commonly found in the fjord such as *Leptohalysis scotti*, *Liebusella goesi*, *Nonionellina labradorica* and *Textularia earlandi* is not documented in previous studies.

A high abundance of denitrifying foraminifera in both oxic and anoxic marine environments play an important role in the nitrogen cycle (Risgaard-Petersen et al., 2006; Piña-Ochoa et al., 2010; Bernhard et al., 2012; Glock et al., 2013; Xu et al., 2017). Previous estimates of foraminifera contributions to denitrification range from 1 to 90 % (Dale et al., 2016; Xu et al., 2017). Estimates of foraminifera contribution to benthic denitrification are limited by the high spatial and temporal variability of sediment geochemistry and distribution of denitrifying foraminifera, which poses particular methodological challenges. Marine sediments often include chemical micro-heterogeneities (Aller et al., 1998; Stockdale et al., 2009), which can be averaged within the volume of a sediment slice. Moreover, sediment core slicing or centrifugation can induce cell lysis, which can induce a bias in porewater nitrate concentrations (Risgaard-Petersen et al., 2006). To characterize these microenvironments at submillimeter/ millimeter scales, new approaches have to be used. Recently, a 2D-DET (two Dimensions Diffusive Equilibrium in Thin-film) technique combining colorimetry and hyperspectral imagery was developed to obtain the distribution of nitrite and nitrate in sediment porewater at millimeter resolution in two dimensions (Metzger et al., 2016). This method avoids mixing of intracellular nitrate and nitrate contained in the sediment porewater.

The present study aims to examine how the NIS *Nonionella* sp. T1 and the other denitrifying species affect the nitrogen cycle by comparing two stations with contrasting oxygen and nitrate environments subjected to hypoxic events. The objectives of the paper are: (1) to characterize the density of the living benthic foraminifera at two contrasted stations; (2) to measure the denitrification rate of the NIS *Nonionella* sp. T1 and (3) to quantify its contributions to benthic denitrification; (4) to discuss the probable future impact of the NIS *Nonionella* sp. T1 on the foraminifera fauna and the nitrogen cycle in the Gullmar Fjord.

2 Material and Methods

2.1 Site description and sampling conditions

The Gullmar Fjord is 28 km long, 1-2 km wide and located on the Swedish West coast (Fig. 2). The fjord undergoes fluctuations between cold and temperate climates (Svansson, 1975; Nordberg, 1991; Polovodova Asteman and Nordberg, 2013; Polovodova Asteman et al., 2018). The fjord is stratified (Fig. 2 d) in four water masses (Svansson, 1984; Arneborg, 2004). Hypoxia events in the fjord have been linked to the influence of the North Atlantic Oscillation (NAO) (Nordberg et al., 2000; Björk and Nordberg, 2003; Filipsson and Nordberg, 2004). Several monitoring stations are located in the fjord: Släggö (65 m depth), Björkholmen (70 m depth) and Alsbäck (117 m depth), the hydrographic and nutrient data were obtained from the SMHI's publically available data-base SHARK (Svenskt Havsarkiv, www.smhi.se). Since 2010, the threshold of hypoxia ([O_2] < 2 mg L^{-1}, i.e. 63 µmol L^{-1}) in Alsbäck station (red squares, Fig. 3) is reached typically in late autumn and winter. Deep-water exchanges usually occur in late water-early spring. However, the duration of hypoxia varies between years and hypoxia events also occurred in the summer 2014 and 2015, due to lack of deep-water exchange. The frequency of hypoxic events has increased in the fjord (Nordberg et al., 2000; Filipsson and Nordberg, 2004).

Two sampling cruises were conducted in the Gullmar Fjord on board R/V *Skagerak* and *Oscar von Sydow*, respectively. The 2017 cruise (GF17) took place between 14th and 15th November 2017 and two stations were sampled (GF17-3 and GF17-1, Fig. 2 c and d) to define the living foraminifera fauna and the sediment geochemistry at two contrasted stations. The 2018 cruise (GF18) took place on the 5th September 2018 with the focus to collect living *Nonionella* sp. T1 for O_2 respiration and denitrification rates measurements. Only one station (at the same position as GF17-3) was sampled.

GF17-3 (50 m water depth) is located closest to the mouth of the fjord (58°16'50.94"N/ 11°30'30.96"E) with bottom waters from Skagerrak (blue diamond, Fig. 3) and GF17-1 (117 m depth) close to the deepest part of the fjord (58°19'41.40"N/11°33'8.40"E) near Alsbäck monitoring station in the middle of the stagnant basin (red square, Fig. 3). In November 2017, CTD profiles indicated the water mass structures at both stations (Fig. S1). Bottom water at GF17-3 station was oxic with a dissolved oxygen content of 234 µmol L^{-1}. The dissolved oxygen content decreased strongly with depth at the GF17-1 station reaching 9 µmol L^{-1} at the seafloor, which is below the severe hypoxia threshold.

2.2 Foraminifera sampling and processing

During the 2017 cruise, two sediment cores per station (1A, 1C and 3A, 3C for GF17-1 and GF17-3 stations respectively) were immediately subsampled with a smaller cylindrical core (Ø 8.2 cm) and sliced every 2 mm up to 2 cm and every 5 mm from 2 to 5 cm to study living foraminifera distribution. The samples were incubated without light for 10–19 hours in ambient seawater with Cell Tracker Green (CMFDA, 1 mM final concentration) at *in situ* temperatures (Bernhard et al., 2006) and then fixed with ethanol 96°. Fixed samples were sieved (> 355, 150, 125 and 100 µm) and the > 100 µm fraction, the most commonly fraction used for foraminiferal analyses in the Gullmar Fjord (see Charrieau et al., 2018 and references therein) was examined using an epifluorescence microscope equipped for fluorescein detection (i.e., 470 nm excitation; Olympus SZX13). In the present study, the foraminifera distribution will be described highlighting the NIS *Nonionella* sp. T1.

2.3 Geochemical sampling and processing

One core from the shallow GF17-3 station was reserved for O_2 microelectrode profiling. Oxygen concentration was measured in the dark with a Clark electrode (50 µm tip diameter, Unisense ®, Denmark) within the first 5 mm depth at a 100 µm vertical resolution. Due to technical problems, no oxygen profiling was done at the GF17-1 station.

One core per station was dedicated for geochemical analyses, they were carefully brought to Lund University (Sweden) and stored at the sampling site temperature (10°C) until further analysis the next day. Overlaying water of the GF17-3 core was gently air bubbled to maintain the oxygenated conditions recorded at this station. Overlaying water of the GF17-1 core was bubbled with N_2 gas passed through a solution of carbonate/bicarbonate to avoid pH rise due to degassing of CO_2 by N_2 bubbling.

Nitrite/Nitrate were analyzed using the 2D-DET method from Metzger et al. (2016). In brief, for each core, a DET (Diffusive Equilibrium in Thin films) gel probe (16 cm x 6.5 cm and 0.1 cm thickness) was hand-made prepared. The gel probe was inserted into the sediment and left for 5 hours to allow for a diffusive equilibration time between the gel and porewaters; After equilibration, the gel was removed of the core and laid on a first NO_2^- reagent gel. After 15 mn at ambient temperature the pink coloration must appear were nitrite is detected. A reflectance analysis photograph of the nitrite gels fauna was taken with a hyperspectral camera (HySpex VNIR 1600). The next step was to convert existing nitrate into nitrite with the addition of a reagent gel of vanadium chloride (VCl_3). After 20 min at 50°C, additional pink is interpreted as porewater nitrate concentration. Followed by the acquisition of another hyperspectral image and converted into false colours through a calibrated scale of concentrations, the final image was cropped to avoid border effects. Each pixel (190 µm x 190 µm) was decomposed as a linear combination of the logarithm of the different end-member spectra using ENVI software (unmixing function) (Cesbron et al., 2014; Metzger et al., 2016). Nitrite and nitrate detection limit is 1.7 µmol L^{-1} (Metzger et al., 2016). Nitrate production/consumption zones for each station were estimated by extracting the average and standard deviation of the 290 vertical 1D profiles ((5.5 cm width x 1 pixel) / 0.019 cm for 1-pixel size) on the 2D gels and modelling using PROFILE software (Berg et al., 1998)).

2.4 Oxygen respiration and denitrification rates measurements of the NIS *Nonionella* sp. T1

The two cores sampled in the 2018 cruise (GF18) at the shallower GF17-3 station were carefully transported at *in situ* temperature (8 °C) and stored for three days at the Department of Geosciences, Aarhus University (Denmark). *Nonionella* sp. T1 specimens were picked under *in situ* temperature and collected in a Petri dish, containing a thin layer of sediment (32 µm) to check their vitality. Only living, active *Nonionella* sp. T1 specimens were picked and cleaned several times using a brush with micro-filtered, nitrate-free artificial seawater.

Oxygen respiration rates were measured, following the method developed by Høgslund et al. (2008) using a Clark type oxygen microsensors (50 µm tip diameter, Unisense ®, Denmark) (Revsbech, 1989) calibrated by a two-point calibration using air-saturated water at *in situ* temperature (8 °C) and sodium ascorbate solution (to strip O_2 out of the system) as zero. Then, a pool of 5 living *Nonionella* sp. T1 was transferred into a glass microtube (inner diameter 0.5 mm, height 7.5 mm) that was fixed inside a 20 ml test tube mounted in a glass-cooling bath (8 °C). A motorized micromanipulator was used to measure O_2 concentration profiles along a distance gradient that ranged from 200 µm of the foraminifera to 1200 µm using 100 µm steps. Seven O_2 concentration profiles were generated with one incubation containing the pool of *Nonionella* sp. T1. Negative controls were done by measuring O_2 rates from microtube with empty foraminifera shells and blanks with empty microtube. Oxygen respiration rates were calculated with Fick's first law of diffusion, $J = -D * dC/dx$, where J is the flux, dC/dx is the concentration gradient obtained by profiles and D is the free diffusion coefficient of oxygen at 8 °C for a salinity of 34 (1.382×10^{-5} cm^{-2} s^{-1}, Ramsing and Gundersen, 1994). The seven O_2 respiration rates were calculated as the product of the flux by the cross section area of the microtube (0.196 mm^2). Then, the average O_2 respiration rate was divided by the 5 *Nonionella* sp. T1 presented in the microtube to obtain the respiration rate per individual.

The same pool of *Nonionella* sp. T1 specimens as for the O_2 respiration rates was used for denitrification rate measurements. Denitrification rates were measured as it is described in Risgaard-Petersen et al., (2006). In this method, denitrification is stopped at the N_2O production by acetylene inhibition that can be measured with a N_2O microprobe (50 µm tip diameter, Unisense ®, Denmark). Thus, N_2O was measured as the end product instead of N_2 (Risgaard-Petersen et al., 2006).

Nitrous oxide flux was estimated from the chemical gradient profiled from the pool of *Nonionella* sp. T1 inserted in a microchamber. The N_2O production was multiplied by two because two moles of NO_3^- are required for the production of one mole of N_2O (Risgaard-Petersen et al., 2006). The microchamber is porous to gases and is bathed in a sodium ascorbate solution that maintains oxygen concentration at zero within the microchamber. The microchamber was filled with an oxygen/nitrate-free solution of artificial seawater saturated with acetylene (to inhibit N_2O transformation into N_2) containing 5 mM of Hepes buffer (to maintain the pH stable). Calibration was performed using the standard addition method by successive injections of a N_2O saturated solution in order to have 14 µM steps of final concentration. Negative controls were done by checking the absence of O_2 from microchamber with empty foraminifera shells and blanks with empty microchamber. Then, the pool of *Nonionella* sp. T1., was transferred to the microchamber with a micropipette. The N_2O concentration profiles were repeated seven times on the pool of *Nonionella* sp. T1. The source of nitrate during denitrification comes from intracellular nitrate storage of *Nonionella* sp. T1 (not measured in this study).

Since O_2 respiration and denitrification rates are linked to cytoplasmic volume or biovolume (BV) (Geslin et al., 2011; Glock et al., 2019), the specimens from the pool of *Nonionella* sp. T1 were measured (width (a) and length (b) Fig. 4) using a micrometer mounted on a Leica stereomicroscope (MZ 12.5) to estimate the average BV. The volume of the shells was estimated by using the best resembling geometric shape, a spheroid prolate ($V = \frac{4}{3}\pi\left(\frac{a}{2}\right)^2\left(\frac{b}{2}\right)$). Then, according to Hannah et al., (1994) 75 % of the measured entire volume of the shell was used corresponding to the estimated cytoplasmic volume. To compare the size of the *Nonionella* sp. T1 sampled in the 2017 cruise (GF17, study of the fauna) with the *Nonionella* sp. T1 samples in the 2018 cruise (GF18, denitrification rate measurements), 5 specimens sampled in the 2017 cruise were also measured.

2.5 Contributions of the NIS *Nonionella* sp. T1 to diffusive oxygen and nitrate uptake

The following estimated contributions to sediment diffusive oxygen and nitrate uptake were performed mainly on the dominant denitrifying species, *Nonionella* sp. T1. The size of the *Nonionella* sp. T1 specimens sampled during the two cruises differed markedly (Table 1). Thus, we need to correct the denitrification rate of *Nonionella* sp. T1 specimens from the 2017 cruise to take into account the difference of shell size. Thus, the measured *Nonionella* sp. T1 denitrification rate (2018 cruise) was normalized by specimen BV (2017 cruise) using the relationship: $\ln(y) = 0.68 \ln(x) - 5.57$, where y is the denitrification rate (pmol ind^{-1} d^{-1}) and x is the shell BV (μm^3) ((Geslin et al., 2011; Glock et al., 2019; Equation S1). The corrected *Nonionella* sp. T1 denitrification rate is multiplied by the *Nonionella* sp. T1 specimens counted found in each denitrifying zones defined by PROFILE modelling. Then, two calculation approaches were discussed to estimate *Nonionella* sp. T1 contributions to benthic denitrification: (A) to divide the *Nonionella* sp. T1 denitrification rate by the nitrate porewater denitrification rate estimated from PROFILE modelling, then the second calculation (B) to divide the *Nonionella* sp. T1 denitrification rate by the total denitrification from PROFILE plus the *Nonionella* sp. T1 denitrification rate. In the first approach (A) we suggest *Nonionella* sp. T1 use only the nitrate in the sediment porewater. In the second approach (B) we suggest that the foraminifera use both intracellular and porewater nitrate pools for denitrification.

3 Results

3.1 The NIS *Nonionella sp.* T1 oxygen respiration and denitrification rates in the Gullmar Fjord

The O_2 respiration rates measured in the pool of *Nonionella* sp. T1 specimens collected in the 2018 cruise (GF18) were 169 ± 11 pmol O_2 $indiv^{-1}$ d^{-1} with an average BV of 1.3 ± 0.7 10^{+06} μm^3 (BV details, Table 1). The denitrification rate, measured on the same pool of specimens, was 21 ± 9 pmol N $indiv^{-1}$ d^{-1}.

The *Nonionella* sp. T1 average BV collected in the 2017 cruise (GF17-3) was 4.0 ± 0.6 10^{+06} μm^3, i.e. more than three times larger the *Nonionella* sp. T1 average BV from the 2018 cruise (1.3 ± 0.7 10^{+06} μm^3). As denitrification rates and foraminifera BV are linked (see method), the measured denitrification rate was corrected using the BV of Nonionella sp. T1 from the 2017 cruise. Thus, the *Nonionella* sp. T1 corrected denitrification rate was 38 ± 8 pmol N $indiv^{-1}$ d^{-1} (Equation S1).

3.2 The NIS *Nonionella* sp. T1 and foraminifera fauna regarding porewater nitrate micro-distribution

The bottom water at GF17-3 station was oxic (Fig. S1, $[O_2]$ = 234 µmol L^{-1}) and the measured oxygen penetration depth (OPD) in the sediment was 4.7 ± 0.2 mm (n = 3). No nitrite was revealed on the gel (< 1.7 µmol L^{-1}), only nitrate was detected. Bottom water average NO_3^- concentration was 14.6 ± 2.3 µmol L^{-1} and nitrate concentration decreased with depth in the sediment (Fig. 5 c, d). Nitrate concentration ranged between 13.1 ± 3.2 to 11.7 ± 3.4 µmol L^{-1}, from the water-sediment interface to the OPD. Nitrate concentration decreased strongly after the OPD from 11.7 ± 3.4 to 2.8 ± 0.9 µmol L^{-1} until 4.0 cm depth. From 4.0 to 5.0 cm depth, NO_3^- concentration was very low with an average value of 2.7 ± 0.9 µmol L^{-1} (Fig. 5 c, d). The PROFILE parameters (Berg et al., 1998) used on laterally averaged nitrate porewater vertical distribution of both stations are available in Table S1. Thus, the PROFILE modelling of the averaged nitrate porewater profiles revealed one nitrification zone from 0 to 1.2 cm depth and two denitrifying zones (red line, Fig. 5 d). The first denitrification zone occurred between 1.2 to 3.6 cm depth with a nitrate consumption of 3.92 E^{-05} nmol cm^{-3} s^{-1} and the second smaller consumption zone was from 3.6 to 5 cm depth (1.53 E^{-06} nmol cm^{-3} s^{-1}). The total denitrification rate from 1.2 to 5 cm depth was 4.07 E^{-05} nmol cm^{-3} s^{-1} (Fig. 5 d).

The total densities of living foraminifera were similar between the cores GF17-3A and 3C (Ø 8.2 cm, 5 cm depth) with 1256 individuals and 1428 individuals, respectively (Fig. 5 a and b; Table S2, GF17-3A and 3C). *Nonionella* sp. T1 was the main denitrifying species, accounting for 34 % of the total living fauna in the core GF17-3A and 74 % in GF17-3C (Fig. 5 a, b; Table S3). One other candidate to denitrification, *Stainforthia fusiformis*, was in minority: 1 % of the total fauna in both cores (Fig. 5 a, b; Table S3, GF17-3A and 3C). The other known denitrifying species previously reported in the Gullmar Fjord, *Globobulimina turgida* (Risgaard-Petersen et al., 2006) and *Globobulimina auriculata* (Whoele et al., 2018) were absent. Three non-denitrifying species (Piña-Ochoa et al., 2010; Xu et al., 2017; Glock et al., 2019) were dominant in the cores GF17-

3A and 3C: *Bulimina marginata* (37 and 5 %, respectively), *Cassidulina laevigata* (9 and 5 %) and *Leptohalysis scotti* (11 and 9 %).

The density and the micro-distribution of *Nonionella* sp. T1 differed between the two cores (Fig. 5 a and b; Table S2, GF17-3A and 3C). In the core GF17-3A and 3C respectively, *Nonionella* sp. T1 density showed large variability from the water-sediment interface to 1.2 cm depth (Table S2) where *Nonionella* sp. T1 relative abundance accounted for 18 % and 50 % of the fauna in the nitrification zone (Table S3, GF17-3A and 3C). In the first denitrifying zone from 1.2 cm to 3.6 cm the *Nonionella* sp. T1 relative abundance represented 27 % and 78 % of the fauna. In the second denitrifying zone, the *Nonionella* sp. T1 relative abundance increased from 3.6 to 5 cm depth and dominated the fauna by 60 % and 98%. The relative abundance of the denitrifying candidate, *Stainforthia fusiformis*, was a minor component in each zones of both cores and did not exceed 2 % (Table S3, GF17-3A and 3C). The three non-denitrifying species (e.g. *B. marginata*, *C. laevigata* and *L. scotti*) also dominated the fauna of both cores GF17-3A and 3C (Table S2 and S4). From the water-sediment interface to 1.2 cm depth *B. marginata* accounted for 42 % and 12 %, *C. laevigata* 16 % and 13 % and *L. scotti* 6 % and 11 %, respectively. In the first denitrifying zone (1.2-3.6 cm depth) *B. marginata* accounted for 34 % and 2 %, *C. laevigata* 7 % and 2% and *L. scotti* 25 % and 13 %, respectively. In the second denitrifying zone (3.6-5 cm depth) *B. marginata* accounted for 34 % and 0 %, *C. laevigata* was absent and *L. scotti* 5 % and 1 %, respectively.

Due to severe hypoxia at the GF17-1 station, oxygen was assumed to be below detection limit within the sediment. No nitrite was detected at this station (< 1.7 µmol L^{-1}). Average NO_3^- concentration in the bottom water reached 5.7 ± 1.0 µmol L^{-1} (Fig. 5 g and h). Nitrate concentrations decreased from the sediment surface (4.2 ± 1.0 µmol L^{-1}) to 1.6 cm (1.8 ± 0.6 µmol L^{-1}) and then average nitrate concentration remained below the detection limit (1.7 µmol L^{-1}). However, a patch with higher nitrate concentration was visible on the left part of the gel between 2.0 and 3.0 cm depth. A 1D vertical profile passing through this patch (white line, Fig. 5 g) was extracted from the 2D image and the maximal nitrate concentration of the patch was above the detection limit with a value of 6.5 µmol L^{-1} at 2.3 cm depth (blue squares profile, Fig. 5 h). The PROFILE modelling (Table S1) of the laterally averaged nitrate vertical distribution revealed at the sampling time one denitrifying zone from the surface to 1.6 cm depth with a nitrate consumption of 2.71 E^{-05} nmol cm^{-3} s^{-1} (red line, Fig. 5 h). Below 1.6 cm depth, nitrate concentration was below the detection limit (hatched grey zone, Fig. 5 h), thus no PROFILE modelling was done after this depth.

Living foraminifera showed different total densities and a large difference in species distribution between the two cores GF17-1A and 1C (Fig. 5 e, f; Table S2), with 1457 individuals and 786 individuals, respectively (Ø 8.2, 5 cm depth). *Nonionella* sp. T1 represented a low relative abundance of the total fauna with 5 % in the core GF17-1A and was almost absent (1 %) in GF17-1 C (Table S3). The known denitrifying *G. auriculata* was minor in the fauna 1 % and 2%. The denitrifying candidate *S. fusiformis* was also found in the cores GF17-1A and 1C reaching only 3% of the total fauna (Figure 5 e, f; Table S3). The other denitrifying candidate *B. pseudopunctata*, was almost absent of the total fauna 0 % and 2 % (Table S3). The same three non-denitrifying species as for the oxic station were also dominant in both cores GF17-1A and 1C: *B. marginata* (64 and 30 %), *C. laevigata* (16 and 15 %) and *L. scotti* (4 and 36 %).

In the denitrifying zone (0-1.6 cm) *Nonionella* sp. T1 relative abundance was low, with 2 % in the core GF17-1A and was almost absent from the fauna in GF17-1C. In the core GF17-1A, *Nonionella* sp. T1 relative abundance reached 26 % of the fauna between 1.4 and 2.5 cm depth (Fig. 5 e, GF17-1A), whereas it was almost absent from the rest of the core GF17-1A and was absent from the core GF17-1C (Table S3). In the cores GF17-1A and 1C, *S. fusiformis* reached respectively 2 % and 3 % in the denitrifying zone (0-1.6 cm). In the rest of the cores from 1.6 to 5 cm depth, *S. fusiformis* represented 4 and 1 % of the fauna, respectively. The three other non-denitrifying species dominated both cores GF17-1A and 1C. In the denitrifying zone (0-1.6 cm depth) *B. marginata* accounted for 66 % and 35 %, *C. laevigata* 19 % and 19 % and *L. scotti* 4 % and 24 %. From 1.6 to 5 cm depth, *B. marginata* dominated the fauna by 61 % and 11 %, *C. laevigata* 5 % and 2 % and *L. scotti* 6 % and 75 %, respectively.

4 Discussion

4.1 The NIS *Nonionella* sp. T1 density in comparison with other species from the Gullmar Fjord

The presence and relative abundance of NIS *Nonionella* sp. T1 in the Gullmar Fjord and in the Skagerrak-Kattegat strait has been documented during the last decades. The earliest SEM observations of specimens resembling *Nonionella* sp. T1 morphotype in the deepest part of the fjord date back to summer 1993 (identified as *Nonionella turgida*, Gustafsson and

Nordberg, 2001). The invasive characteristics of *Nonionella stella* was firstly revealed by Polovodova Asteman and Schönfeld, (2015). Then, *Nonionella stella* was identified as *Nonionella* sp. T1 morphotype also described as NIS and potentially invasive species in the Oslofjord by Deldicq et al. (2019). The estimated introduction date of *Nonionella* sp. T1 into the deepest part of the Gullmar Fjord is 1985 according to Polovodova Asteman and Schönfeld, (2015). The relative abundance of *Nonionella* sp. T1 in the deepest fjord station was less than 5 % between 1985 and 2007 (Polovodova Asteman and Schönfeld, 2015 and references within). At the GF17-1 hypoxic station, the *Nonionella* sp. T1 relative abundance was between 1-5 % (Table S3, GF17-1A and 1C). Thus, the *Nonionella* sp. T1 relative abundance in the deepest part of the fjord seems to remain stable. In contrast to station GF17-1, the GF17-3 oxic station was sampled for the first time in this study. In this station closer to the mouth of the fjord than GF17-1, the relative abundance of *Nonionella* sp. T1 varied between 34 and 74 % (Table S3, GF17-3A and 3C). Previous studies showed an increase in the relative abundance of *Nonionella* sp. T1 morphotype in the Skagerrak-Kattegat region (near the entrance of the Gullmar Fjord). The *Nonionella* sp. T1 represented 10 % of the fauna in June 2013 (Polovodova Asteman and Schönfeld, 2015). The Öresund strait linking the North Skagerrak, the Kattegat and the Baltic Sea, showed an increase in *Nonionella* sp. T1 relative abundance from 1 % to 14 % observed between 1998 and 2009 (Charrieau et al., 2019). The foraminifera fauna in the Gullmar Fjord has changed over the last decennium and *Nonionella* sp. T1 seemed to become an invasive species in the Gullmar Fjord oxic shallow water area.

The foraminifera fauna found at the GF17-1 station in the deepest part of the fjord differed from previous studies (Nordberg et al., 2000; Filipsson and Nordberg, 2004; Risgaard-Petersen et al., 2006; Polovodova Asteman and Nordberg, 2013; Polovodova Asteman and Schönfeld, 2015). Indeed, until the early 1980s, the foraminifera fauna in the deepest part of the fjord was dominated by a typical Skagerrak – Kattegat fauna (*Bulimina marginata, Cassidulina laevigata, Hyalinea balthica, Liebusella goësi*, *Nonionellina labradorica* and *Textularia earlandi*) (Nordberg et al., 2000). However, the fauna changed. *S. fusiformis* and *B. pseudopunctata* became the major species (Nordberg et al., 2000; Filipsson and Nordberg, 2004). Further studies by Polovodova Asteman and Nordberg, (2013) demonstrated that at least until 2011 *S. fusiformis*, *B. pseudopunctata* and *T. earlandi* dominated the fauna. Foraminifera fauna described in the present study differs, it is the consequence of the occurrence of numerous severe hypoxic events in the fjord (Fig. 3) due to lack of deep-water exchange. In November 2017 *S. fusiformis* did not exceed 3 % of the fauna (Table S3, GF17-1A and 1C), *B. pseudopunctata* reached only

2 % in the core GF17-1C (Table S3, GF17-1C) and *T. earlandi* was a minor species < 1 %. Then, in November 2017 *B. marginata, C. laevigata* and *L. scotti* were the dominant species in the fjord. The *Elphidium clavatum-selseyensis* species complex (following the definition from Charrieau et al., 2018), *H. baltica*, *N. labradorica*, and *T. earlandi* were present in low relative abundance (< 5 %, Table S3). Namely, *G. turgida* reached 37 % of the foraminifera fauna in August 2005 at the deepest station (Risgaard-Petersen et al., 2006); whereas in November 2017 this species was minor. The decreasing in relative abundance of *S. fusiformis* and *B. pseudopunctata* must be interpreted with caution since our study used the > 100 µm fraction whereas some of the previous studies used > 63 µm. We also wet picked the specimens and used Cell Tracker Green to identify living foraminifera, which might affect the results compared to Rose Bengal studies of dry sediment residuals. The relative abundance of the potential invasive *Nonionella* sp. T1 has increased according to the study of Polovodova Asteman and Schönfeld, (2015) in the oxic part of the fjord. The two non-denitrifying species *B. marginata* and *C. laevigata* described as typical species of the Skagerrak-Kattegat fauna (Filipsson and Nordberg, 2004) have again increased markedly in the fjord. It is evident that the foraminifera fauna in the Gullmar Fjord is presently very dynamic with considerable species composition shifts.

4.2 Foraminifera ecology considering nitrate micro-distribution

Our study showed, for the first time, *Nonionella* sp. T1 dominated the foraminifera fauna in the Gullmar Fjord, this at the GF17-3 oxic station despite some spatial variability (Fig. 5 a, b; Table S2; S4). *Nonionella* sp. T1 density increased with sediment depth below the oxic zone (Fig. 5 a – d; Table S2), which could be explained by its preference to respire nitrate rather than oxygen. This would be following the hypothesis of using nitrate as a preferred electron acceptor suggested by Glock et al., (2019). *Nonionella* sp. T1 distributions could be explained by its capacity to store nitrate intracellularly before porewater nitrate was denitrified by other organisms such as bacteria. At this station, *Nonionella* sp. T1 distributions may be explained as: following the oxic zone (Fig. 5 c, d; from the surface to OPD) *Nonionella* sp. T1 respires oxygen (169 ± 11 pmol O_2 $indiv^{-1}$ d^{-1}). Deeper in the hypoxic zone containing nitrate (Fig. 5 c, d; from OPD to 3.6 cm depth), *Nonionella* sp. T1 accumulates intracellular nitrate and respires nitrate (38 ± 8 pmol N $indiv^{-1}$ d^{-1}). In the hypoxic zone where the nitrate porewater is depleted (Fig. 5 c, d; from 3.6 to 5 cm depth) *Nonionella* sp. T1 respires on its intracellular nitrate reserves to survive (Fig. 5 a, b; from

3.5 to 5 cm depth). When the intracellular nitrate reserve runs out, *Nonionella* sp. T1 can migrate to an upper zone where nitrate is still present in the sediment to regenerate its intracellular nitrate reserve (Fig. 5 a, b; from 1.2 to 3.5 cm depth).

Hypoxia occurred approximately at least one month before the sampling cruise in the deepest part of the fjord (Fig. 3). When hypoxia is extended to the water column, nitrification both in the water column and the sediment is reduced or even stopped, as oxygen is almost absent (Fig. 1 b; Childs et al., 2002; Kemp et al., 2005; Conley et al., 2007; Jäntti and Hietanen, 2012). Under this condition, the coupled nitrification-denitrification processes are strongly reduced (Kemp et al., 1990). At the GF17-1 station, no nitrification in superficial sediment was showed by our data and nitrate was low but still detectable in the bottom water. Nitrate can diffuse from the water column into the sediment, and thereby generate the denitrification zone as modelled by PROFILE between the surface and 1.6 cm depth (Fig. 5 h).

The rare presence of the NIS *Nonionella* sp. T1 and other denitrifying species as *Globobulimina auriculata*, *Bolivina pseudopunctata* and *Stainforthia fusiformis* in the hypoxic station indicates that sediment chemical conditions turned unfavorable towards denitrification during prolonged hypoxia. Instead, the non-denitrifying species *Bulimina marginata*, *Cassidulina laevigata*, and *Leptohalysis scotti* dominated in this hypoxic environment. Their survival could be due to seasonal dormancy (Ross and Hallock, 2016; LeKieffre et al., 2017) and propagules which can disperse and reproduce when environmental conditions turn favorable again (Alve and Goldstein, 2003). The suspected deep nitrification zone (blue square profile, Fig. 5 h) could explain the presence of nitrate micro-niches deeper in the sediment and might explain the patchy distribution of *Nonionella* sp. T1 also at the hypoxic site (see Fig. 5 e; Table S2, GF17-1A). Therefore, deep nitrate production in these micro-environments could favor the presence of *Nonionella* sp. T1, which can be attracted by this nitrate source of electron acceptor to respire (Nomaki et al., 2015; Koho et al., 2011). This deep nitrification zone could be a result of an aerobic or anaerobic process. An aerobic nitrification zone in deep sediment can be formed by macrofaunal activity (burrowing activity) that introduce some oxygen deeper into anoxic sediment (Aller, 1982; Karlson et al., 2007; Nizzoli et al., 2007; Stief, 2013; Maire et al., 2016). This nitrification zone could also be due to an anaerobic process. The Gullmar Fjord is Mn-rich (Goldberg et al., 2012) and metal-rich particles can be bio-transported into the anoxic sediment, thus allowing ammonium oxidation into NO_3^- by Mn and Fe-oxides in the absence of oxygen deeper in the sediment (Aller, 1994; Luther et al., 1997).

4.3 Contributions and potential impacts of the NIS *Nonionella* sp. T1 to benthic denitrification in the Gullmar Fjord

If we consider that *Nonionella* sp. T1 is denitrifying the nitrate from sediment porewater (approach A, Table 2; see method 2.5) its contribution to benthic denitrification in the oxic station would be 47 % in the core GF17-3A and would reach 100 % in the core GF17-3C. If we consider that *Nonionella* sp. T1 also uses its intracellular nitrate pool for denitrification (approach B), its contribution to benthic denitrification would be 32 % in the core GF17-3A and would reach 50 % in the core GF17-3C (Table 2). These two calculation approaches highlight the difficulties and the importance of knowing the concentration of environmental nitrate and foraminifera intracellular nitrate at the same time to estimate the contributions of foraminifera to benthic denitrification. Moreover, in this study there is no data on anammox process which contributes also in the total denitrification (Brandes et al., 2007). The results reported in previous studies as Engström et al., (2005) do not allow us to extrapolate their data at our oxic station, located at the entrance of the fjord. Thus, we assume that our estimate of denitrification is conservative, since the possible contribution of anammox is not included in the calculation. However, despite these uncertainties *Nonionella* sp. T1 contribution to benthic denitrification supports the hypothesis that this non-indigenous denitrifying foraminifer play a major role in the benthic nitrogen cycle for sediments.

At the hypoxic station, the opposite was shown where the estimated contribution of *Nonionella* sp. T1 to benthic denitrification was below 1 % whatever the calculation approach. The estimated contributions of the other denitrifying foraminifera found in the hypoxic station were low. Foraminifera contributed to almost 5 % of benthic denitrification in the hypoxic station. Compared to the oxic station, the NIS *Nonionella* sp. T1 and the other denitrifying species contributions to benthic denitrification were small in a prolonged hypoxic station of the Gullmar Fjord.

Overall, the Gullmar Fjord is well oxygenated except for the deepest basin where oxygen goes down when there is no deep water exchange (Fig. 3 c). Therefore, the GF17-3 oxic station could be considered representative of the Gullmar Fjord benthic ecosystem. *Nonionella* sp. T1 is not the most efficient denitrifying species compared to *Globobulimina turgida* (42 pmol N ind^{-1} d^{-1}, with BV = 1.3 10^{+06} μm^3) and also less efficient than *Nonionella* cf. *stella* from Perú. However, *Nonionella* sp. T1 high density could accelerate sediment denitrification and participate to increase the contrast between the two hydrographic conditions. Indeed, an increase in contrast due to oxygenation conditions: oxic vs severe hypoxia induced a gap in the availability of nitrate for anaerobic facultative metabolisms in the sediment. In the oxygenated part of the fjord, high contribution to benthic denitrification (estimated between 50 and 100%) by *Nonionella* sp. T1 could contribute to a potential de-eutrophication of the system by increasing the nitrogen loss. Primary production (PP) of the Gullmar Fjord is dominated by diatoms bloom in spring and autumn (Lindahl and Hernroth, 1983). Since the 1990s, Lindahl et al. (2003) observed an increase in PP of the Gullmar Fjord, therefore a potential eutrophication. This increase in PP also shown in the adjacent Kattegat could be related to the nitrogen input loading from the land and atmosphere (Carstensen et al., 2003). Lindahl et al. (2003), argued that PP of the Gullmar Fjord was due to climatic forces resulting from a strong positive North Atlantic Oscillation (NAO) index, which increased the availability of deep-water nutrients (Kattegat nitrate-rich) and due to warmer ocean. The benthic denitrification of the Gullmar Fjord produces nitrogen unassimilable by primary producers. Moreover, denitrifying foraminifera intracellular nitrate becomes unavailable to the system and can be bio-transported and permanently sequestered in sediments (Glock et al., 2013; Prokopenko et al., 2011). Thus, denitrifying foraminifera including *Nonionella* sp. T1 could help counterbalance a potential eutrophication of the system via nitrogen loss (Seitzinger, 1988).

Whereas, in the hypoxic parts of the fjord, nitrate and nitrite rapidly exhausted become scarce, resulting in a decrease in denitrification. The consequence is a decrease of denitrifying foraminifera fauna. The increase of ammonium in anoxic sediment resulting by a decrease in nitrification, denitrification and anammox processes does not allow the nitrogen elimination from the sediment to the water column. Thus, potentially promoting an ammonium accumulation in the deep fjord parts subjected to prolonged severe hypoxia (Fig. 1). Moreover, the low availability of nitrate in the sediment would possibly increase the benthic transfer towards the water column of reduced compounds such as manganese and iron produced deeper in the sedimentary column by other anaerobic metabolisms (Hulth et al., 1999). These new results demonstrate that the role of denitrifying foraminifera is underestimated in the nitrogen cycle and overlooking this part of the meiofauna may lead to a misunderstanding of environments subject to hydrographic changes.

5 Conclusion

This study revealed a drastic change in living foraminifera fauna due to several hypoxic events that occurred in the last decennium in the Gullmar Fjord. For the first time, the non-indigenous species (NIS) *Nonionella* sp. T1 dominated up to 74 % the foraminifera fauna at a station with oxygenated bottom waters and high nitrate content in sediment porewater. This NIS can denitrify up to 50-100 % of the nitrate porewater sediment under oxic conditions in the fjord. Whereas, under prolonged hypoxia, nitrate depletion turns environmental conditions unfavorable for foraminifera denitrification, resulting in a low density of *Nonionella* sp. T1 and other denitrifying species. Thus, foraminifera contribution to benthic denitrification was negligible (~ 5 %) during prolonged seasonal hypoxia in the fjord. Moreover, the potential invasive denitrifying *Nonionella* sp. T1 could impact the nitrogen cycle under oxic conditions by increasing the sediment denitrification and could counterbalance potential eutrophication of the Gullmar Fjord. Thus, our study demonstrated that the role of denitrifying foraminifera is underestimated in the nitrogen cycle especially in oxic environments.

Figures list

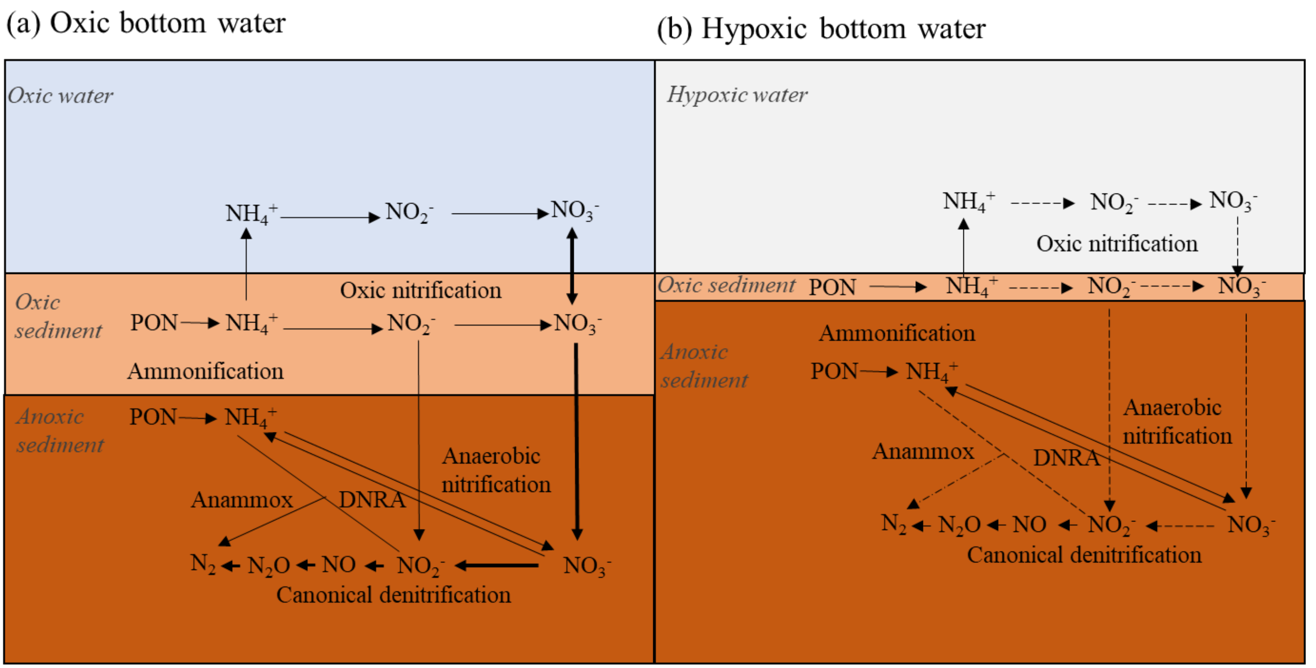

Figure 1. Simplified nitrogen cycling in marine sediments when the bottom water is oxic (a) and hypoxic (b). Chemical formulae: PON (particulate organic nitrogen), NH_4^+ (ammonium), NO_3^- (nitrate), NO_2^- (nitrite), NO (nitrogen oxide), N_2O (nitrous oxide), N_2 (nitrogen). The bold/dotted arrows indicate reactions advantaged/reduced by oxygen and nitrate presence/depletion. See text for more details. Modified from Jantti and Hietanen, (2012).

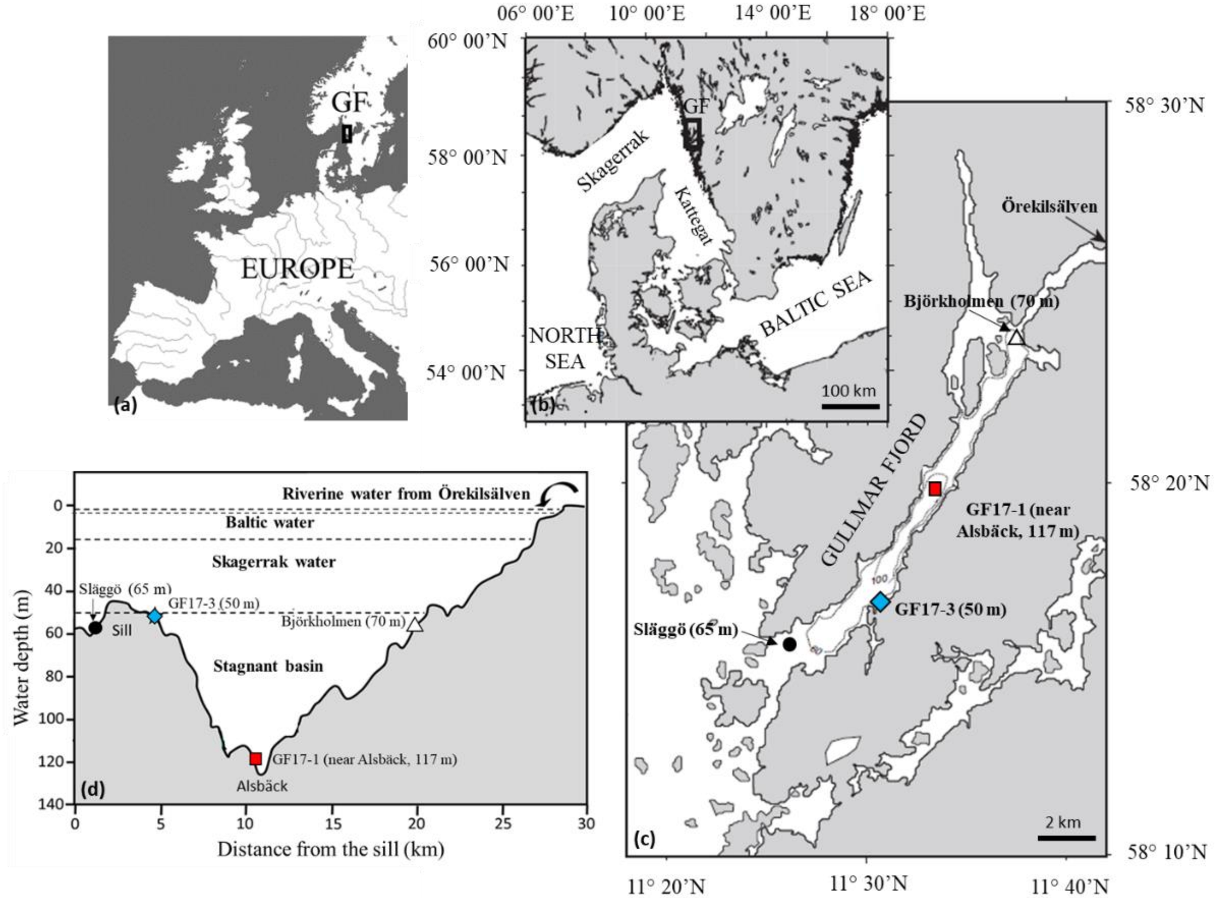

Figure 2. (a-c) Location of studied stations in the Gullmar Fjord (Sweden); blue diamond: GF17-3 oxic station (50 m depth); red square: GF17-1 hypoxic station (117 m depth); dark circles: monitoring stations Släggö (65 m depth) and Björkholmen (70 m depth). (d) Transect from the sill with four Gullmar Fjord water masses and studied stations (modified from Arneborg et al., 2004).

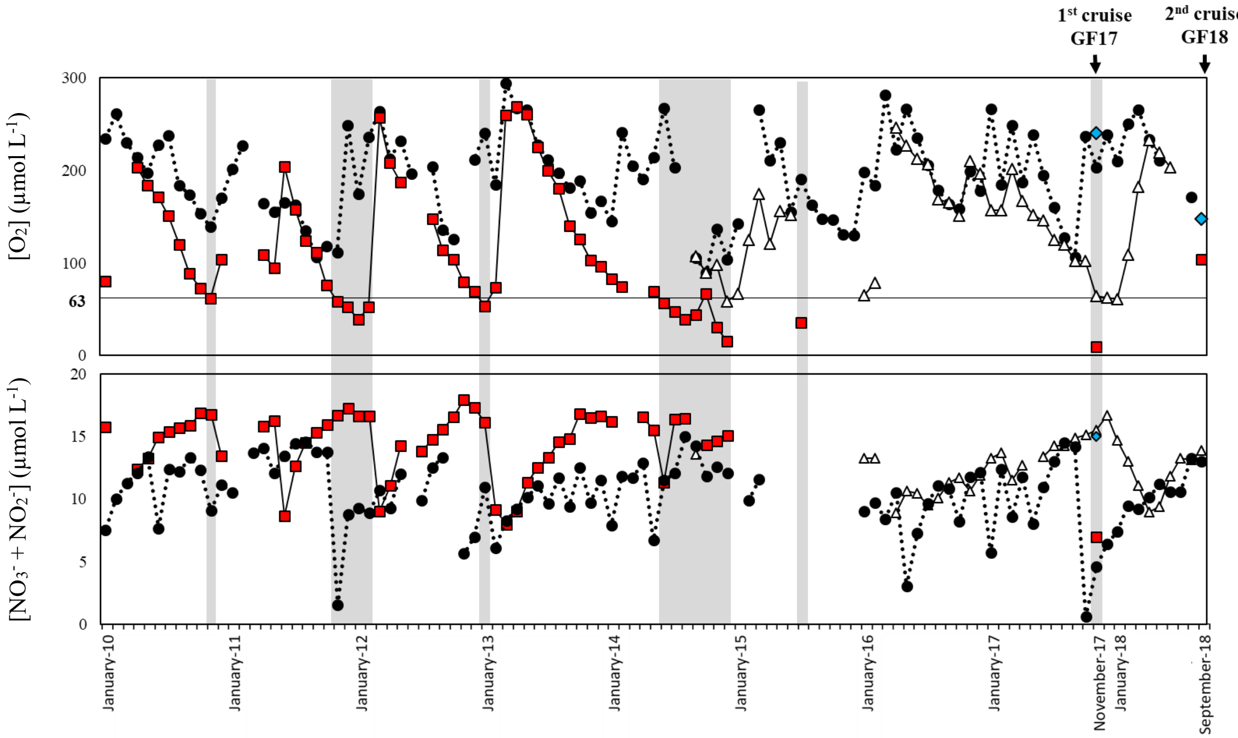

Figure 3. Record from January 2010 to September 2018 of bottom water oxygen ([O_2]) and nitrite + nitrate ([NO_3^- + NO_2^-]) measurements from the monitoring stations Släggö (65 m depth; black dot), Björkholmen (70 m depth; white triangle) and the sampling stations GF17-1 (Alsbäck, 117 m depth; red square) and GF17-3 (50 m depth; blue diamond). The arrows indicate the date of the two sampling cruises: the 2017 cruise (14th, 15th November 2017) and the 2018 cruise (5th September 2018). The grey zones indicate hypoxia threshold ([O_2] < 63 µmol L^{-1}).

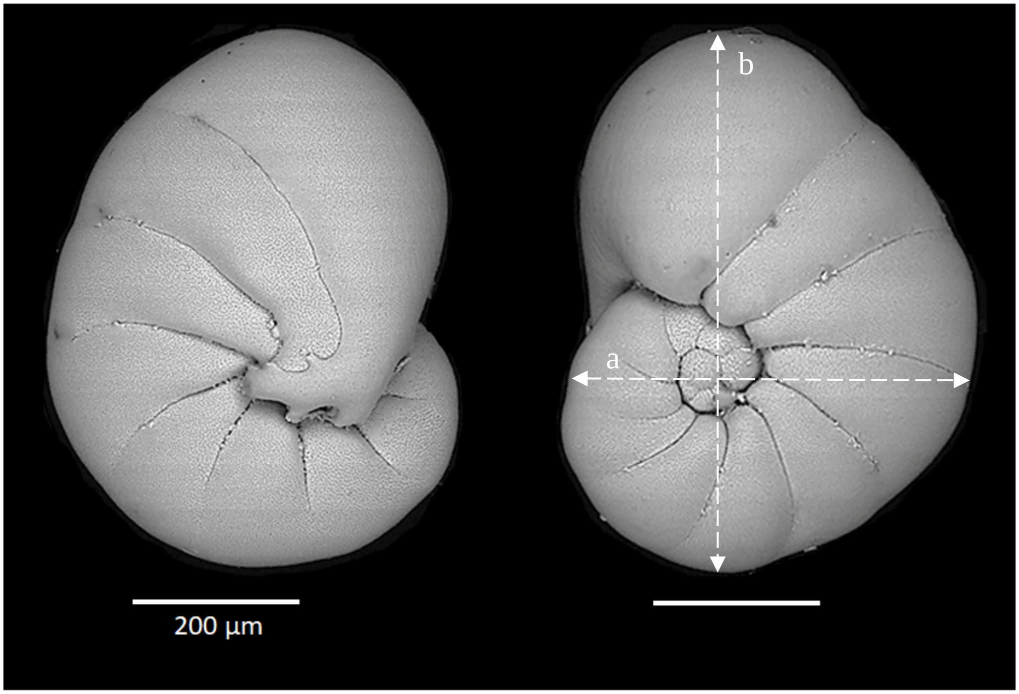

Figure 4. Scanning Electronic Microscope images of a *Nonionella* sp. T1 from the GF17-3 oxic station in the Gullmar Fjord. White lines (a, b) correspond to measured distances serving for a spheroid prolate volume model.

Table 1. Total shell volume (µm^3) and the biovolume (BV, µm^3) corresponding to 75% of the total shell volume measured on the pool of five *Nonionella* sp. T1 from the 2017 and the 2018 cruises in the Gullmar Fjord. Abbreviations: sd (standard deviation), ind. (individual).

Nonionella sp. T1	1st cruise total shell volume	1st cruise BV	2nd cruise total shell volume	2nd cruise BV
ind. 1	$6.7\ 10^{+06}$	$5.0\ 10^{+06}$	$3.1\ 10^{+06}$	$2.3\ 10^{+06}$
ind. 2	$4.5\ 10^{+06}$	$3.4\ 10^{+06}$	$2.4\ 10^{+06}$	$1.8\ 10^{+06}$
ind. 3	$5.1\ 10^{+06}$	$3.8\ 10^{+06}$	$1.4\ 10^{+06}$	$1.0\ 10^{+06}$
ind. 4	$4.9\ 10^{+06}$	$3.7\ 10^{+06}$	$9.2\ 10^{+05}$	$6.9\ 10^{+05}$
ind. 5	$5.8\ 10^{+06}$	$4.4\ 10^{+06}$	$6.2\ 10^{+05}$	$4.7\ 10^{+05}$
Average (µm^3)	$5.4\ 10^{+06}$	$4.0\ 10^{+06}$	$1.7\ 10^{+06}$	$1.3\ 10^{+06}$
sd (µm^3)	$0.8\ 10^{+06}$	$0.6\ 10^{+06}$	$1.0\ 10^{+06}$	$0.7\ 10^{+06}$

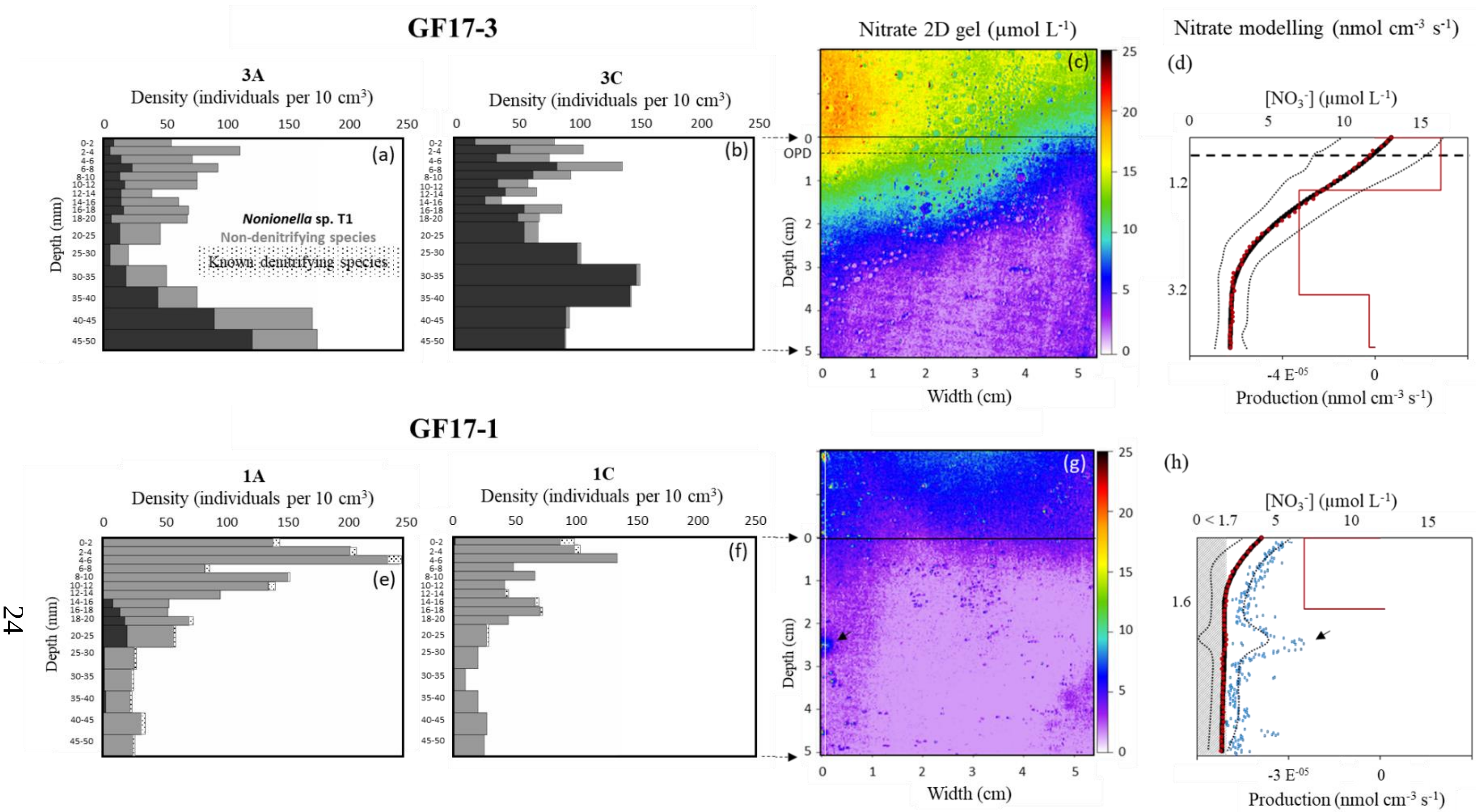

Figure 5. Micro-distributions of living foraminifera densities in GF17-3 oxic station (a, b) and in GF17-1 hypoxic station (e, f). *Nonionella* sp. T1 specimens are in black, the sum of the non-denitrifying species in grey colors and the small dots (e, f) show the other denitrifying species (known and potential candidates). The maps of porewater nitrate 2D gels are presented for stations GF17-3 (c) and GF17-1 (g). The sediment-water interface is represented by a black line at 0 cm depth (c, g) and the Oxygen Penetration Depth (OPD) is represented by the dashed line in bold at 4.7 ± 0.2 mm depth (c). Nitrate 1D profiles (d and h, black dots) are calculated using the average value of each pixel line of the nitrate distribution image (290 pixels wide), the standard deviation is represented by two fine dotted lines (c and g respectively). The corresponding best-fitting concentration profiles (red dots, d and h) and the production zones (red line) are modelled with PROFILE. The 1D profile corresponding to x = 1 mm (white line, g) is represented with a blue square profile (h) and the deep nitrate spot is indicated by a black arrow. The hatched grey zone (h) represents the detection limit of the nitrate 2D gel (<1.7 µmol L^{-1}).

Table 2. Summary of the NIS *Nonionella* sp. T1 contributions to benthic denitrification in the Gullmar Fjord. The porewater denitrifications zones come from PROFILE modelling (Fig. 5 d, h). To estimate the contributions of *Nonionella* sp. T1 the counted specimens per zones was used. Two different approaches were used to estimate the contribution of *Nonionella* sp. T1: (A) divided the *Nonionella* sp. T1 denitrification rate by the nitrate porewater denitrification rate estimated from PROFILE modelling, then the second approach (B) divided the *Nonionella* sp. T1 denitrification rate by the denitrification rate from PROFILE plus the *Nonionella* sp. T1 denitrification rate. The calculations are detailed in Equation S2.

Stations	Sediment depth interval of denitrification (cm)	*Nonionella* sp. T1 (counted specimens per zone)	Nitrate porewater denitrification rates (nmol cm^{-3} s^{-1})	*Nonionella* sp. T1 denitrification rates (nmol cm^{-3} s^{-1})	*Nonionella* sp. T1 contribution (%), approach A	*Nonionella* sp. T1 contribution (%), approach B
GF17-3A	1.2 to 5	841	4.07 E^{-07}	1.90 E^{-05}	47	32
GF17-3C	1.2 to 5	1807	4.07 E^{-07}	4.06 E^{-05}	100	50
GF17-1A	0 to 1.6	3	2.71 E^{-05}	6.72 E^{-08}	0	0
GF17-1C	0 to 1.6	12	2.71 E^{-05}	2.69 E^{-07}	1	0

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Author contributions

C.C. participated in the sampling cruise, did the foraminifera taxonomy, contributed to 2D gel experiments and analyses by hyperspectral camera. C.C. did the nitrate and oxygen respiration measurements and wrote the present manuscript. E.G. participated in the sampling cruise, contributed to foraminifera analysis, scientific discussions. E.M. participated in the sampling cruise, managed with A.M. the 2D gels experiments, and contributed to hyperspectral camera treatments and scientific discussions and manuscript rewriting. H.L.F managed with A.M the sampling cruise and contributed to foraminifera taxonomy and scientific discussions and manuscript rewriting. N.R.P. managed the oxygen and nitrate respiration measurements and contributed to the scientific discussions. P.L. managed hyperspectral treatments for 2D gels and contributed to scientific discussion. M.G. participated in the 2D gel lab experiments and hyperspectral treatments. T.J. participated to the sampling cruise, contributed to 2D gels experiments and scientific discussions and manuscript rewriting. B.J. contributed to scientific discussion and manuscript rewriting. A.M. managed the sampling cruise and 2D gels experiments and contributed to hyperspectral camera treatments and scientific discussions and manuscript rewriting.

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Competing interests

The authors declare no competing interest.

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constance.choquel@gmail.com on behalf of the coauthors

Dear Editor and referees,

Thank you for your interest in our work and your comments to improve the paper "Denitrification by benthic foraminifera and their contribution to N-loss from a fjord environment". Please find the revised manuscript attached. The main corrections performed to the manuscript are highlighted in yellow.

Briefly, we have changed the title of the manuscript, the new title highlights the denitrification of foraminifera and their impact on the nitrogen cycle. The abstract has been adapted accordingly. In the first paragraph of the introduction a contextualization of the importance of the nitrogen cycle in semi-enclosed environments subject to hypoxia has been added. Then, a paragraph in discussion 4.3 has been added to inform readers about the eutrophication state of Gullmar Fjord. Discussion sections formerly 4.2 and 4.3 have been merged under the name " 4.2 Foraminifera ecology considering nitrate micro-distribution". To take into account the remarks of the short comment and the referees, we have changed the term "invasive" *Nonionella* sp. T1 by non-indigenous species (NIS). The term "invasive" is introduced because it is cited in the existing literature. The potential invasiveness of *Nonionella* sp. T1 in the Gullmar fjord is mentioned later in the discussion sub-section 4.1. Figure 4 of the material and method about the 2D gel method has been removed as potential interested readers can consult the original paper that details the procedure. A conversion and a unit error have been found for the denitrification rates (nmol cm^{-3} s^{-1}). The final contribution results remain unchanged as the conversion error was done for both denitrification rates for foraminifera and cores (see changes Fig. 5, Table 1, Annex Equation S2, and associated text). For more details on minor changes please refer to the replies to referees.

Best regards.

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Dear Referee 1,

Thank you for your constructive comment and your interest in our work. I agree with the majority of the suggestions that you bring to our study. Indeed, this study targeted the importance of the contributions of denitrifying benthic foraminifera regarding contrasted oxygen and nitrate conditions at two different sites in the Gullmar Fjord. Indeed, the introductory part on the nitrogen cycle deserves to be better contextualized with more general bibliographical references. I think it is premature to engage in the description of the effects of denitrification of forams on primary production and nitrogen fixation. I prefer to remain cautious about the prospects of the discussion so as not to make too precise speculation.

Question 1: In my opinion, the title should include the regional characteristics including Gullmar Fjord or the North Sea rather than a generalized focus on invasive species' contribution to nitrate uptake. Or the overall discussion of this MS should include more of; what does this mean? This invasive species is increasing in numbers in the region (maybe in other areas too?) which is capable of such contribution to N dynamics and we are expecting to see in the future. The observation of its increase in the region is valuable. Nevertheless, I am not sure this is exactly the message of this specific study.
Answer 1: I suggest a novel title as "Total nitrate uptake by benthic foraminifera in the Gullmar Fjord"

Question 2: Do authors think before the invasion of Nonionella sp. T1 benthic denitrification was overall less than their observations in this study or it has been overall the same values, but the other species are simply losing the competition now in the region? Is there any indication or previous study focusing on that? if this is the first time observation on this specific topic in this region, the authors should emphasize it even more.
Answer 2: Station GF17-3 (50 m) was sampled for the first time in this study. There is therefore no retreat on the benthic denitrification and the assemblages of foraminifera at this precise location of the Fjord.

Question 3: Please provide references for benthic foraminifera taxonomy in supplementary material, considering which publication (maybe even which figure) was used for identification of the species listed in Table S3 and S4.
Answer 3: Yes I will add them. I looked at Charrieau et al., (2018)

Question 4: Abstract: Line 14: there is no flow/connection between the first 2-3 sentences. It would be better to focus on first the importance of invasive species in certain regions or the importance of oxygen, nitrate dynamics in such regions. I think authors should decide how to formulate the most important message of this MS. Line 18: micro-distribution: microhabitat instead? Line 19: worth to mention Gel methodology already here for least confusion of 2D geochemistry concept. The next sentence also needs a reshape giving a broader idea of these contrasting sites. Oxygenated overlying and bottom waters with high nitrate content in porewaters vs hypoxic bottom waters where porewater is nitrate scarce.

Answer 4: I will focus the abstract on the importance of the contribution of denitrifying forams in the Gullmar fjord according to the contrasting geochemical conditions in oxygen and nitrates. Rewrite the sentence concerning the methodology of 2D gels.

Question 5: Introduction: First sentence: I am confused with nomenclature, unit choice, and conversion of values here. There are many studies focusing on different values for the term hypoxia so I highly recommend citing the publication that the authors followed. This is also valid for unit choice, I am familiar with dissolved oxygen concentration units of mL/L and umol/kg or umol/L. Generally, 2 ml/l is circa 90 umol/l. Most of the studies concerning benthic foraminifera in low oxygen environments focus on these units. I just wonder which study the authors decided to follow in this case.

Answer 5: I use only the unit µmol/L in the study. The hypoxia threshold used is 63 µmol/L cited by Breitburg 2018.

Question 6: Line 33: contrasted dissolved O2 conditions: Over what time interval? a year? Different seasons? or different sampling sites? I know this information will be mentioned later but it would be nice to give the information here already.
Answer 6 : Yes, to be re-specified. Two contrasting oxygen stations, one hypoxic in the deep basin (GF17-1) at the end of autumn 2017 and an oxic station at the entrance to the fjord.

Question 7: Line 44: "total denitrification". Overall, denitrification together with anammox is also called N-loss. I recommend authors have a look at some other reviews on marine N cycle: Galloway et al., 2004, Gruber and Sarmiento, 1997; Gruber and Galloway, 2008. Maybe even Sigman et al 2009 (is in the direction of N isotope chemistry but is a nice review). These are reviews that would give a bit more insight and overview of the marine N cycle with perspective to open sea/ocean. There are many publications on coastal systems and while investigation on N2 loss and its impact on eutrophication I came across to Seitzinger 1988 I think should be included either to the introduction or the discussion to make the findings of this study more pronounced. It is worth mentioning the potential benefit of benthic denitrification to eutrophication already in the introduction giving examples from previous studies.

Answer 7: Some of these references can support the introductory part of the state of the art of the nitrogen cycle in marine sediments in order to contextualize more broadly the importance of identifying the sources and outputs of nitrogen from a system (Galloway, 2004). In most coastal environments such as the Baltic Sea the loss of nitrogen through denitrification exceeds the supply of nitrogen through nitrogen fixation. These sink regions of the ocean are the areas associated with the anoxic regions (Grubber and Sarmiento 1997). When benthic denitrification exceeds nitrogen fixation, eutrophication can be mitigated via nitrogen loss (Seitzinger 1988). The Gullmar Fjord would be a sink region.
In the last part of the discussion (4.4) it is possible to briefly provide more details on the eutrophication state of Gullmar Fjord. Primary production in Gullmar Fjord is dominated by diatoms bloom in spring and autumn (Lindahl and Hernroth, 1983). Since the 1990s Lindahl et al. (2003) observed the increase in primary production of the Gullmar fjord, therefore a potential eutrophication of the Fjord. This increase

in original productivity also shown in the adjacent Kattegat could be related to the nitrogen input loading from the land and atmosphere (Carstensen et al. (2003)). Lindahl et al. (2003), argued that primary production production of the Gullmar fjord was due to climatic forces resulting from a strong positive North Atlantic Oscillation (NAO) index, which increased the availability of deepwater nutrients (Kattegat nitrate-rich) and due to warmer ocean surface. The benthic denitrification of Gullmar Fjord makes it possible not to supply the system with nitrogen available for primary producers. Denitrifying foraminifera including *Nonionella* sp. T1 could thus help counterbalance this eutrophication by increasing the loss of N_2. Glock et al., (2013) also supported denitrifying forams in OMZ contributed to N-loss (until 46%). Then, foraminifera intracellular nitrates become unavailable to the system and can be bio-transported and permanently sequestered in sediments (Glock et al., 2013; Prokopenko et al., 2011).

<u>Question 8:</u> Line 48: nitrification cannot process under low oxygen conditions. How low? Please indicate the values here.
Answer 8: According to Mortimer et al., (2004). Once the oxygen in the sediment is no longer detected (close to 0 µmol / L) the nitrification also becomes undetectable.

<u>Question 9:</u> Section 2 Methods Suggestion for site or expedition indicator throughout the text: Instead of 1st and 2nd cruise, authors could use years, e.g., 2017 and 2018.
Answer 9: Yes indeed it may be clearer using the dates of the missions

<u>Question 10:</u> Line 109: (see previous studies) please indicate references instead.
Answer 10: Nordberg and al., 2000; Filipsson and Nordberg, (2004)

<u>Question 11:</u> Line 127: is there a special reason for the choice of 100 um fraction? Whereas well accepted fractions are 63, 125, and 150 um?
Answer 11: In the previous studies in the Gullmar Fjord, the size fraction > 100 µm has most commonly been used for foraminiferal analyses (see Charrieau et al., 2018).

<u>Question 12:</u> Line 140 and figure 4: Is Figure 4 needed? Is this method described here the first time and different from Metzger et al., 2016?
Answer 12: This is the same method as Metzger et al., 2016 but since the steps in this method can be difficult to follow for non-specialists I find the diagram helps to easily visualize the method.

<u>Question 13:</u> Line 202: I find Table S1 rather important for this MS. What about involving it to the main MS but not only in supplementary information?
Answer 13: I'm not convinced I think this table is better in extra method.

<u>Question 14:</u> 4. Discussion: Line 301: I think it should be GF17-1A and 1C in the parenthesis.
Answer 14: ok

Question 15: Line 309: (our results) data not shown and presented? If so, please mention or indicate where this information comes from. In the same line, it would be better to mention some of the previous studies showing differences too.

Answer 15: Ok

Question 16: I recommend changing the titles for the section 4.2 and 4.3 to ": : :T1/foraminifera habitat in relation with the nitrate micro-distribution: : :" since there might be other factors having an impact on the ecology of these species, it would be better to keep the focus on nitrate and oxygen in these sections of the discussion.

Answer 16: I suggest to merge the two parts 4.2 and 4.3

4.3 The foraminifera ecology considering the nitrate micro-distribution

Inside first paragraph about oxic station and a second paragraph about hypoxic station.

Question 17: Line 395: once again discussion on benthic N loss contribution to eutrophication: I think this needs a broader discussion and requires some references. Moreover, does N2 flux from sediment promote N2 fixation, and thus, e.g., cyanobacterial activity? Are there studies focusing on N2 fix vs N loss in Gullmar Fjord or similar settings? I think considering these would improve the discussion significantly.

Answer 17: it's difficult to answer this question without getting too speculative

The question here suggests that nitrogen supply via benthic denitrification of the forams could be captured by N_2-fixing cyanobacteria and participate in their development. Significant cyanobacteria blooms are already known in the Baltic Sea (Boesch 2003 Swedish agency report). In the Gullmar fjord there are few studies on cyanobacteria (Croot, 2003) the evolution of N_2-fixation by these cyanobacteria in Gullmar Fjord is not obvious and lack of data. Benthic denitrification of the forams may participate in the N pool to be fixed by cyanobacteria but I think this hypothesis is too speculative, then cyanobacteria in Gullmar Fjord do not appear to be a major threat to the system at this time.

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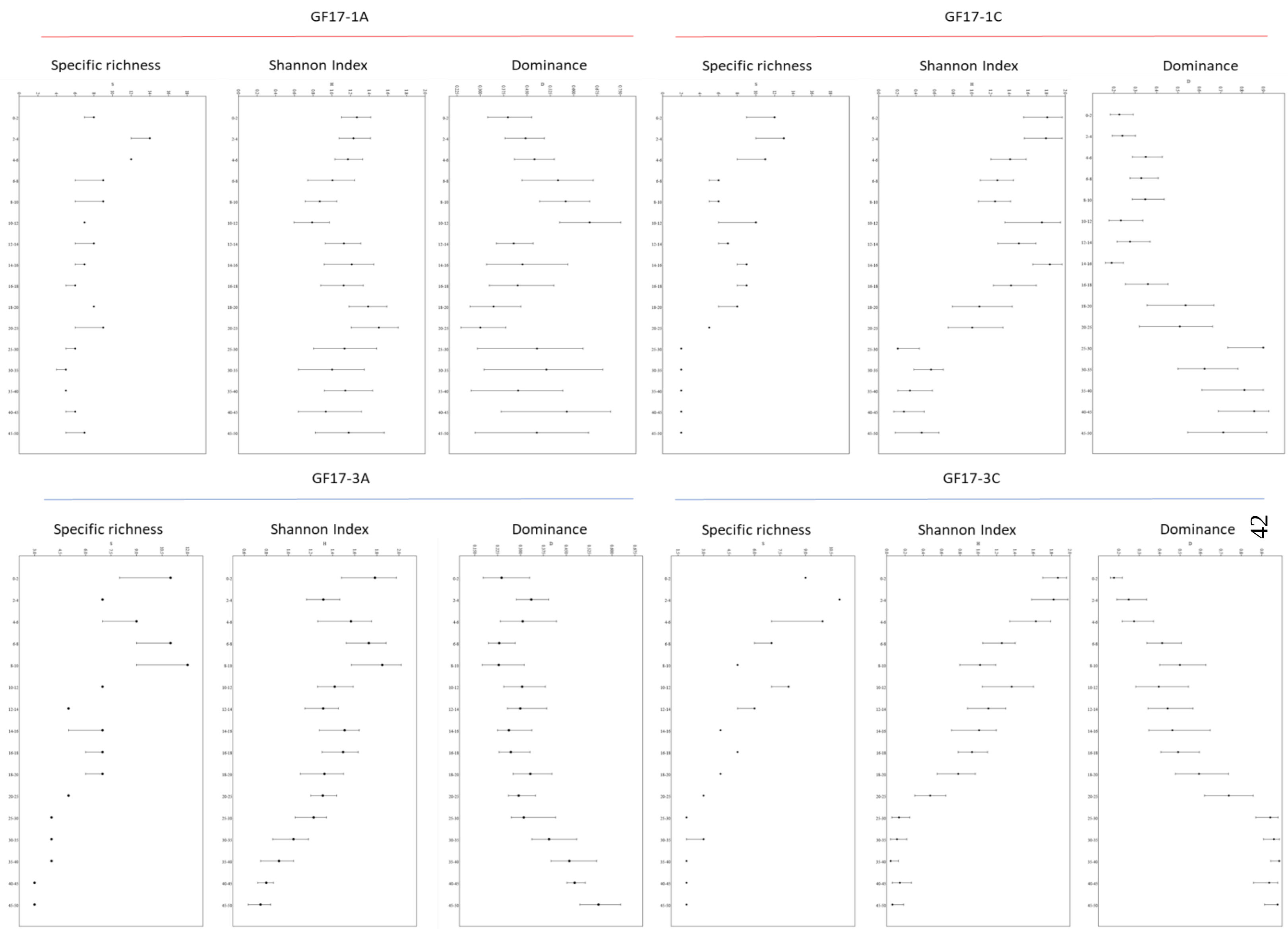
Dear Referee 2,

Thank you for your constructive comment and your interest in our work. I agree with the majority of the suggestions that you bring to our study. The status of *Nonionella* sp T1 remains unclear. I am to follow the recommendations made by V. Bouchet by introducing *Nonionella* sp. T1 as Non-Indigenous Species then, in discussion I will discuss its invasiveness in Gullmar Fjord. Indeed, the dominance of *Nonionella* sp. T1 could be harmful for the Foraminifera diversity species. I am aware that this study must be followed by a long bio-monitoring > 63 µm (seasonal, different depths stations) to validate the ongoing change in Gullmar Fjord fauna.

Question 1: The title of the study implies, that the work focuses on total nitrate uptake of a specific benthic foraminifer. However, the emphasis of the first part in the discussion of this study implies a thorough taxonomic investigation of the Fjord fauna, which is not the case in this study. I agree with the authors,

that there is an ongoing change in the benthic foraminiferal community of the Gullmar Fjord. But to verify this trend and to discuss its consequences, a longer-term monitoring study observing seasonal fluctuations of the benthic foraminiferal community together with environmental parameters at several stations within the fjord is necessary. Further, a more detailed comparison with previous literature would be necessary. I think the authors should point out, that such monitoring studies (including the 63 – 125µm size fraction) are important for the future, specifically considering the new observations of this study.
Answer 1: I agree that a long monitoring would be necessary to validate the change in fauna and include a study with a smaller fraction.

Question 2: I agree with the author of the short comment considering the invasive status of *Nonionella* sp. T1. Certainly, this species is proven to be non-indigenous. However, the actual invasive status of this species is not proven yet. It is not yet clear, if the occurrence of Nonionella sp. T1 is responsible for the disappearance of any other species in the Fjord, nor is there any evidence, that this species is harmful for the ecosystem of the Gullmar Fjord. On the contrary, the authors point out, that this species might even be of advantage for the trophic status of the fjord. It is important to stick with correct ecological terminology to avoid confusion in further research. I would recommend to change the term 'invasive' to 'non-indigenous'.
Answer 2: I agree with V. Bouchet comment. I will introduce *Nonionella* sp. T1 as a Non-Indigenous Species (Deldick et al., 2019). Then, in the discussion I will mention the invasive character of this species in the Gullmar Fjord in view of its strong increase in density at the entrance to the Fjord. There is no evidence that *Nonionella* sp. T1 can harm the ecosystem, however *Nonionella* sp. T1 could affect the fauna of foraminifera. Indeed, the specific richness (S) and the Shannon index (H) decrease with sediment depth sediment in the GF17-3 station while the dominance due to Nonionella increases (see graphs GF17-3A and 3C). In the hypoxic station, the dominance is driven by *Cassidulina laevigata* and *Bulima marginata* which dominated the fauna.


GF17-1A
GF17-1C
GF17-3A
GF17-3C
Specific richness
Shannon Index
Dominance

Additionally, I would like to add a few technical corrections and minor remarks:

Introduction:

Question 3: Line 29: ‘and thereby to survive’ should be ‘and thereby survive’

Answer 3: ok

Question 4: Line 32: ‘This study focus on...’ should be ‘This study focuses on...’

Answer 4: Ok

Material and Methods:

Question 5: Line 127: ‘Fixed samples were sieved and the > 100 m fraction was examined...’ Did you remove any larger meiofauna e.g. by sieving through a larger sieve (5 mm, 2 mm, 1mm)? If so, this should be mentioned too, since adults of larger denitrifying genera e.g. *Globobulimina* often cannot pass through a 1 mm sieve.

Answer 5: the sieves used are

>355	355-150	150-125	125-100

No 1 mm sieve was used there should be no loss of *Globobulimina*.

Discussion:

Question 6: Line 292: I would consider to change the title of this section into something like: ‘Abundance of Nonionella sp. T1 in comparison with other species’

Answer 6: yes I will change the title to be more careful about the change of fauna.

Question 7: Line 315: I think there is something a little bit wrong with this sentence. Should it be something like: ‘That the foraminiferal fauna described in the present study differs, is the consequence...’

Answer 7: I will rewrite better this sentence.

Question 8: Line 327: Did Polodova Asteman and Schönfeld (2015) sample the same location at the oxic part of the fjord?

Answer 8: No, they sampled in the deep Alsback station which was oxic at the time of the sampling in August 2013 and July 2014. They sampled a station in the Skagerrak near the mouth of the fjord in June 2013, I compared my oxic station with this data out of the Fjord.

Question 9: Line 359: Could propagules also be a reason for the survival or re-appearance of the non-denitrifying species in the hypoxic part of the fjord?

Answer 9 : Yes, propagules can disperse and reproduce when environmental conditions are favourable according to Alve and Goldstein, 2003. However, there is no change in density of *Nonionella* sp. T1 at the Alsback station from the densities found by Polovodova Asteman and Schönfeld (2015). It would be interesting to look again at this Alsback station to see if there is an evolution of the densities of *Nonionella* sp. T1 and if there is a seasonality of denitrifying foraminifera depending on the oxygenation conditions (hypoxic vs oxic).

Question 10: Line 392: I would be careful with this consideration, because other well oxygenated areas of the Fjord might be dominated by other species - depending on depth or other environmental parameters.

Answer 10: Yes to bring more weight to this hypothesis it would be necessary to make several oxic stations at different depths in the Fjord.

Question 11: Figure 6: It should be ‘Depth (mm)’ for GF17-3A and 3C and GF17-1A and 1C and not Depth (cm).
Answer 11: ok