RESEARCH ARTICLE

# The nitrogen cycle in anaerobic methanotrophic mats of the Black Sea is linked to sulfate reduction and biomass decomposition

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## Abstract

Anaerobic methanotrophic (ANME) mats host methane-oxidizing archaea and sulfate-reducing prokaryotes. Little is known about the nitrogen cycle in these communities. Here, we link the anaerobic oxidation of methane (AOM) to the nitrogen cycle in microbial mats of the Black Sea by using stable isotope probing. We used four different $^{15}$N-labeled sources of nitrogen: dinitrogen, nitrate, nitrite and ammonium. We estimated the nitrogen incorporation rates into the total biomass and the methyl coenzyme M reductase (MCR). Dinitrogen played an insignificant role as nitrogen source. Assimilatory and dissimilatory nitrate reduction occurred. High rates of nitrate reduction to dinitrogen were stimulated by methane and sulfate, suggesting that oxidation of reduced sulfur compounds such as sulfides was necessary for AOM with nitrate as electron acceptor. Nitrate reduction to dinitrogen occurred also in the absence of methane as electron donor but at six times slower rates. Dissimilatory nitrate reduction to ammonium was independent of AOM. Ammonium was used for biomass synthesis under all conditions. The pivotal enzyme in AOM coupled to sulfate reduction, MCR, was synthesized from nitrate and ammonium. Results show that AOM coupled to sulfate reduction along with biomass decomposition drive the nitrogen cycle in the ANME mats of the Black Sea and that MCR enzymes are involved in this process.

## Introduction

The stratified Black Sea becomes anoxic at water depths below 70–100 m, providing ideal conditions to study anaerobic marine nutrient cycles (Kuypers *et al.*, 2003; Lam *et al.*, 2007). In organic-rich ecosystems such as the Black Sea, the consumption of dioxygen coincides with the depletion of other electron acceptors such as nitrate, oxidized metals or sulfate. This minimizes possible energy gains for microbial communities. The highly reduced

redox state of the Black Sea is reflected in the high abundance of methane, sulfide, and ammonium in the water column as well as in sediments.

Previous studies have demonstrated that anaerobic oxidation of methane (AOM) is the central biological redox process in the Black Sea (Reeburgh *et al.*, 1991). AOM, performed by anaerobic methanotrophic (ANME) consortia, also contributes significantly to carbon and sulfur cycles in marine environments worldwide (Reeburgh, 2007) but its role in the nitrogen cycle is under debate (Oremland, 2010; Bowles & Joye, 2011). In the nitrogen cycle, under anoxic conditions, the main processes are nitrate reduction, ammonium assimilation, biomass decomposition, dinitrogen fixation (diazotrophy) and anaerobic ammonium oxidation (ANAMMOX, Fig. 1). Nitrate and dioxygen were reported to be absent at the water depth of the AOM Black Sea mats (230 m) but both reported sampling spots were far from the mats' location (Kuypers *et al.*, 2003; Lam *et al.*, 2007). In contrast, dissolved dioxygen is required for nitrification and was found in the water column near the sediment surface of the western Black Sea shelf in water depths of 134 and 142 m (Friedl *et al.*, 1998). *Calditrix* – a genus comprising reported nitrate reducers (Miroshnichenko *et al.*, 2003) – was detected in 16S rRNA and 16S rDNA clone libraries of the pink and the black type of the Black Sea mats (Friedrich *et al.*, 2007). Both findings make nitrate a possible electron acceptor for AOM in the Black Sea mats of the north-western shelf region.

The first evidence for a direct link between methane oxidation and nitrate reduction came from Raghoebarsing *et al.* (2006), who showed that methane oxidation was directly coupled to the reduction of nitrate. Recently, the microorganisms involved were shown to possess the intriguing ability to produce intrinsic dioxygen by cleaving two moles of nitric oxide to one mole of dinitrogen and one mole of dioxygen to drive methane oxidation aerobically (Ettwig *et al.*, 2010). This opened the discussion whether this type of AOM coupled to nitrate reduction should be regarded as aerobic or anaerobic (Oremland, 2010). Aerobic methane oxidation involves monooxygenases that were not discovered in the Black Sea AOM mats (Meyerdierks *et al.*, 2010). AOM requires methyl coenzyme M reductase (MCR) enzymes (Krüger *et al.*, 2003; Shima *et al.*, 2012) which are permanently disabled by dioxygen (Mahlert *et al.*, 2002).

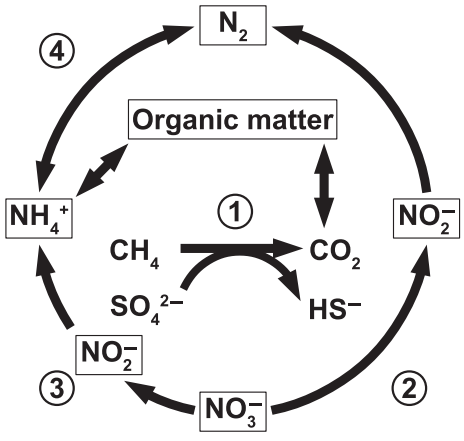



**Fig. 1.** The simplified anaerobic nitrogen cycle. Note that not all steps were observed in the Black Sea AOM mats. The AOM reaction (1) provides energy and carbon ($CO_2$; Wegener *et al.*, 2008) for these mats. Sulfate reduction stimulates nitrate reduction to dinitrogen (2) but not DNRA. (3) DNRA was stimulated by organic matter decomposition, which also releases ammonium. Ammonium was also provided in negligible amounts by dinitrogen fixation (4).

Oxidation of methane is not the only energy-providing pathway in methane-rich ecosystems. A secondary pathway is nitrate reduction coupled to biomass decomposition as observed at cold seeps in the Gulf of Mexico (Bowles & Joye, 2011). It was postulated that nitrate reduction to dinitrogen linked to biomass decomposition is generally important in such environments. Reduced sulfur such as sulfide or zero-valent sulfur (Milucka *et al.*, 2012) supplied by AOM coupled to sulfate reduction may serve as an electron shuttle for nitrate reduction. Indeed, the anaerobic oxidation of sulfide by nitrate reducers naturally occurs in sulfide-rich marine transition zones (Eisenmann *et al.*, 1995; Fossing *et al.*, 1995). A second route of nitrate reduction is dissimilatory nitrate reduction to ammonium (DNRA; Sørensen, 1978; Tiedje, 1988; Kimura, 2000). Numerous sulfate reducers are capable of DNRA, among them *Desulfovibrio* and *Desulfobulbus* species (Kimura, 2000). The latter was frequently detected in AOM-influenced environments (Knittel & Boetius, 2009).

Methane-dependent uptake of $^{15}$N-ammonium into amino acids (and hence into the biomass) of ANME consortia was shown previously but rates were not determined (Krüger *et al.*, 2008). Diazotrophy as an alternative means of nitrogen uptake in ANME consortia was demonstrated but its relevance in AOM settings remained unclear (Orphan *et al.*, 2009). Nitrogen fluxes in microbial communities can be tracked by stable isotope probing (SIP) of different biomolecules (e.g. nucleic acids or proteins). Protein-SIP can be used to obtain functional and phylogenetic information of microbial consortia involved in the nitrogen cycle (Jehmlich *et al.*, 2010). In spite of the higher sensitivity of protein-SIP compared with DNA-SIP, the limit of detectable $^{15}$N incorporation is *c.* 5 at.%. The incorporation is also affected by the lifetime of proteins, which might hamper the analysis when it is very long. Such low biosynthesis rates – a consequence of the extremely

long doubling times in AOM consortia of up to 7 months – are typical in AOM sediments (Girguis *et al.*, 2003; Nauhaus *et al.*, 2007; Dale *et al.*, 2008; Milucka *et al.*, 2012) and also in the AOM Black Sea mats (Krüger *et al.*, 2008).

In general, the Black Sea AOM mats are characterized by two phenotypically and phylogenetically different mat types adapted to their natural habitat in and around the carbonate chimneys where methane is the primary energy source (Michaelis *et al.*, 2002). In previous reports, the archaeal community of the inner pink mats hosted mainly ANME-1 archaea while ANME-2 archaea were the major part of the outer black ring (Blumenberg *et al.*, 2004; Knittel *et al.*, 2005). Whether the higher availability of methane (for the inner pink mats closer to the gas streams) or sulfate (for the outer black mats in direct contact with the seawater) determines the activity and the distribution of certain ANME groups is unclear but important for estimating protein synthesis rates.

It has been hypothesized that methanogenic archaea are capable of methanogenesis and methanotrophy (Hoehler *et al.*, 1994; Lloyd *et al.*, 2011; and citations therein). In fact, MCR enzymes of ANME archaea make up 7% of the extractable proteins of the AOM mats (Krüger *et al.*, 2003). These enzymes were shown to catalyse anaerobic methanotrophy as reverse methanogenesis (Scheller *et al.*, 2010). Hence, they are essential for the AOM mats and are therefore a primary target of the above described protein-SIP investigations.

While previous studies in AOM-driven systems have focused on carbon (Wegener *et al.*, 2008; Holler *et al.*, 2011) and sulfur (Holler *et al.*, 2011; Milucka *et al.*, 2012) fluxes, nitrogen – an essential nutrient – received less attention. Two publications reported incorporation of $^{15}$N-labeled nitrate, ammonium, dinitrogen, cyanide or amino acids (glycine and leucine) into AOM-associated cells using a combined catalyzed reporter deposition fluorescence *in situ* hybridization/nano secondary ion mass spectrometry approach (Dekas *et al.*, 2009; Orphan *et al.*, 2009). However, these reports lacked an estimation of turnover and biosynthesis rates, and the demonstration of the *de novo* synthesis of enzymes involved in AOM.

The present work elucidates major nitrogen fluxes and their interaction with AOM coupled to sulfate reduction. We also confirm the biosynthesis of MCR enzymes of ANME cells in the Black Sea mats and show that methanotrophs are actively involved in the nitrogen cycle. We fed four different $^{15}$N-labeled nitrogen compounds to the mats and traced nitrogen incorporation into the biomass. By using $^{15}$N protein-SIP, we demonstrated the *de novo* synthesis of MCR subunits.

## Materials and methods

### Chemicals

Inorganic salts for preparation of artificial seawater media (Widdel & Bak, 1992) were purchased from VWR (Germany). Vitamins and trace elements for incubation media were purchased from Sigma-Aldrich (Germany). All labeling (isotope) substrates were purchased from CAMPRO Scientific (Germany). All chemicals were purchased in the highest available purity. A detailed composition of the medium is provided in the Supporting Information.

### Sample collection

Samples were collected from carbonate chimneys erecting from the seafloor of the Black Sea at a water depth of 230 m (Michaelis *et al.*, 2002) during R/V *Poseidon* cruise 317 leg 2 in 2004 and R/V *Meteor* cruise M72 leg 1 in 2007 at 44°46.46′N, 31°59.50′E. To minimize oxygen contamination, all sampling was conducted under nitrogen atmosphere. We separated pink from black mats using sterile scalpels. Then, the mats were transferred into 1-L glass bottles containing filter-sterilized and nitrogen-sparged Black Sea water. Subsequently, these bottles were sealed with butyl rubber stoppers and plastic screw caps and then flushed with methane. After shipping in cooled containers, the bottles were gently shaken at 4–12 °C. When sulfide concentrations exceeded 12 mM, the medium was replaced by fresh artificial seawater medium (for details see Supporting Information).

### Quantification of *mcr*A genes

Genes coding for MCR proteins (*mcr*A) were quantified by specific quantitative PCR (qPCR) assays as described in the Supporting Information.

### Medium preparation and inoculation

An overview over the experimental setup is displayed in Supporting Information Table S1. To avoid oxygen contamination, all manipulations were performed under an oxygen-free nitrogen atmosphere. Dissolved air was removed from the medium by purging with helium until air concentrations were < 1% (v/v) in the headspace (verified by gas chromatography). Remaining oxygen in the medium was reduced by adding 1 mM $Na_2S$ (final concentration). For sulfate reduction experiments, 17 mM $MgSO_4$ was added. In incubations without sulfate, the sulfate was replaced by $MgCl_2$ in equimolar amounts. Autoclaved controls were set up only when helium was

used instead of methane. As an inoculum, a 1-to-1 slurry (v/v) of homogenized mat material and medium without electron acceptors or ammonium was prepared as described elsewhere (Nauhaus *et al.*, 2005). If not indicated otherwise, incubations were inoculated with a mix of black mats. After inoculation of 16 mL medium with 4 mL slurry under $N_2$ atmosphere (using an oxygen-free glove box), the headspaces were flushed with helium for *c.* 20 min. However, after flushing the headspace with helium, *c.* 25% (v/v) dinitrogen remained (determined by gas chromatography as described in the Supporting Information). The microcosms were prepared in 56-mL serum bottles sealed with butyl rubber stoppers and aluminium crimp caps. Except for the controls (single incubations), all incubations were set up in quadruplets. Twenty millilitres of medium contained about 2 mL mat material. The incubation time for $^{15}$N-nitrate incubations was 37 days, for $^{15}$N-ammonium incubations it was 66 days and for incubations with $^{15}$N-dinitrogen it was 129 days.

Three $^{15}$N-nitrogen sources were separately added to the batch incubations: $^{15}N_2$ [17% (v/v) final concentration], $K^{15}NO_3$ (5 mM final concentration) or $^{15}NH_4Cl$ (5 mM final concentration). Additionally, ANAMMOX was tested using $Na^{15}NO_2$ and $^{14}NH_4Cl$ or $Na^{14}NO_2$ and $^{15}NH_4Cl$ in equimolar concentrations of either 0.1 or 5 mM final concentration (Meyer *et al.*, 2005). The test for ANAMMOX with sulfate, iron(III) and manganese (IV) is described in the Supporting Information. To adjust the osmolarity, the missing molar amount of salt was replaced by NaCl. The headspace was filled with either helium or unlabeled methane. A complete data set of the experiment was obtained after 1 year of incubation during which total biomass, ammonium and gas were analysed. Re-feeding using $^{15}$N-substrates was required in $^{15}$N-nitrate cultures because protein incorporation rates were very slow while nitrate reduction was relatively fast (protein incorporation rates are displayed in Table S2). All measurements to calculate rates, except protein incorporation rates, were conducted before re-feeding the batch reactors. Details of the re-feeds and medium exchanges are provided in Table S1.

To demonstrate methane oxidation to $CO_2$ in the presence of nitrate (AOM with nitrate as electron acceptor), a second batch reactor experiment with 10% (v/v) $^{13}$C-labeled or unlabeled methane and $^{15}$N-nitrate (5 mM) was carried out, with 17 mM sulfate or without sulfate. This experiment is termed 'double label experiment' in the following.

## Analyses of inorganic parameters

Sulfide concentrations were determined photometrically by the formation of copper sulfide which was measured biweekly (Cord-Ruwisch, 1985). Ammonium, nitrate and nitrite were detected using Merckoquan quick tests (Merck, Germany). Nitrate and nitrite reduction were measured weekly during the first month of incubation but reduction rates were determined by measuring $^{15}$N-dinitrogen after 9 days and $^{15}$N-ammonium after the first 14 days. To determine $^{15}$N-ammonium in the supernatants, 2 mL culture broths were centrifuged, the supernatants were filtered through 0.45-μm polytetrafluoroethylene filters (Sartorius, Germany), acidified with 3.5% (w/v) HCl, final concentration, and evaporated at 60 °C overnight under vacuum. The $^{15}$N-enriched remainder was diluted (*c.* 1 : 1) using a known amount of unlabeled ammonium sulfate and then weighed in tin capsules to obtain a total of *c.* 2–3 mg. The powder was combusted in tin capsules at 1020 °C in a Euro EA Elemental Analyzer (Eurovector, Italy) directly coupled via a ConFlo III-interface (Thermo Fisher Scientific, Germany) to an isotope ratio mass spectrometer (IRMS, MAT 253, Thermo Fisher Scientific). $^{15}$N-incorporation for all products was achieved when the 300-fold magnified $^{30}N_2$ mass peak area was greater than the $^{28}N_2$ mass peak area in the IRMS. The analyzed supernatant was free from $^{15}$N-nitrate, which was tested using Merckoquant nitrate tests. The detection limit of 0.12 mM was 2.4% of the 5 mM $Na^{15}NO_3$ added to the medium. It was therefore less than one order of magnitude lower than the average total $^{15}$N concentration (26%) in the supernatant of the reactors tested for DNRA. Turnover rates were calculated as described in the Supporting Information. In principle, all $^{15}$N atoms in the analyte were considered to be metabolic products of the investigated substrate and subtracted from the unlabeled background.

For the double labeling experiment, $^{15}$N-nitrate reduction to $^{15}$N-dinitrogen was measured after 49 days by headspace sampling. $^{13}$C-methane oxidation to $^{13}CO_2$ was measured after 79 days. The separation of $CO_2$ from the labeled methane by precipitation in a saturated BaOH solution was required for a reliable $\delta^{13}C$ determination of the headspace $CO_2$ (Siegert *et al.*, 2011). For all isotope measurements, the mean of the resulting $\delta^{13}C$ or $\delta^{15}N$ values was calculated within 95% confidence intervals of four individual batch cultures when available. Unlabeled methane and dead controls were prepared as single incubations.

## Protein analyses

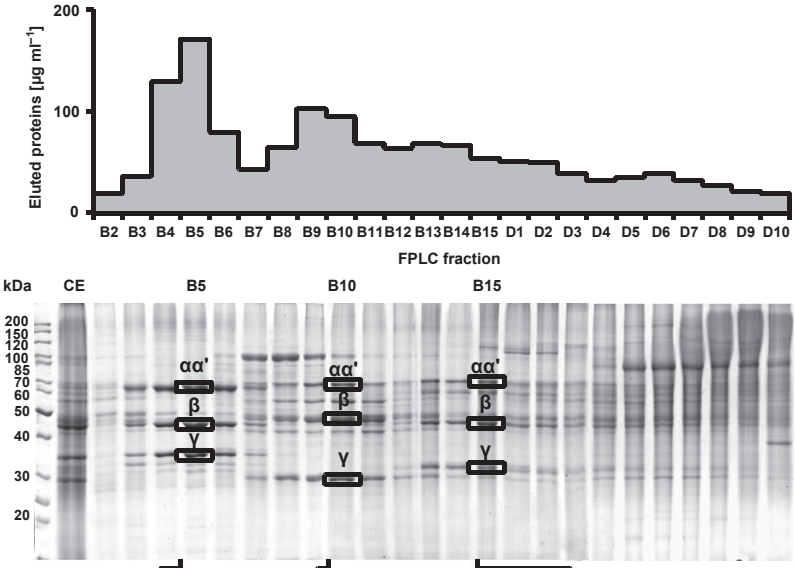
The extraction and purification of mat proteins was conducted according to a modified protocol of Basen *et al.* (2011). Proteins were extracted from 2 g wet mat material by triplicate ultra-sonication on ice for 10 min each, after suspension of the mat material in 50 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS)/KOH

buffer (pH 7). The obtained homogenate was centrifuged for 15 min at 10 000 ***g*** and 4 °C. The supernatant was again centrifuged for 1 h at 120 000 ***g*** and 4 °C. Protein fractions were obtained by fast protein liquid chromatography (FPLC) separation in an ÄKTA purifier 10 (GE Healthcare) using a HiTrap Q HP 5 mL column (GE Healthcare). The instrument was operated at 1 mL $min^{-1}$ using a 50 mM MOPS/KOH buffer at pH 7. The NaCl gradient was increased over 84 min from 0.2 to 0.6 M.

To identify MCR proteins, 10 μL of each FPLC fraction were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein bands of interest were excised from gel lanes. In-gel tryptic cleavage was performed as described previously (Jehmlich *et al.*, 2009). The highest purity of MCR subunits was found in lane B5 (Fig. 2). Thus, these subunits were operationally named B5-MCR. Peptides were reconstituted in 0.1% (w/v) formic acid. To identify target proteins, a nano ultra-performance liquid chromatograph (nano-UPLC) coupled LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific) was used as described by Bastida *et al.* (2010). Chromatography was performed by using 0.1% (w/v) formic acid in solvents A (100% water) and B (100% acetonitrile), with peptides eluted over 30 min with a 8–40% solvent B gradient using a nano-UPLC system (nanoAcquity, Waters, Germany) coupled to the mass spectrometer. Continuous scanning of eluted peptide ions was carried out between 150 and 2000 *m/z*, automatically switching to MS/MS collision-induced dissociation mode on ions exceeding an intensity of 3000. The raw data were processed for database search as described by Bastida *et al.* (2010) using *Bacteria* and *Archaea* sequence entries of NCBInr (National Center for Biotechnology Information) as criteria for taxonomic identification.

## Analyses of isotope incorporation into proteins

The detection of $^{15}N$ in the proteins by LTQ Orbitrap MS (Jehmlich *et al.*, 2008; Bastida *et al.*, 2010) failed due to the low label incorporation rates of the mats. In our




| excised band | B5-1 | B10-1 | B15-1 |
|---|---|---|---|
| GI no | 40217436 | 34223964 | 52549158 |
| reference | Krüger et al. 2003 | Hallam et al. 2003 | Hallam et al. 2004 |
| subunit | alpha | alpha | alpha |
| next relative isolate | n/a | n/a | n/a |
| excised band | B5-2 | B10-2 | B15-2 |
| GI no | 40217434 | 116753885 | 34305112 |
| reference | Krüger et al. 2003 | Copeland et al. unpublished | Hallam et al. 2003 |
| subunit | beta | beta | beta |
| next relative isolate | n/a | Methanosaeta thermophila PT | Methanosarcina mazei |
| excised band | B5-3 | B10-3 | B15-3 |
| GI no | 40217435 | 10946205 | 52549157 |
| reference | Krüger et al. 2003 | Gosh et al. unpublished | excised band |
| subunit | gamma | gamma | gamma |
| next relative isolate | n/a | Methanosarcina barkeri | n/a |

**Fig. 2.** FPLC elution profile (top) and SDS-PAGE chromatography (centre) of the soluble fraction of Black Sea black mats. MCR-containing bands are highlighted. The NaCl gradient was increased over 84 min from 0.2 to 0.6 M. Database hits against *Archaea* are indicated in the table (bottom). The genome of *Methanosarcina mazei* was published by Deppenmeier *et al.* (2002). Note that protein concentrations refer to FPLC-eluted proteins and are not cytosolic concentrations. Unpublished data were obtained by database access (NCBI) only. The subunits are indicated with Greek letters. CE, crude extract before FPLC fractionation; n/a, not applicable because no cultured isolate was found in the database

case, the minimum detectable incorporation was *c.* 5 at.% $^{15}$N (Jehmlich *et al.*, 2010). Therefore, we used an alternative method. Precipitated, nearly pure MCR subunits were analyzed for their isotopic composition in an elemental analyser (EA) as described above. The detection limit was below 0.1 at.% $^{15}$N incorporation.

FPLC fractions (2 mL each) and total soluble protein extracts (500 μg) were precipitated using 20% (v/v) trichloroacetic acid by incubation at 4 °C overnight. After centrifugation (13 000 ***g***, 4 °C, and 15 min), pellets were dissolved with 200 μL molecular-grade water and the 5-fold volume (1 mL) of 100% cold acetone was added. After incubation for 15 min, protein pellets were obtained by centrifugation as described above. Acetone was removed and the remainders were dried for 1 h at 37 °C. Protein pellets were gently dissolved in 40 μL molecular grade water and transferred into tin capsules. Then, 10 μL of pure acetone was added and the liquids were evaporated for 3 h at 60 °C. Dry protein pellets were analyzed by combustion of the sample in tin capsules in an EA similar to total biomass and ammonium measurements (Jung *et al.*, 1997).

$^{15}$N-incorporation rates into MCR-dominated FPLC fractions (identified by SDS-PAGE and LC-MS/MS analyses, see above) were calculated using the $\delta^{15}$N values compared with a respective dead control (see Supporting Information). In the case of MCR proteins, rates were not calculated in moles because the exact concentration of MCR subunits in a semipurified MCR fraction was unknown. However, the proportion of newly synthesized MCR proteins relative to all extracted MCR proteins (i.e. present before the experiment) per day could be estimated. This value is expressed as a percentage of all respective MCR subunits per day (% day$^{-1}$). Absolute rates expressed in ng cm$^{-3}$ day$^{-1}$ were estimated as described in the Supporting Information. A detailed description of how isotopic values were used to calculate rates is given in the Supporting Information.

### Statistical analysis

When given, errors are standard deviations of four individual reactors or samples. Errors of gene copy numbers are standard deviations of three individual measurements. Errors of samples within 95% confidence intervals are maxima and minima. To test the null hypotheses, heteroscedastic one-tailed Student's *t*-tests were used. The null hypothesis was that a significant difference between the two populations tested did not exist. A resulting *P*-value $> 0.05$ was assumed to confirm a null hypothesis (no difference) and a *P*-value $\leq 0.05$ was defined as indicating a significant difference.

## Results

### Sulfate reduction and methane oxidation

AOM is coupled to sulfate reduction in the AOM mats of the Black Sea. Sulfide formation with or without methane was observed only in incubations with $^{15}$N-dinitrogen or $^{15}$N-ammonium (Table 1). In the pink mats, sulfate reduction to sulfide was driven by methane but with lower rates. While the presence of methane had no effect in $^{15}$N-dinitrogen incubations, it accelerated sulfide production in $^{15}$N-ammonium incubations four-fold (Table 1). $^{15}$N-nitrate reduction inhibited sulfide production irreversibly. No sulfide release was detected until the end of the experiment, even after all nitrate was depleted.

To show that methane oxidation to $CO_2$ is linked to nitrate reduction via sulfate reduction, we carried out a double labeling experiment with $^{13}$C-labeled methane as electron donor and $^{15}$N-nitrate as electron acceptor – both in the presence and in the absence of sulfate. In this experiment, $^{13}$C-methane was oxidized to $^{13}CO_2$ and $^{15}$N-nitrate was reduced to $^{15}$N-dinitrogen, showing that methane was oxidized to $CO_2$ with nitrate as electron acceptor (Table 2). This was not the case in killed incubations or incubations without sulfate, indicating that sulfate was required for AOM with nitrate.

### Distribution of ANME-1 and ANME-2 communities

We used qPCR assays targeting *mcr*A genes to estimate the distribution of the ANME-1 and ANME-2 communities in the AOM mats of the Black Sea (see Supporting Information). In the pink mats, ANME-1 *mcr*A genes were present at $5 \times 10^7 \pm 6 \times 10^6$ copies cm$^{-3}$ more than three orders of magnitude more abundant than ANME-2 *mcr*A genes at $1 \times 10^4 \pm 9 \times 10^2$ copies cm$^{-3}$. In the black mats, the difference in gene copies was less than one order of magnitude, with $2 \times 10^6 \pm 2 \times 10^5$ copies cm$^{-3}$ of ANME-1 *mcr*A genes compared with $6 \times 10^5 \pm 6 \times 10^4$ copies cm$^{-3}$ of ANME-2 *mcr*A-genes.

### Microbial turnover of $^{15}$N substrates

#### $^{15}$N-nitrate reduction

To test the effect of AOM-coupled sulfate reduction on nitrate reduction, $^{15}$N-nitrate was added to reactors containing (or lacking) sulfate or methane. $^{15}$N-nitrate-derived nitrogen was incorporated into the biomass (Table 1). $^{15}$N-nitrate was also reduced to ammonium (DNRA) and dinitrogen in all reactors, except the killed controls. When methane and sulfate were added,

**Table 1.** Overview of the experimental setup and corresponding results

| Mat | Substrates | | Sulfide produced | | | Total biomass $^{15}$N incorp. | | | $^{15}$N-ammonium produced | | | $^{15}$N-dinitrogen produced | | | Total $^{15}$N-nitrate reduction | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Rate ($\mu$mol cm$^{-3}$ day$^{-1}$) | Error$_n$ | $n$ | Rate (nmol N cm$^{-3}$ day$^{-1}$) | Error$_n$ | $n$ | Rate (nmol N cm$^{-3}$ day$^{-1}$) | Error$_n$ | $n$ | Rate (nmol N cm$^{-3}$ day$^{-1}$) | Error$_n$ | $n$ | Rate [nmol N cm$^{-3}$ d$^{-1}$] | Error$_n$ | $n$ |
| Black | Nitrate | | | | | | | | | | | | | | | | |
| | − Sulfate | No methane | −0.101 | ±0.098 | 2 | 113 | ±12 | 2 | 203 | +148/−101 | 3 | 107 | ±1 | 3 | 310 | +148/−101 | 3 |
| | | Methane | −0.048 | +0.054/−0.103 | 3 | 194 | ±8 | 2 | 225 | +34/−20 | 3 | 104 | ±6 | 2 | 329 | +34/−20 | 3 |
| | + Sulfate | No methane | −0.043 | ±0.107 | 2 | 88 | ±2 | 2 | 187 | ±6 | 2 | 132 | +17/−19 | 4 | 319 | +17/−19 | 4 |
| | | Methane | −0.044 | +0.036/−0.040 | 3 | 323 | +33/−18 | 3 | 148 | ±5 | 2 | 632 | ±185 | 2 | 780 | ±185 | 2 |
| | Ammonium | | | | | | | | | | | | | | | | |
| | − Sulfate | No methane | −0.197 | ±0.011 | 2 | 16 | +11/−13 | 3 | | | | | | | | | |
| | | Methane | −0.243 | +0.009/−0.015 | 3 | 76 | ±10 | 2 | | | | | | | | | |
| | + Sulfate | No methane | 0.288 | +0.127/−0.106 | 3 | 123 | ±21 | 2 | | | | | | | | | |
| | | Methane | 1.247 | ±0.079 | 2 | 177 | ±3 | 2 | | | | | | | | | |
| | Dinitrogen | | | | | | | | | | | | | | | | |
| | − Sulfate | No methane | −0.112 | +0.005/−0.006 | 3 | 0.021 | +0.006/−0.004 | 3 | 1.01 | +0.06/−0.07 | 3 | | | | | | |
| | | Methane | −0.089 | +0.015/−0.022 | 3 | 0.098 | ±0.014 | 2 | 1.09 | +0.16/−0.11 | 3 | | | | | | |
| | + Sulfate | No methane | 0.375 | ±0.001 | 2 | 0.029 | ±0.007 | 2 | 0.75 | +0.12/−0.17 | 3 | | | | | | |
| | | Methane | 0.405 | ±0.056 | 2 | 0.133 | ±0.012 | 2 | 0.58 | +0.05/−0.03 | 3 | | | | | | |
| Pink | Ammonium | | | | | | | | | | | | | | | | |
| | + Sulfate | No methane | 0.078 | n/a | 1 | | | | | | | | | | | | |
| | | Methane | 0.589 | +0.046/−0.044 | 4 | | | | | | | | | | | | |

The mean was calculated from $n$ reactor values within 95% confidence limits of (2$\sigma$) the standard deviation. Total nitrate reduction is the sum of DNRA and nitrate reduction to dinitrogen where the error is the highest error of both. The original number of reactors was four. Error margins are high and low values and not standard deviations. Data from all four reactors are presented in Table S6.

**Table 2.** Anaerobic oxidation of $^{13}C$-methane in the presence of $^{15}N$-nitrate, and the recovery of label in the headspace

| Incubation time (days) | | 79 | | | 49 | |
|---|---|---|---|---|---|---|
| Substrates | $\delta^{13}C_{CO_2}$ (‰ vs. VPDB) | Error$_n$ | $n$ | $\delta^{15}N_{N_2}$ (‰ vs. air) | Error$_n$ | $n$ |
| $-SO_4^{2-}$ | | | | | | |
| $^{13}CH_4$ | −8.8 | −4.4/+2.8 | 3 | 2591 | ±44 | 2 |
| $^{12}CH_4$ | −21.4 | n/a | n/a | 2007 | n/a | n/a |
| Killed | 9.6 | n/a | n/a | 64 | n/a | n/a |
| $+SO_4^{2-}$ | | | | | | |
| $^{13}CH_4$ | 287.0 | ±5.25 | 2 | 2370 | ±516 | 2 |
| $^{12}CH_4$ | −18.2 | n/a | n/a | 2893 | n/a | n/a |
| Killed | 18.5 | n/a | n/a | 43 | n/a | n/a |

Errors are maximum and minimum deviations from the mean of $n$ samples within 95% confidence limits of (2σ) standard deviation, calculated from quadruplet incubations. $n$ is the number of incubations within these confidence intervals. n/a, not applicable because single incubations were set up. VPDB, Vienna PeeDee Belemnite. Killed incubations were killed with formaldehyde.

$^{15}N$-nitrate-derived nitrogen incorporation rates were significantly elevated compared with incubations where sulfate or methane or both substrates were missing. The $P$-values of heteroscedastic one-tailed Student's $t$-tests were 0.002, 0.0005, and 0.0002, respectively. Sulfate alone did not increase $^{15}N$-nitrate-derived nitrogen incorporation rates (Table 1). However, the addition of methane alone (no sulfate present) did significantly stimulate incorporation rates compared with incubations lacking only methane or methane and sulfate ($P = 0.0001$ and 0.0009, respectively). In the case of nitrate reduction to dinitrogen, $^{15}N_2$-production rates were significantly higher when sulfate and methane were added compared with the other incubations (all $P = 0.02$). Sulfate alone (no methane present) increased rates of nitrate reduction to dinitrogen ($P = 0.04$). For the addition of methane alone, such an increase was not observed.

DNRA rates were not significantly elevated when sulfate and methane were added (Table 1). Student's $t$-test $P$-values were 0.07 or 0.13, for only sulfate or neither substrate, respectively. $^{15}N$-ammonium formation rates were significantly higher than the biomass incorporation rates, implying that the source of the released ammonium was supplemented nitrate and not biomass. DNRA rates were significantly lower than biomass incorporation rates only when sulfate and methane were added ($P = 0.0006$ between $^{15}N$-biomass incorporation and $^{15}N$-ammonium release). Thus, DNRA was not the dominating nitrate-reducing process when AOM coupled to sulfate reduction was driving the system. In contrast, $^{15}N$-ammonium release was significantly higher than $^{15}N$-biomass incorporation rates when only sulfate but no methane was added (Table 1). This indicates biomass decomposition. The $^{15}N$-ammonium release rate was significantly lower when methane and sulfate were added compared with the incubations that had methane only ($P = 0.04$). All other differences among the DNRA incubations were not significant (all $P > 0.08$). Notably, there was no significant difference between the incubations which contained methane and sulfate compared with the incubation that had sulfate only. The total $^{15}N$-nitrate turnover rate (the sum of total biomass incorporation, ammonium and dinitrogen release) doubled from on average 451 nmol N cm$^{-3}$ day$^{-1}$ for the incubations lacking either sulfate, methane or both to 1103 nmol N cm$^{-3}$ day$^{-1}$ for the incubations with sulfate and methane (Table 1). The ratio between the three ways of nitrate conversion – assimilatory nitrate reduction, DNRA and nitrate reduction to dinitrogen – changed from *c.* 1.0 : 1.8 : 0.9 (without methane and without sulfate) to 1.0 : 0.5 : 2.0 (with sulfate and methane).

### *$^{15}N$-ammonium turnover*

All incubations supplemented with $^{15}N$-ammonium incorporated the label into the biomass (Table 1). Based on photometric ammonium tests, total ammonium (undifferentiated $^{15}N$ and $^{14}N$) was excreted into the medium at different rates (Fig. 3). The addition of sulfate and methane significantly accelerated the incorporation of

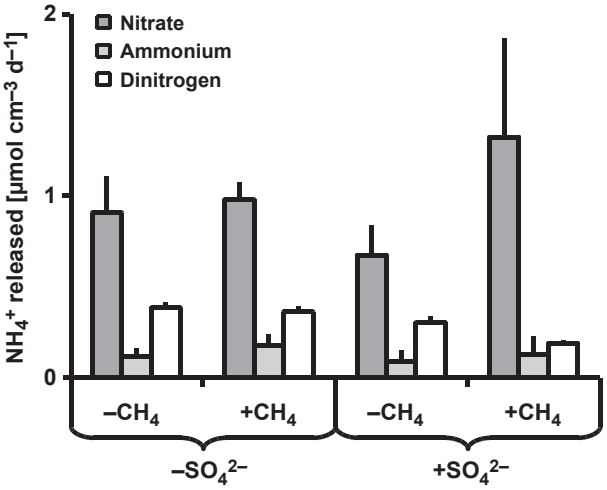



**Fig. 3.** Total ($^{14}N$ and $^{15}N$) ammonium release. The ammonium concentrations were measured using quantitative photometric quick tests and include $^{15}N$- and $^{14}N$-ammonium. All substrates were $^{15}N$-labeled. The ammonium concentrations from these total ammonium measurements were used to calculate the fraction of $^{15}N$-ammonium in $^{15}N$-nitrate and $^{15}N$-dinitrogen incubations (see also Supporting Information). Error bars are (2σ) standard deviations of four individual reactors.

$^{15}$N-ammonium into the biomass (Table 1). Student's *t*-tests for the incubations with sulfate and methane against incubations without sulfate or methane, as well as without both substrates, resulted in *P*-values of 0.0001, 0.04 and 0.002, respectively. Sulfate alone (no methane present) significantly stimulated $^{15}$N-ammonium incorporation rates compared with incubations without sulfate and without methane ($P = 0.02$). This was different for incubations without sulfate in which only methane was present (when compared with incubation with sulfate alone, $P = 0.07$). Nonetheless, ammonium was not oxidized under the conditions tested. Neither $^{15}$N-dinitrogen nor $^{15}$N-nitrate were produced in the presence of the electron acceptors sulfate, ferrihydrite or manganese dioxide (data in Table S1). However, sulfide was formed in incubations with ammonium but without methane, indicating biomass decomposition (Table 1). Reduced iron or manganese were not detected. While $^{15}$N-nitrite was reduced to $^{15}$N-dinitrogen (both atoms labeled), ANAMMOX coupled to nitrite reduction (only one atom labeled) was not observed. These $\delta^{15}$N values could not be calculated exactly because the mass 30 was too high.

### $^{15}$N-dinitrogen fixation (diazotrophy)

On average, $^{15}$N-dinitrogen fixation rates were three orders of magnitude lower than incorporation rates of $^{15}$N-nitrate-derived nitrogen and $^{15}$N-ammonium (Table 1). The addition of sulfate and methane significantly stimulated the $^{15}$N-dinitrogen fixation rates. Student's *t*-test *P*-values against incubations lacking sulfate, methane or both substrates were 0.02, 0.0001 and 0.0001, respectively. The addition of sulfate alone (no methane present) did not accelerate $^{15}$N-dinitrogen fixation rates significantly compared with incubations without methane and sulfate ($P = 0.5$). However, when methane was added without sulfate, the incorporation rates were significantly higher compared with incubations that did not contain methane ($P = 0.001$).

### Incorporation of $^{15}$N into MCR subunits

Methyl coenzyme M reductase enzymes are essential for the methane-oxidizing mats because they catalyze the first step of AOM (Krüger *et al.*, 2003; Scheller *et al.*, 2010). Therefore, $^{15}$N-incorporation into MCR subunits was examined. A greater variation of the $\delta^{15}$N-values in MCR enzymes in incubations without labeled substrates (–11.0‰ to 16.5‰) compared with total proteins (–5.6‰ to 2.8‰) was found (Table S3). Several types of MCR subunits were identified (Fig. 2). To estimate MCR synthesis rates, the fraction B5 and its neighboring fractions, containing the highest concentration of MCR subunits, were selected. $^{15}$N-incorporation into B5-MCR proteins derived from $^{15}$N-ammonium was exclusively found in the black mats ($\delta^{15}$N 1759‰), but not in the pink mats ($\delta^{15}$N 9.0‰). Therefore, only the black mats were investigated further. $^{15}$N-nitrate and $^{15}$N-ammonium were utilized for B5-MCR synthesis at comparable rates (Fig. 4). Exceptions were the incubations with methane but without sulfate, where the incorporation rates were five times higher for $^{15}$N-nitrate-derived nitrogen than for $^{15}$N-ammonium. This effect was similar when $^{15}$N-substrates were considered to be the only nitrogen source, compared with the mix of $^{14}$N and $^{15}$N as it naturally occurs in the biomass (for a further explanation of these two models, see the Supporting Information).

## Discussion

The results of this study revealed the most important nitrogen sources in AOM mats of the Black Sea by quantifying the turnover rates for three $^{15}$N-labeled nitrogen species. Nitrate reduction and ammonium assimilation dominated the overall nitrogen cycle of these AOM mats while dinitrogen fixation was negligible. We demonstrated *de novo* synthesis of proteins and MCR enzymes in these mats under laboratory conditions.

### Nitrogen cycling

#### Nitrate reduction

The assimilation of $^{15}$N-derived from $^{15}$N-nitrate into biomass was usually at least twice the rate of $^{15}$N-ammonium

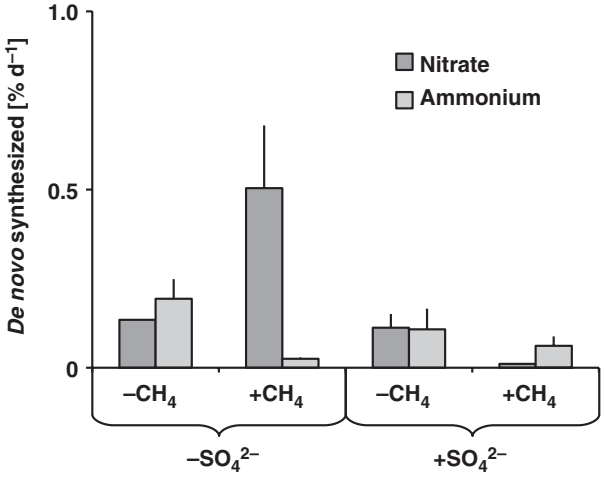



**Fig. 4.** Relative synthesis rates of the MCR subunits taken from an FPLC fraction where these subunits were dominant (see Fig. 2). Rate calculations are described in the Supporting Information. Error bars are standard deviations of rates calculated individually from $\delta^{15}$N values of whole FPLC fractions that contained the B5-MCR subunits. These were usually one or two preceding or successive FPLC fractions, e.g. fraction B4 (for $\delta^{15}$N values of these fractions see Table S4).

assimilation (Table 1), except for the incubations with sulfate but without methane. Furthermore, $^{15}$N-nitrate was reduced to dissolved $^{15}$N-ammonium and gaseous $^{15}$N-dinitrogen. Nitrite, an intermediate of nitrate reduction, was also reduced to dinitrogen in the ANAMMOX test (Table S1). Simultaneously, $^{13}$C-methane was oxidized to $^{13}CO_2$ in the presence of sulfate in a double label experiment with $^{15}$N-nitrate (Table 2). When sulfate was not given to the reactors no $^{13}CO_2$ enrichment was observed. When measured after 9 days, rates of nitrate reduction to dinitrogen were six times higher in reactors supplemented with sulfate and methane (Table 1, all $P = 0.02$). Sulfate was evidently required for nitrate reduction to dinitrogen when coupled to AOM (Table 2), which indicates that AOM and nitrate reduction were coupled in short-term reactions. After 49 days, $^{15}N_2$-enrichment was observed in all $^{15}$N-nitrate incubations, showing that mat decay contributed to nitrate reduction to dinitrogen and that both processes occurred concomitantly.

Sulfate had a stimulating effect on nitrate reduction, indicating a coupling with AOM. Nitrate reduction presumably required the presence of reduced sulfur compounds, perhaps sulfides (Eisenmann *et al.*, 1995; Fossing *et al.*, 1995). Thauer (2011) suggested that (organic) sulfur compounds might be electron shuttles in microbial AOM consortia, and our results support the hypothesis of a sulfur-based electron shuttle in the Black Sea mats. In our experiments, inorganic sulfide was not detected in nitrate incubations (Table 1). A plausible explanation for the absence of detectable sulfide is the oxidation of sulfide to sulfate with nitrate as electron acceptor and the subsequent re-reduction in the AOM process. An intermediate reaction may be polysulfide dismutation (Milucka *et al.*, 2012). The oxidation of intermediate polysulfide (zero-valent sulfur) to sulfate with nitrate as electron acceptor may have occurred before the polysulfide was passed to dismutating partners that release sulfide. This could suggest that ANME archaea possess the ability to reduce nitrate, which is in good agreement with the observed *de novo* synthesis of MCR enzymes in the absence of sulfate (Fig. 4 and Supporting Information). Zero-valent sulfur may have acted as a permanent sulfur sink and redox mediator. Indeed, the oxidation of zero-valent sulfur with nitrate is highly exergonic under standard conditions and energetically favored compared with polysulfide dismutation (Table 3). The discovery of polysulfide dismutation in samples from the Mediterranean mud volcano Isis was possible because in these samples the stoichiometry of methane oxidation and sulfate reduction was not exactly 1-to-1 (Milucka *et al.*, 2012). In our Black Sea mats, the stoichiometry was exactly 1-to-1 as determined by radiotracer experiments (Michaelis *et al.*, 2002), supporting a direct reduction to sulfide without polysulfide as intermediate. This sulfide was probably the electron donor for nitrate reduction. In conclusion, our results suggest that nitrate reduction depends on supply of reduced sulfur compounds acting as electron shuttles in which AOM is the energy-providing process.

**Table 3.** Energy gains under physiological standard conditions for anaerobic oxidation of zero-valent sulfur by nitrate compared with the dismutation of $HS_2^-$

| Net reaction | $\Delta G^o$/mole S (kJ mol$^{-1}$) |
|---|---|
| $3NO_3^- + 4S^0 + 7H_2O \rightarrow 3NH_4^+ + 4SO_4^{2-} + 2H^+$ | −325.5 |
| $6NO_3^- + 5S^0 + 2H_2O \rightarrow 3N_2 + 5SO_4^{2-} + 4H^+$ | −548.0 |
| $4HS_2^- + 4H_2O \rightarrow SO_4^{2-} + 7HS^- + 5H^+$ | −25.4 |

The energy gain for $HS_2^-$ dismutation was taken from Milucka *et al.* (2012).

It is noteworthy that $C_1$ carbon compounds can be excluded as electron shuttles in AOM systems such as our Black Sea mats (Nauhaus *et al.*, 2005; Wegener *et al.*, 2008; Meulepas *et al.*, 2010). Nitrate reduction was also observed in the absence of sulfate but was not linked to AOM (Tables 1 and 2). This suggests that mechanisms of electron transport from AOM to nitrate such as direct electron transfer without molecular diffusion or transport, as shown for *Geobacter*/*Pelobacter* consortia (Summers *et al.*, 2010) or for microbial corrosion producing iron sulfides (Enning *et al.*, 2012), are unlikely.

Recently, it has been reported that nitrate reduction coupled to biomass decomposition occurred at high rates at cold seeps in the Gulf of Mexico (Bowles & Joye, 2011). Nitrate reduction without methane played a role in our experiments as well. The $^{15}$N-ammonium release rate was higher than incorporation of $^{15}$N-nitrate-derived nitrogen with sulfate but without methane (Table 1). This indicates biomass decomposition in the absence of methane. However, the previously reported rates of 11–32 nmol cm$^{-3}$ day$^{-1}$ were approximately one order of magnitude lower in the Gulf of Mexico than in our Black Sea mats. Our mats formed a thick biofilm containing complex carbon compounds. Moreover, in the absence of nitrate, ammonium was released even when it had been added to the medium (Fig. 3). This is indicative of biomass decomposition. In contrast, DNRA rates were not significantly faster when methane and sulfate were added (Table 1). In this case it was nitrate reduction to dinitrogen. Under non-AOM conditions (either sulfate or methane or both were missing), DNRA rates exceeded incorporation rates (Table 1). This means that biomass decomposition was faster under non-AOM conditions and coupled to DNRA. Under AOM conditions (methane and sulfate

present), the significantly higher $^{15}N$ biomass incorporation rates in incubations compared with DNRA ($P = 0.0006$) indicate that $^{15}N$-nitrate-derived nitrogen was used for biomass synthesis. In conclusion, biomass decomposition and AOM were concurrent processes during nitrate reduction. This agrees well with the observations in the Gulf of Mexico (Bowles & Joye, 2011).

## Ammonium assimilation

All energy conservation requires a redox gradient from a more negative to a more positive redox potential. This is the case during AOM when coupled to sulfate reduction with an approximate theoretical energy gain of −32 kJ per mole methane. Methane is the most important energy source for the Black Sea mats but it requires an electron acceptor. The mats are adapted to sulfate reduction as electron-accepting process. Here, we elaborated lines of evidence that nitrate reduction was coupled to sulfate reduction when methane was the energy source (Tables 1–3). When organic matter of the mats was the energy source (no methane present), sulfate reduction was required as an intermediate process. That is, in the presence of ammonium and sulfate without methane, sulfide was produced by the mats (Table 1). We may conclude that ammonium was the electron donor for sulfate reduction, as was suspected in marine sediment cores (Schrum *et al.*, 2009; Wehrmann *et al.*, 2011). However, all tests for anaerobic ammonium oxidation ruled out ammonium as energy source. None of the electron acceptors tested [nitrite, manganese(IV), iron(III) and sulfate] resulted in $^{30}N_2$ formation from $^{15}N$-ammonium (Table S1). This indicates that ammonium was exclusively used for the mats' anabolism.

A stimulating effect of methane on assimilatory nitrate reduction and on ammonium incorporation was observed (Table 1). Ammonium assimilation was also significantly dependent on the presence of sulfate. This dependence of ammonium uptake on sulfate may have been the result of overall faster metabolic turnover rates, most likely driven by growth of sulfate-reducing microorganisms. AOM-coupled sulfate reduction could have potentially been limited by reduced ammonium supply. This ammonium limitation might occur *in situ* spatially or temporarily restricted, especially when AOM rates are higher under strongly elevated methane partial pressures (*in situ c.* 200 times higher) resulting in higher metabolic and growth rates than in our batch experiments. In our experiments, ammonium was never limiting, probably due to biomass decomposition (Fig. 3). When sufficient biomass is available *in situ* as well, this situation can be extrapolated to the natural habitat.

## $^{15}N$-dinitrogen fixation (diazotrophy)

Compared with $^{15}N$-ammonium incorporation rates and assimilatory $^{15}N$-nitrate reduction rates, the $^{15}N$-dinitrogen fixation rates were almost three orders of magnitude lower and therefore not relevant as nitrogen source (Table 1). This is also reflected in the very low label recovery from soluble proteins in $^{15}N$-dinitrogen incubations (Table S4). At these low rates, diazotrophy depended on AOM-coupled sulfate reduction (Table 1). The diazotrophic rate increased four-fold with sulfate and methane present compared with incubations lacking both. Diazotrophy is highly energy consuming, with an investment of 16 moles ATP to produce 1 mole of ammonium. Therefore, it is not surprising that a biological system operating close to the energetic limit has reserved this capacity only for nitrogen-limited conditions.

The wet mats had a nitrogen content of 2.1% (w/w). The ammonium concentrations in the diazotrophic incubations were between 1 and 6 mM (17–102 mg $L^{-1}$). Hence, biomass decay was sufficient to provide enough ammonium for the mats' N-pool to inhibit diazotrophy (Kessler *et al.*, 2001). Since biomass decomposing processes constantly released ammonium (Fig. 3), the ammonium concentrations could not be decreased experimentally to concentrations where N became limiting, thus favoring diazotrophy. Nevertheless, *nif*H-homologues – marker genes indicating the potential to synthesize diazotrophic nitrogenases – were detected in the Black Sea mats but the *nif*I (also *gln*B) ammonium sensor-encoding genes (Kessler *et al.*, 2001) have not been reported in a metagenomic analysis of the Black Sea mats (Meyerdierks *et al.*, 2010). While it may also be the case that the metagenome was incomplete and that the ammonium reporters were not detected, the absence of this ammonium reporter is, however, not indicative for the absence of ammonium inhibition on diazotrophy. In contrast, it could explain why diazotrophy was observed at all, although at low rates. Moreover, it seems possible that dinitrogen-fixing cells naturally inhabit an ecological niche within their respective consortium. In such a niche other nitrogen sources, such as biomass, ammonium, or nitrate, could have been depleted, for example within methane and/or dinitrogen-rich fluids or inside cavities of carbonate precipitations. Ammonium limitation can also occur because it precipitates with bicarbonate or reacts with the biomass. As a result, inert dinitrogen may diffuse faster than ammonium, making dinitrogen more easily available. Since sufficient nitrogen supply is essential for any metabolic activity, diazotrophy could be an emergency survival strategy.

### Synthesis of MCR enzymes

It is commonly accepted that consortia of ANME *Archaea* and *Bacteria* of the *Desulfosarcina/Desulfococcus* cluster perform AOM (Knittel & Boetius, 2009). ANME *Archaea* oxidize methane using MCR enzymes (Shima *et al.*, 2012). *Desulfosarcina/Desulfococcus* clusters of the Black Sea mats release sulfide from AOM-coupled sulfate reduction in 1-to-1 stoichiometry (Michaelis *et al.*, 2002). In AOM consortia of the Mediterranean mud volcano Isis, intermediate polysulfide dismutation was the energy-conserving process in *Desulfosarcina/Desulfococcus* clusters and the stoichimetry was < 1 mole methane oxidized per mole sulfide produced (Milucka *et al.*, 2012). The relation of ANME-2 to ANME-1 *mcr*A gene copies was 1 : 3500 in the pink Black Sea mats compared with 1 : 3 in the black mats. While this indicates that both types of MCR were produced in the mats, ANME-2 MCR proteins were presumably more abundant in the black mats than in the pink mats. The AOM activity of both mat types was measured via sulfate reduction rates (measured as sulfide production) because AOM and sulfate reduction in the Black Sea mats are linked by a 1-to-1 stoichiometry (Michaelis *et al.*, 2002). Higher AOM activity was found in the black mats (Table 1). Accelerated $^{15}$N-incorporation rates in the black mats were probably a result of their higher AOM and sulfate reduction activity.

We used the key enzyme of AOM, MCR, to demonstrate its involvement in the nitrogen cycle. Because this enzyme was highly abundant in our Black Sea mats (Fig. 2), this allowed us to provide direct evidence for the coupling of AOM to nitrogen assimilation and growth. MCR enzymes are the only enzymes reported to catalyze AOM directly (Krüger *et al.*, 2003; Scheller *et al.*, 2010; Shima *et al.*, 2012). Three different main types of MCR enzymes were found in the Black Sea mats (Fig. 2). Because only enzymes of lane B5 were pure enough for isotopic measurements, we further examined these B5-MCR subunits. This type of MCR was previously reported to account for up to 7% of the extracted proteins of the Black Sea AOM mats (Krüger *et al.*, 2003). Besides providing qualitative evidence for $^{15}$N-nitrogen incorporation into the B5-MCR subunits, we intended to estimate *de novo* synthesis rates of this protein. Relative incorporation rates into B5-MCR enzymes – an indicator for the *de novo* synthesis of this protein – were highest when methane was added without sulfate (Fig. 4): for possible reasons why see the Supporting Information.

$^{15}$N-nitrate-derived nitrogen was incorporated in MCR enzymes in all nitrate-reducing incubations (Fig. 4). This showed that MCR proteins were the methane-oxidizing enzymes under nitrate-reducing conditions with sulfate required. Further support for sulfate reduction as an intermediate step comes from a recent finding that ANME archaea accumulated reduced zero-valent polysulfides derived from sulfate (Milucka *et al.*, 2012). Indeed, such zero-valent sulfur also served as electron donor for deep marine nitrate-reducing *Epsilonproteobacteria* such as *Sulfurimonas paralvinellae* (Takai *et al.*, 2006). This observation suggests that classical AOM catalyzed by MCR enzymes was the methane-oxidizing process and not dioxygen forming NO dismutation (Ettwig *et al.*, 2010). Moreover, MCR enzymes are sensitive to dioxygen (Mahlert *et al.*, 2002). The intrinsic production of dioxygen from nitric oxide (NO) would have deactivated MCR enzymes catalyzing AOM in the Black Sea mats. This was not the case (Table 2).

$^{15}$N-ammonium was assimilated into the total biomass and the B5-MCR enzymes (Table 1 & Fig. 4). For methanotrophic consortia the assimilation of ammonium into biomass and amino acids was reported previously (Krüger *et al.*, 2008). Here, the active incorporation into an essential catalytic enzyme, demonstrating its *de novo* synthesis, is shown for the first time. The rate of *de novo* synthesized B5-MCR enzymes was dependent on the incubation conditions (Fig. 4). Relative B5-MCR incorporation rates of $^{15}$N-ammonium increased when neither sulfate nor methane was supplied. Since various MCR enzymes catalyze bidirectional reactions, a switch from methanotrophy to methanogenesis could have been a reason (Krüger *et al.*, 2003; Scheller *et al.*, 2010; Holler *et al.*, 2011). This may require structural changes in the enzymatic machinery, and perhaps the synthesis of new subunits. In the presence of sulfate but the absence of methane, less B5-MCR enzymes may have been synthesized (Fig. 4) because the energetically favored biomass-degrading sulfate reducers might have out-competed the now purely methanogenic ANME archaea (Bertram *et al.*, 2013) for substrate utilization.

$^{15}$N-dinitrogen was not utilized for total protein synthesis or for B5-MCR synthesis (see Tables S3 and S4). The reason for this was presumably the inhibition of diazotrophy by ammonium as discussed above.

## Conclusions

AOM-coupled sulfate reduction and biomass decomposition were the overall driving reactions for nitrogen incorporation into the biomass of AOM Black Sea microbial mats. Nitrate reduction and AOM could have been coupled by sulfide or zero-valent sulfur as a mediator within AOM consortia. Nitrate reduction to dinitrogen was driven by AOM coupled to sulfate reduction, while DNRA was independent of AOM. The discovery of AOM-dependent nitrate reduction is surprising for microbial communities

which inhabit what should be a nitrate-free zone of the Black Sea, and an explanation for this remains elusive.

Biomass decomposition was more important in the Black Sea mats than anticipated. This indicates that microbial life in the mats can be sustained by carbon and nitrogen sources that are already present. Since biomass decomposition liberates bio-available nitrogen, diazotrophy played an insignificant role in the Black Sea mats. The synthesis of MCR proteins was dependent on incubation conditions and elevated when sulfate was absent. Enzymes of this type were apparently the only proteins responsible for AOM, even when nitrate was the only terminal electron acceptor.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** *De novo* synthesis of amino acids from $^{15}$N-nitrate derived nitrogen in pink and clack mats.

**Fig. S2.** Estimated absolute synthesis rate of type 1 MCR proteins.

**Fig. S3.** Relative synthesis rate of type 1 MCR proteins.

**Table S1.** Test for anaerobic oxidation of ammonium using the electron acceptors sulfate, ferrihydrite, manganese dioxide and nitrite.

**Table S2.** Re-feeding regime (re/f) and medium exchange (m/ex) during the course of incubation (one year) using $^{15}$N substrates.

**Table S3.** Protein incorporation rate estimations for the black mats based on single incubations.

**Table S4.** Incorporation of the $^{15}$N-dinitrogen, $^{15}$N-nitrate and $^{15}$N-ammonium into total soluble proteins.

**Table S5.** Isotopic data of FPLC precipitates containing mainly MCR subunits and incubation times until protein extraction.

**Table S6.** Corresponding rates and standard deviations (SD) of Table 1 in the main text.

**Table S7.** Anaerobic oxidation of ammonium by manganese dioxide.