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Denitrification as the dominant nitrogen loss process in the Arabian Sea

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Primary production in over half of the world ocean is limited by fixed nitrogen (N) availability. The main loss term from the fixed N inventory is the production of dinitrogen gas (N_2) by heterotrophic denitrification or the more recently discovered autotrophic process, anaerobic ammonia oxidation (anammox). Oceanic oxygen minimum zones (OMZ) are responsible for about 35 % of oceanic N_2 production and up to half of that occurs in the Arabian Sea (AS)[1]. Although conventional denitrification was long thought to be the only loss term, it has recently been argued that anammox alone is responsible for fixed N loss in the OMZs[2, 3, 4]. Here we report that denitrification, not anammox, dominated the N_2 loss term in the AS, the largest and most intense OMZ in the world ocean. In 7 of 8 experiments in the AS, denitrification was responsible for 87 – 99 % of the total N_2 production. The dominance of denitrification was reproducible between two independent isotope incubation methods, the conventional small volume "exetainer" method, and a new method using large volume bags. In contrast, anammox, rather than denitrification, was detected using the exetainer method in the Eastern Tropical South Pacific (ETSP) OMZ, as had been previously reported[3, 5]. The abundance of denitrifying bacteria always exceeded that of anammox bacteria, by up to 7- and 19-fold in the ETSP and AS respectively. Geographic and temporal variability in carbon supply may be responsible for the different contributions of denitrification and anammox in these two OMZs. The large contribution of denitrification to N_2 loss in the AS indicates the global significance of denitrification to the oceanic N budget.

Nitrogen limits biological production in many terrestrial and aquatic environments so it is essential to understand processes that control N availability. The global inventory of fixed N may be out of balance, with losses exceeding inputs[6]. This perceived imbalance has motivated research into inputs via nitrogen fixation and losses via denitrification and anammox. The discoveries of previously unknown marine microbes involved in marine nitrogen fixation[7] and that anammox may be the dominant N_2 loss term in many environments[5,8,9] both point to large uncertainties in our understanding of the global ocean N cycle and thus our ability to predict how it might respond to perturbation.

Denitrification is an anaerobic respiratory process that is found widely in both autotrophic and heterotrophic microbes in all three biological domains. Phylogenetic studies of functional genes involved in denitrification indicate that both the ETSP and AS OMZs (defined as the depth zone where oxygen concentrations are low enough to induce anaerobic metabolism) harbor large and diverse assemblages of heterotrophic denitrifying bacteria[10,11,12]. In the absence of oxygen, denitrifiers respire nitrate (NO_3^-) sequentially to nitrite (NO_2^-), nitric and nitrous oxides (NO, N_2O) and finally to N_2. In consuming organic matter, they regenerate inorganic nutrients such as carbon dioxide (CO_2), ammonium (NH_4^+), and phosphate (PO_4^{3-}), which are necessary to sustain continued primary production. Anammox bacterial assemblages in nature are less diverse than denitrifier assemblages and are represented by only 1 or 2 phylotypes [12,13,14]. In contrast to denitrifying bacteria, anammox bacteria are autotrophs that consume NH_4^+ and NO_2^- in respiration and form biomass by CO_2 fixation. Anammox is thus dependent upon nutrient regeneration by some other process in order to supply the inorganic nutrients and reductants it requires. Denitrification by heterotrophic bacteria seemed to be the obvious supply process for both NH_4^+ and NO_2^-, which are required by anammox, and unlike the wastewater

treatment plants in which anammox was originally identified, are in very low concentrations in most of the ocean. It is therefore difficult to understand how anammox could occur in the ETSP OMZ to the exclusion of denitrification[3,5].

Lam et al[4] suggested that dissimilatory nitrate reduction to ammonium (DNRA) is a potential source of the NH_4^+ in the ETSP and argued that other processes can supply most of the nitrite. A revision of the nitrogen cycle of OMZs was proposed, in which conventional denitrification is unnecessary because nitrite is supplied by either aerobic nitrifying or anaerobic nitrate reducing microbes and ammonium is supplied by DNRA. This revision is difficult to reconcile with numerous reports of abundant and diverse denitrifying bacterial assemblages in OMZs, where their facultative anaerobic metabolism should be favored by plentiful organic supply and lack of oxygen. Although not detected in short term incubations in the ETSP[3,5], denitrification has, however, been detected in longer incubations in exetainers elsewhere[15] and in the ETSP and AS in much larger volume containers[16,17]. These observations provide evidence that viable denitrifying bacteria are present in the major OMZs and this, along with the need for N substrates to support anammox, seems incompatible with the lack of measureable denitrification. To reconcile these findings, we measured denitrification and anammox rates and quantified the abundance of denitrifying and anammox bacteria in the OMZ regions of the ETSP and the AS (Supplementary Fig. 1).

All four stations in both systems exhibited the classic oxygen and dissolved inorganic nitrogen (DIN) profiles usually associated with OMZs (Fig. 1). In the AS pelagic OMZ, we measured denitrification and anammox using two methods: 1) the exetainer method previously used in the ETSP[15] (8 ml volume, 36 - 48 h incubations, He sparged) and 2) incubations in large trace-metal clean trilaminate gas-impermeable bags[18] (~8 L volume with no headspace, 48 h

incubations, ambient gas concentrations). No changes in DIN concentrations were detectable by spectrophotometric methods over the course of the incubations, and transformation rates were detectable only by isotopic measurements of the N_2 pool ($^{29}N_2$ production from $^{15}NH_4^+$ as a tracer for anammox and $^{30}N_2$ production from $^{15}NO_2^-$ for denitrification). Denitrification rates ranged from 0.24 – 25.4 nM N_2 d^{-1} in the exetainers and from 1.3 – 6.5 nM N_2 d^{-1} in the bags (Fig. 2). Anammox rates were generally much lower, 0.24 – 4.32 nM N_2 d^{-1} in the exetainers and 0.12 - 0.21 nM N_2 d^{-1} in the bags. Although the bag and exetainer incubations were performed on different days with water collected from different casts and incubated under different conditions, the difference in rates obtained by the two methods is relatively minor. Denitrification rates calculated from $^{30}N_2$ production include the coupled DNRA-anammox pathway, but the rate of this coupled pathway cannot exceed the anammox rate. For the seven experiments excluding the high anammox rate from 150 m at Station 2, denitrification averaged 93.7 % of the total N_2 production rate.

To quantify the abundance of signature genes representing denitrification, we used degenerate primers[19] for the *nirS* gene (which encodes the heme containing nitrite reductase gene in the denitrification pathway), and for anammox, we used anammox-specific 16S rRNA gene primers[20]. *nirS* abundance measured by quantitative PCR (Q-PCR) in the Arabian Sea ranged from 3.2 – 5.3 x 10^5 copies ml^{-1}, and exceeded anammox abundance by 5.9 – 19.2 fold at the two stations for which rates are reported (Fig. 2). In 2004, at the same stations, we investigated the diversity of the *nirS* genes and found that one clade was dominant at Station 1. This clade, Dom *nirS*, represented 25 % of the sequences in clone libraries and 30% of the total *nirS* abundance by Q-PCR at Station 1 ([12]). In 2007, at the time of our rate measurements, the Dom *nirS* clade was present at up to 20% of the total *nirS* by Q-PCR, similar to the abundance of the anammox 16S

rRNA genes. Neither the total *nirS* primers[19] nor the Dom *nirS* primers[12] amplify the known anammox *nirS* sequences, so these abundances do not include anammox genes. Assuming both *nirS* and the anammox 16S rRNA genes are present in one copy per cell[21,22], *nirS*-type denitrifiers represent a large fraction of the total cells present[12,23] and they are more numerous than anammox bacteria.

In the ETSP, only anammox was detected using the exetainer incubation method[24], although anammox was not detected at all depths (Fig. 2). $No^{30}N_2$ (indicating denitrification) was detected in incubations with $^{15}NO_3^-$ added as a tracer. The range in anammox rates and the lack of denitrification were consistent with previous reports for the ETSP[3,5] in which the same methods were used. Denitrification at a rate similar to rates measured in the bag incubations in the AS were measured in bag incubations at 260 m at Station 24 in the ETSP, although exetainer experiments detected neither anammox nor denitrification at this depth (Fig. 2).

At the same depths in the ETSP where anammox or denitrification was detected, the abundance of denitrifiers containing the *nirS* gene ranged from 3.1 – 5.7 x 10^5 cells ml^{-1}. The Dom *nirS* clade from the AS was not detected by Q-PCR in any samples from the ETSP. Published clone library data[10,4] so far have not identified a clear dominant phylotype in the ETSP system. The abundance of anammox bacteria in the ETSP samples was 0.7 – 1.5 x 10^5 cells ml^{-1}, ~3 – 7- fold less abundant that the *nirS* denitrifiers. At stations 3° north of our Station 9, Hamersley et al[5] reported abundances of anammox bacteria based on Q-PCR and FISH (targeting the 16S rRNA sequence) of up to 1.5 x 10^5 cells ml^{-1}. The similarity between the anammox abundances reported here (Fig. 2) and those reported previously for the same region[5] lends support to the general pattern we observed. The similarity in *nirS* denitrifier abundances

between the AS and ETSP suggests that denitrifiers are important in both systems, but anammox abundance was generally less in the AS, where anammox rates were much lower.

Denitrifier abundances reported here are likely underestimates of the total denitrifier abundance because the *nirS* primers may not detect all *nirS* containing organisms and denitrifiers that use the copper type nitrite reductase, encoded by the *nirK* gene, were not quantified. The *nirS* primers used by Lam et al[4] significantly underestimate the denitrifier abundance, because they do not amplify most of the sequenced *nirS* genes reported previously from OMZ waters in the AS[12] and the ETSP[10].

The rates reported here are the first direct measurements of denitrification in the OMZ of the AS in short term incubations. Denitrification defined as production of $^{29}N_2$ from $^{15}NO_{3\text{-}in}$ large volume incubations reported previously[1] could not distinguish between anammox and denitrification. Nicholls et al[17] inferred denitrification in the AS from rates of N_2O production from $^{15}NO_2$- and $^{15}NO_3$- (and $^{30}N_2$ production from $^{15}NO_2$- mainly after > 2 days); this group also detected only very low rates of anammox. In our study, both exetainers and large bags yielded rates of similar magnitude in incubations of similar length (up to two days). The range of denitrification rates reported here includes the average rate of 9.1±1.1 nM N_2 d^{-1} reported for total denitrification (implicitly including anammox)[1] in the AS, the mean rate of 2.0 nM N_2 d^{-1} $^{15}N2$ production from $^{15}NO_2$ in longer incubations in the AS[17], and the rate of 14.7±3.53 nM N_2 d^{-1} estimated from N_2O consumption in the ETSP[25].

Denitrification rates were much faster than anammox at all depths and all stations except in one exetainer experiment. The significant rates of denitrification that we measured in the AS contrast with rates measured in the ETSP by ourselves and others, in which anammox, but not denitrification, was detected[3,5]. Because identical methods were used in the ETSP and AS, we

suggest that the AS differs from the ETSP in the relative contribution of denitrification and anammox, at least at the times of these experiments. We previously documented that denitrification was limited by availability of organic carbon in the OMZ of the ETSP, but not in the AS[16]. Therefore it seems likely that important differences in carbon availability, in terms of magnitude, composition or temporal supply, may be responsible for these ecosystem scale differences[26] and might also lead to temporally and spatially dynamic distributions of both processes.

The high abundance and diversity of denitrifiers, compared to the lower abundance and lower diversity of anammox organisms, suggests that dynamic assemblages of denitrifiers persist in both OMZs. It is clear, however, that the denitrifier assemblages in the two environments differ in important ways, including the consistent detection of a dominant denitrifier phylotype, which was absent in the ETSP, but which was associated with the core of the OMZ and highest denitrification rates in the AS.

The lack of correlation between bacterial abundances and the respective rates is compatible with the contrasting lifestyles of the two kinds of microbes: versatile opportunistic heterotrophic denitrifying bacteria are probably capable of a much more dynamic activity response to episodic substrate supply than are the more highly constrained slower growing autotrophic anammox bacteria. We hypothesize that episodic organic matter supply supports the denitrifiers in the ETSP and that at times of high organic flux, the relative contribution of denitrification and anammox shifts towards the ratio observed in the AS. The close coupling between aerobic nitrification and anammox, which might be able to supply some of the nitrite required for anammox in the ETSP, is unlikely to be important in the core depths of the OMZ in either the ETSP or the AS. When detected, highest anammox rates generally occur near the

upper edge of the OMZ in the ETSP[5], while we detected denitrification in the core of the OMZ at the depth of the secondary nitrite maximum in the AS and in the ETSP. The conventional nitrogen cycle of the AS must be revised to include anammox, but denitrification is clearly the overwhelmingly dominant flux for fixed N loss the ocean's largest OMZ. Its importance in the AS, which alone is responsible for up to half of the fixed N loss from the pelagic realm[1], indicates that denitrification is a major process in the overall marine N cycle.

METHODS SUMMARY

Sampling was conducted aboard the R/V Knorr in the ETSP in 2005 and the R/V Roger Revelle in the AS in 2007. Nutrient concentrations were determined by standard spectrophotometric methods and oxygen was measured using the CTD electrode. Water for incubations was collected from Niskin bottles, which were plumbed with He during sample removal to avoid atmospheric contamination. Anammox and denitrification rates were measured using the standard method with small volume exetainers[15], in which various isotope additions were made to helium sparged vials and the accumulation of $^{15}N_2$ was measured on a Europa 20/20 mass spectrometer in triplicate vials poisoned at 12, 24, 36, and 48 h. Exactly the same exetainer methods were used as described by[15] in order to obtain results that were exactly comparable to those previously reported. Anammox was quantified from the accumulation of $^{29}N^2$ in the incubation to which $^{15}NH_4^+$ was added as a tracer along with $^{14}NO_2^-$, and denitrification was quantified as the accumulation of $^{29}N_2$ and $^{30}N_2$ in incubations to which $^{15}NO^{2-}$ was added as a tracer. Rates of denitrification and anammox were considered significant if the increase in $^{29}N_2$ or $^{30}N_2$ was linear over the first 36 h of exetainer incubations. The new method used large trilaminate bags with no headspace and no sparging[16]. The same tracer treatments as in the exetainer method were used but at lower concentrations. Degassed tracer solutions were added while filling the bags directly from the Niskin sampling bottles. Single endpoint determinations of $^{29}N^2$ and $^{30}N^2$ were made on subsamples from the 8-L bags after 48 h and analyzed following the method of Emerson et al[27] on a Finnigan Delta XL mass spectrometer. Signature genes for denitrification and anammox were quantified by quantitative PCR.

Methods

Inorganic nutrient concentrations were measured by autoanalyzer and CTD oxygen values were standardized to Winkler titrations.

Methods of DNA extraction, PCR amplification and Q-PCR using SYBR Green for *nirS* and Dom *nirS*[11,12] and anammox 16S rRNA genes[20] (primers AMX368F and AMX820R) have been described previously. Standardization and verification of specificity for Q-PCR assays were performed as previously described[12].

Denitrification and anammox rates in small volumes were measured using the previously described incubation protocol[15]. Water samples were collected from Niskin bottles, which were plumbed with He during sample removal to avoid atmospheric contamination of gases and held at 12°C (<3 h), then transferred to a N_2 flushed glove bag, where the ^{15}N tracers (3 µM final concentration $^{15}NO_2^-$ or $^{15}NH_4^+$) were added. Triplicate aliquots (8 mL) were incubated in 12 mL Exetainers, which were sealed and sparged with He for 5 minutes. Three replicates of each sample were killed with 0.05 mL of 7M $ZnCl_2$ at 12-hour intervals between 0 and 48 hours.

The amount of excess ^{15}N-N_2 produced during the incubations was measured directly in the incubation vials with a continuous flow isotope ratio mass spectrometer (Europa Scientific 20-20), in-line with an automated gas preparation unit (Europa Scientific, ANCA-G Plus). Denitrification was defined as binomially distributed $^{29}N_2$ and $^{30}N_2$ production in the $^{15}NO_2^-$ incubation[28] and anammox was defined as $^{29}N_2$ production in $^{15}NH_4^+$ incubation[29]. Statistical analyses were performed using the JMP software program (SAS). The ^{15}N-N_2 production rates were calculated from the 36-hour (4 time points) slope of the standard least squares regression. Error was calculated as the standard error of the slope. Error for denitrification rates was determined by propagation of error from the slopes of F29 and F30.

Isotope measurements for big bag incubations were made on subsamples collected from the bags into $HgCl_2$ poisoned, pre-evacuated, 300 mL glass flasks equipped with gas tight 9 mm bore Louwers-Hapert single o-ring valves. The amount of $^{28, 29, 30}N_2$ added by denitrification in the $^{15}NO_2^-$-amended incubations was calculated using the following equations:

$$[{}^{30}N_2]_{denit} = \frac{[{}^{30}N_2]_{inital} \updownarrow R^{30}_{final} \cdot [{}^{28}N_2]_{inital}}{\square \; R^{30}_{final} \cdot \square P^{30}_{denit} \square \updownarrow 1}$$

$$[{}^{28}N_2]_{denit} = [{}^{30}N_2]_{denit} \cdot \frac{\square P^{28}_{denit} \square}{\square P^{30}_{denit} \square}$$

$$[{}^{29}N_2]_{denit} = [{}^{30}N_2]_{denit} \cdot \frac{\square P^{29}_{denit} \square}{\square P^{30}_{denit} \square}$$

$$Rate_{denit} = \frac{[{}^{28}N_2]_{denit} + [{}^{29}N_2]_{denit} + [{}^{30}N_2]_{denit}}{Time}$$

$[{}^{x}N_2] = N_{2denit}$, *where* N_{2denit} *the amount of* ${}^{x}N_2$ *added by denitrification*

$[{}^{x}N_2]_{initial}$ = *the initial amount of* ${}^{x}N_2$,

$R^{x}_{final} = {}^{x}N_{2final} = {}^{28}N_{2final}$ *isotope ratio at endpo* int,

P^{x}_{denit} = *probability of making* ${}^{x}N_2$ *by denitrification*

The amount of $_{28,29}N_2$ added by anammox in the $^{15}NH_4^+$-amended incubations, in which there was no measurable ambient $^{14}NH_4^+$, was calculated using the following equations:

$$[{}^{29}N_2]_{ana} = [{}^{28}N_2]_{inital} \cdot R^{29}_{final} \updownarrow [{}^{29}N_2]_{inital}$$

$$[{}^{28}N_2]_{ana} = [{}^{29}N_2]_{ana} \cdot \frac{\square P^{28}_{ana} \square}{\square P2^{ana} \square}$$

$$Rate_{ana} = [{}^{28}N_2]_{ana} + [{}^{29}N_2]_{ana}$$

$[{}^{x}$]

$$= \frac{N_{2\,ana}^{x}}{Time}$$,

where $N_{2\,ana}^{x}$ *the amount of* N_2^x *added by anammox*

P_{ana}^{x} = *probability of making* N_2^x *by anammox*

FIGURE LEGENDS

Figure 1. Chemical profiles for experimental stations in the ETSP and AS. Oxygen (---), nitrate (+), and nitrite ( ) concentrations at two stations in the AS: a) Station 1, 19° N 67° E, bottom depth 3100m; b) Station 2, 15° N 64° E, bottom depth 3930m, and ETSP off Peru: c) Station 9, 15°38 S 75° 08 W, bottom depth 977m; d) Station 24, 12° 15 S 79° 18 W, bottom depth 4899m. Arrows indicate depths where denitrification and anammox rates were measured.

Figure 2. Rates of denitrification and anammox and abundance of signature genes for denitrification and anammox.

Left panel: Denitrification and anammox rates. Anammox (Amx-ex, open bars) and denitrification (Denit-ex, gray bars) rates measured in exetainers; anammox (Amx-bb, hatched bars) and denitrification (Denit-bb, black bars) rates measured in 8-L bags. 3 = no rate detected; = not measured. Error bars = standard error of the slope of $^{29}N_2$ or $^{29}N_2$ plus $^{30}N_2$ accumulation in exetainer incubations.

Right panel: Depth distribution of anammox 16S rRNA genes (Amx), total denitrifier *nirS* genes (Tot *nirS*, black bars) and a dominant *nirS* clade (Dom *nirS*, gray bars). Error bars indicate standard deviations of triplicate Q-PCR assays.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

A figure showing station locations is available in Supplemental Fig. 1.

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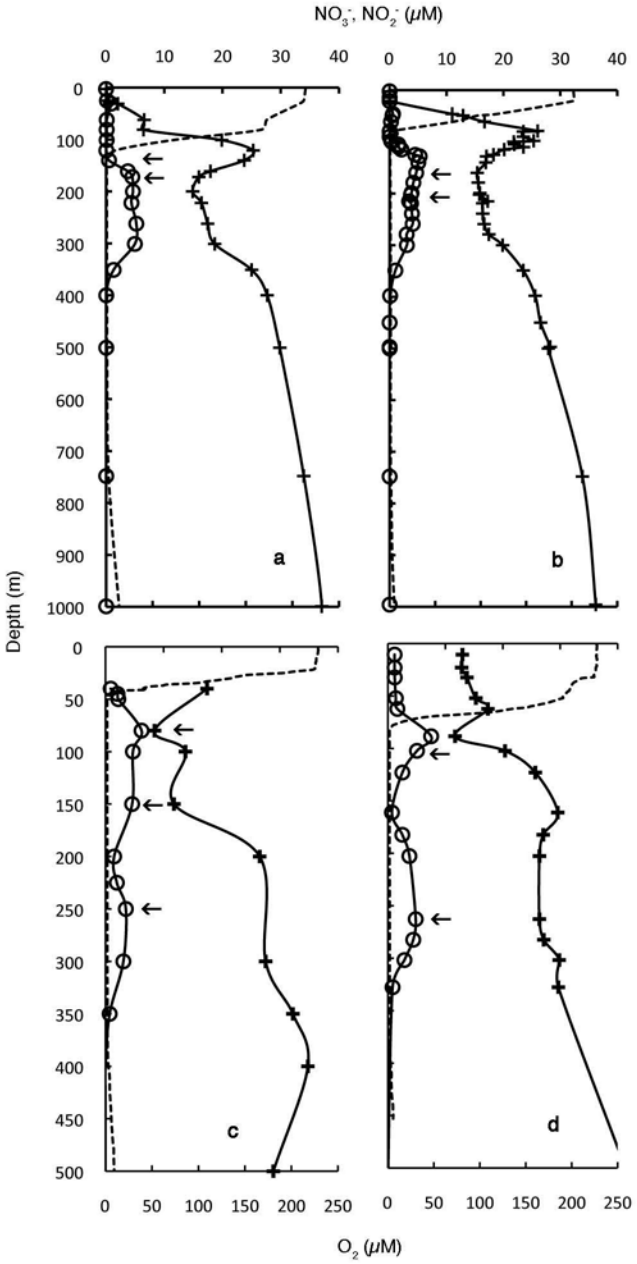
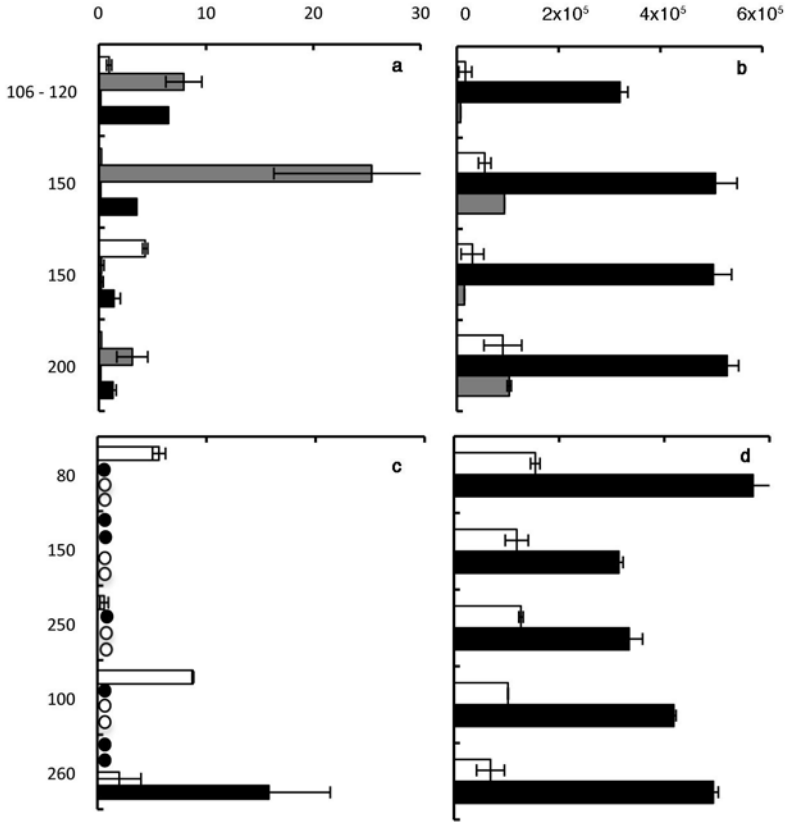
Author contributions

BBW, AHD and AJ designed the AS experiments and participated in all the field work; JJR designed the ESTP and AS exetainer experiments and performed them in the ETSP; SEB and BBW performed the exetainer experiments in the AS; JJR made the exetainer mass spec measurements for both AS and ETSP; BC and AHD made the mass spec measurements for the big bag experiments; BBW, AHD, AJ, SEB and BC carried out the big bag experiments in the AS; HN and AP provided the nitrite and nitrate data for Figure 1a and b; BBW wrote the paper with input from AHD, AJ, SEB, BC and JJR.


NO_3^-, NO_2^- (μM)
0 10 20 30 40
0 10 20 30 40
Depth (m)
0 100 200 300 400 500 600 700 800 900 1000
a
b
0 50 100 150 200 250 300 350 400 450 500
c
d
0 50 100 150 200 250
0 50 100 150 200 250
O_2 (μM)


0
10
20
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106 - 120
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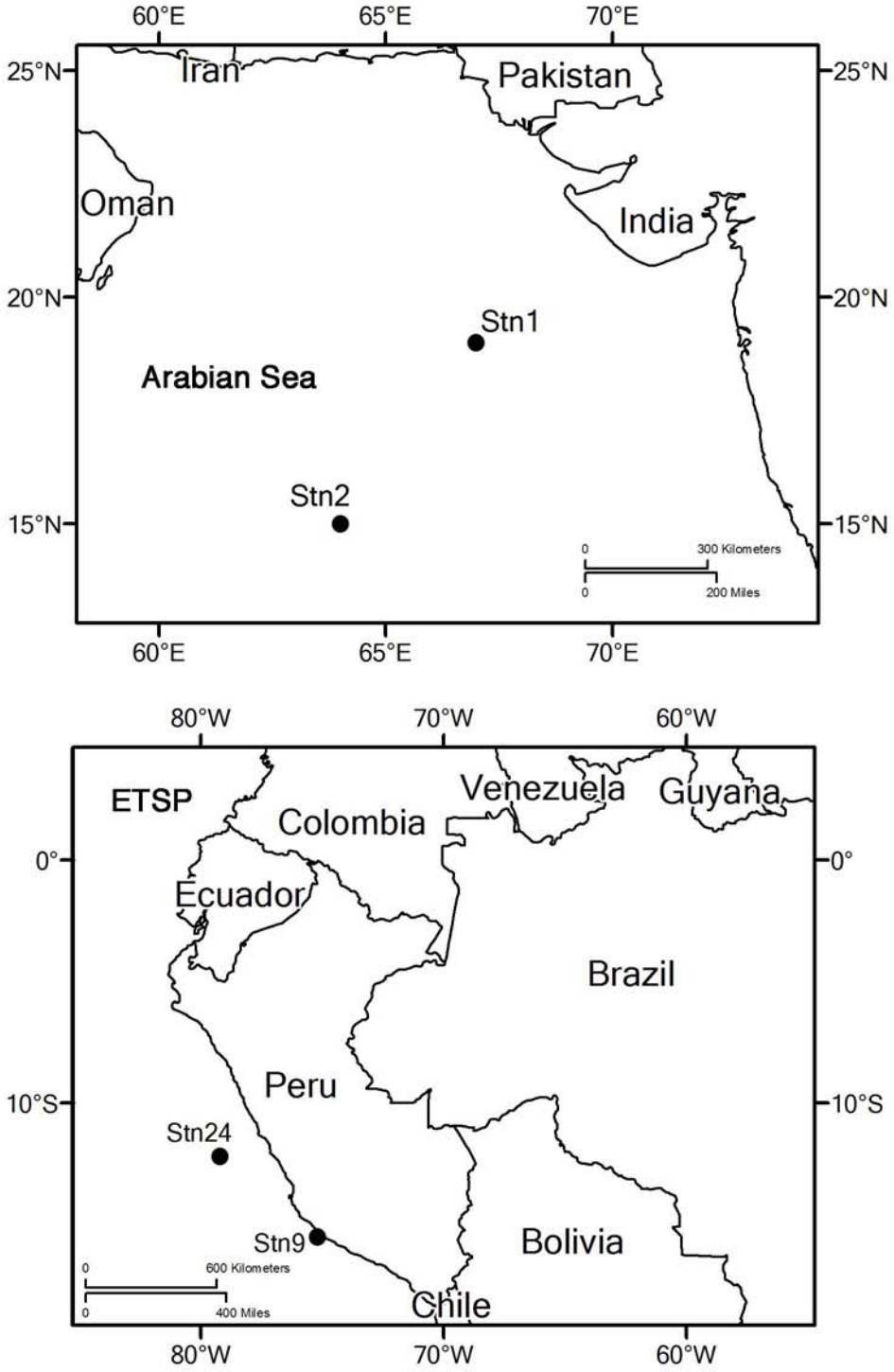
150
250
100
260


60°E
65°E
70°E
25°N
20°N
15°N
Iran
Pakistan
Oman
India
Stn1
Arabian Sea
Stn2
0
300 Kilometers
0
200 Miles
80°W
70°W
60°W
0°
10°S
ETSP
Colombia
Venezuela
Guyana
Ecuador
Brazil
Peru
Stn24
Stn9
Bolivia
Chile
0
600 Kilometers
0
400 Miles