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Methane stimulates massive nitrogen loss from freshwater reservoirs in India

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The fate of the enormous amount of reactive nitrogen released to the environment by human activities in India is unknown. Here we show occurrence of seasonal stratification and generally low concentrations of dissolved inorganic combined nitrogen, and high molecular nitrogen (N_2) to argon ratio, thus suggesting seasonal loss to N_2 in anoxic hypolimnia of several dam-reservoirs. However, ^{15}N -experiments yielded low rates of denitrification, anaerobic ammonium oxidation and dissimilatory nitrate reduction to ammonium—except in the presence of methane (CH_4) that caused ~12-fold increase in denitrification. While nitrite-dependent anaerobic methanotrophs belonging to the NC10 phylum were present, previously considered aerobic methanotrophs were far more abundant (up to 13.9%) in anoxic hypolimnion. Methane accumulation in anoxic freshwater systems seems to facilitate rapid loss of reactive nitrogen, with generally low production of nitrous oxide (N_2O), through widespread coupling between methanotrophy and denitrification, potentially mitigating eutrophication and emissions of CH_4 and N_2O to the atmosphere.

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There is a dearth of information on the biogeochemistry of lakes and reservoirs and how they are impacted by human activities in South Asia, a region that accounts for about a quarter of the world's human population. India is one of the largest consumers of synthetic nitrogen fertilisers in the world (~17 million tons N per year)¹. Altogether with reactive nitrogen release from other sources (e.g., fossil fuel combustion and waste disposal), anthropogenic nitrogen is predicted to result in the eutrophication of aquatic bodies including the coastal ocean² and thus intensification of seasonal hypoxia to anoxia. On the one hand, intensified anoxia facilitates greater loss of reactive nitrogen through microbial reduction of nitrate (NO_3^-) to molecular nitrogen (N_2). On the other hand, it promotes the production of two potent greenhouse gases—methane (CH_4) and nitrous oxide (N_2O). Nitrogen loss from anaerobic aquatic environments had long been believed to occur through heterotrophic denitrification ($\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2$), until anaerobic ammonium oxidation (anammox) ($\text{NO}_2^- + \text{NH}_4^+ \rightarrow \text{N}_2 + 2\text{H}_2\text{O}$) was discovered to be an important component of the nitrogen cycle^{3,4}. More recently, nitrite-dependent anaerobic methane oxidation (N-DAMO) ($3\text{CH}_4 + 8\text{NO}_2^- + 8\text{H}^+ \rightarrow 3\text{CO}_2 + 4\text{N}_2 + 10\text{H}_2\text{O}$) has been found as yet another N_2 production pathway⁵. This is potentially an environmentally significant process not only because of its role in nitrogen loss, but also due to its consumption of CH_4 and constraints on the formation of N_2O , which would otherwise be produced through canonical denitrification⁶. However, potential of N-DAMO has so far largely been inferred from enrichment and molecular detection in environmental samples of the bacteria known to mediate this process – *Candidatus* Methyloirabilis oxyfera⁵ and its relatives in the phylum NC10^{7–16}. All field studies conducted so far have focussed on soils and sediments with just a few exceptions. In one case, NC10 bacteria have been reported from the water column of a dam-reservoir¹¹; while in two other studies they were found within the oceanic oxygen minimum zone (OMZ) of the eastern tropical North Pacific^{17,18}. More direct chemical evidence has emerged from anaerobic incubations of sediment from Lake Constance¹⁵ and of wetland soils in southeastern China¹⁰ spiked with $^{14}\text{CH}_4$ / $^{13}\text{CH}_4$ and NO_2^- that led to the production of $^{14}\text{CO}_2$ / $^{13}\text{CO}_2$, and from high resolution microprofiling of dissolved oxygen, CH_4 , NO_3^- and NO_2^- with and without the addition of NO_3^- in sediment cores of Lake Constance¹⁶. In the few cases where NC10 bacteria were detected in the water columns, albeit at low abundance, N-DAMO activity was not demonstrated. We present here results of incubation experiments with ^{15}N -labelled NO_3^- and NO_2^- in the presence and absence of CH_4 along with a much larger data set, the first of its kind, on rates of denitrification, anammox and dissimilatory nitrate/nitrite reduction to ammonium (DNRA) from a number of dam-reservoirs in India. We also present results of a very large number of N_2O measurements in the reservoirs, the first such report from any freshwater system in South Asia. The results of these measurements in conjunction with molecular data are used to gain insights into pathways of nitrogen loss from Indian freshwater reservoirs.

Results

Seasonal stratification and its impact on water chemistry.

Summer warming of surface waters resulted in strong stratification and consequent oxygen depletion in the hypolimnia of all 15 sampled reservoirs that are located over a wide latitudinal range (9.8°N–31.4°N—Supplementary Fig. 1, Supplementary Table 1) and differ in size, geology, climate and influence from human activities. In all cases, with the exception of two Himalayan reservoirs (Bhakra-Nangal and Tehri), and Rihand and Supa located in the Indo-Gangetic Plain and the Western Ghats,

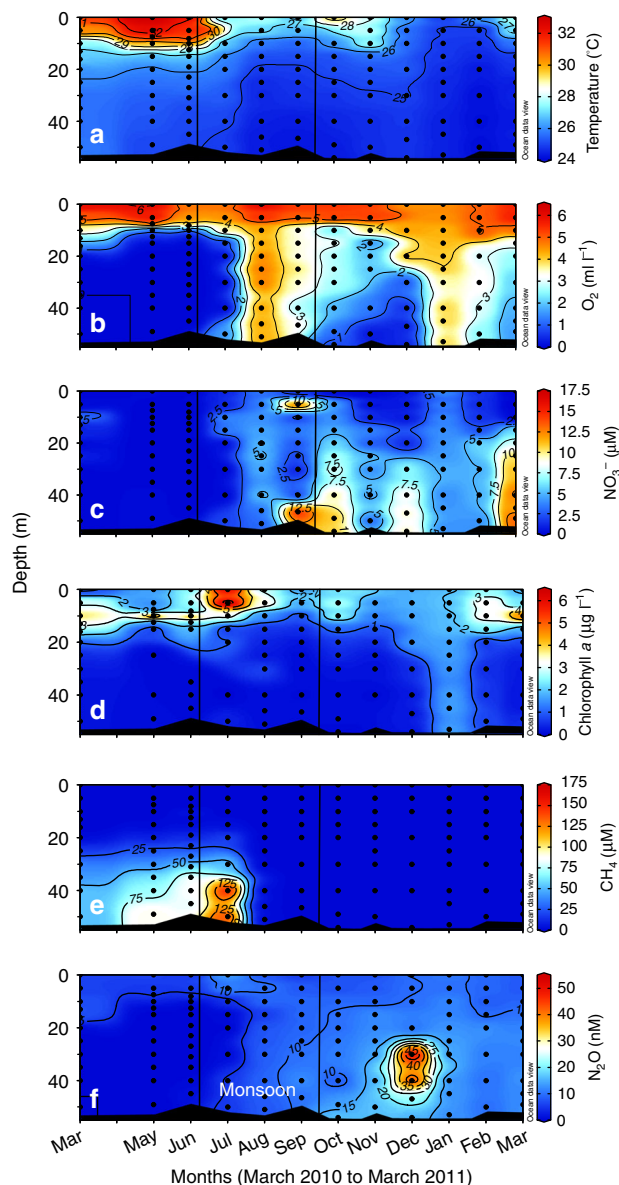


Fig. 1 Annual cycles of physico chemical variables in Tillari Reservoir. Temperature (a), dissolved oxygen (b), nitrate (c), chlorophyll a (d), methane (e), and nitrous oxide (f) were measured once a month from March 2010 to March 2011. The summer monsoon period (June–September) is demarcated by black vertical lines

respectively, dissolved oxygen concentration fell below the detection limit (Supplementary Table 2). The latter two reservoirs were probably not sampled at the peak of oxygen depletion. The anoxic hypolimnia were characterised by low NO_x^- ($\text{NO}_3^- + \text{NO}_2^-$) concentrations (<5 μM) in a majority of reservoirs and accumulation of CH_4 (Figs. 1, 2 and 3, Supplementary Fig. 2, Supplementary Table 2; also see ref. 19). The highest CH_4 concentration measured in the present study was ~207 μM in Tillari Reservoir, further reinforcing the view that CH_4 accumulation in Indian dam-reservoirs is generally less than reported from several other tropical reservoirs¹⁹. Measurements of nitrogen to argon ratio (N_2/Ar) in four of these systems in summer (Supplementary Table 1) revealed excess N_2 in anoxic hypolimnia (up to 13.6 μM) seemingly produced from the reduction of NO_3^- to N_2 . These included the two most-frequently sampled reservoirs, Markandeya and Tillari (Supplementary Figs. 3 and 4), which experience

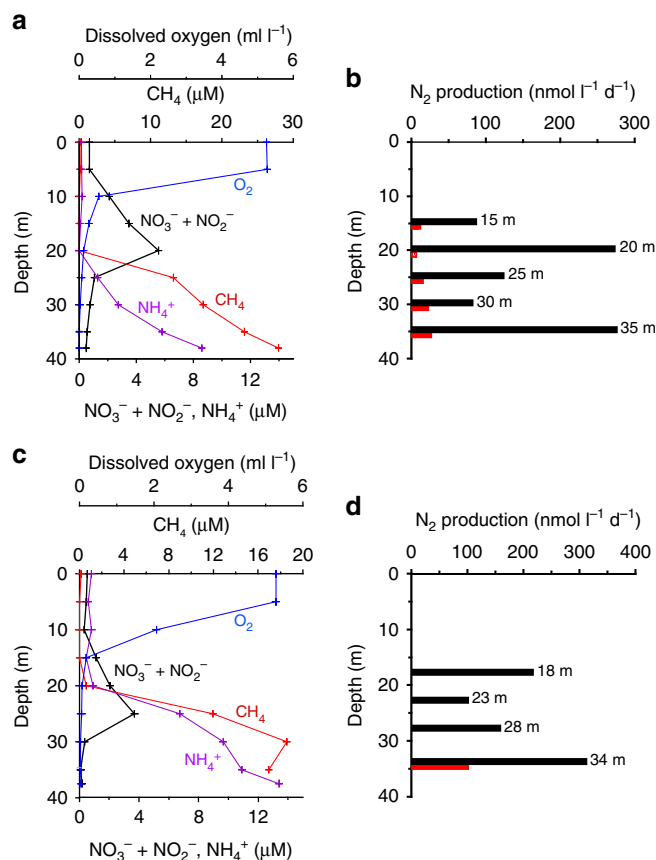


Fig. 2 Water characteristics and denitrification rate in Tillari Reservoir. Depthwise variations in concentrations of dissolved oxygen, nitrate (+nitrite), ammonium and methane on 31.05.2011 (a), and 03.06.2011 (c). Total denitrification rate measured by isotope pairing experiments (black bars - with methane; red bars—without methane) on 31.05.2011 (b), and 03.06.2011 (d)

contrasting degrees of anthropogenic impact. The Tillari Reservoir, located in relatively pristine foothills of the Western Ghats, was visited on an almost monthly basis from March 2010 to July 2014. The time-series for key parameters for the first 12 months are shown in Fig. 1. The next most frequently sampled reservoir—Markandeya Reservoir located in the Deccan Plateau—is much more affected by human activities (runoffs from agricultural fields and much denser population centres in the drainage basin of the Markandeya River). Although seasonal changes in water chemistry in this reservoir were similar to those observed in the Tillari Reservoir, the amplitude of variability was much larger. The maximum NO_3^- concentration ($\sim 150 \mu\text{M}$), for example, is about ten times higher than that recorded in the Tillari Reservoir. The reservoirs are dimictic—i.e., the water column is vertically mixed during winter and the southwest monsoon (June–September), when the reservoirs are recharged with water and nutrients (Fig. 1 and Supplementary Fig. 5). As expected, NO_3^- regenerated from organic matter began to accumulate as oxygen levels declined in the hypolimnion after the establishment of stratification in early spring of 2011 in Tillari Reservoir (Fig. 1). The NO_3^- concentration in the hypolimnion averaged around $12 \mu\text{M}$ on 10.03.2011. It decreased dramatically once the hypolimnion became anoxic, averaging around $2 \mu\text{M}$ on 31.05.2011 (data not shown). In 2010, however, when the first observation was made in Tillari Reservoir on 31 March, anoxia was already fully developed and the peak NO_3^- concentration measured was only about $1 \mu\text{M}$. This pattern of NO_3^- buildup within the hypolimnion in late

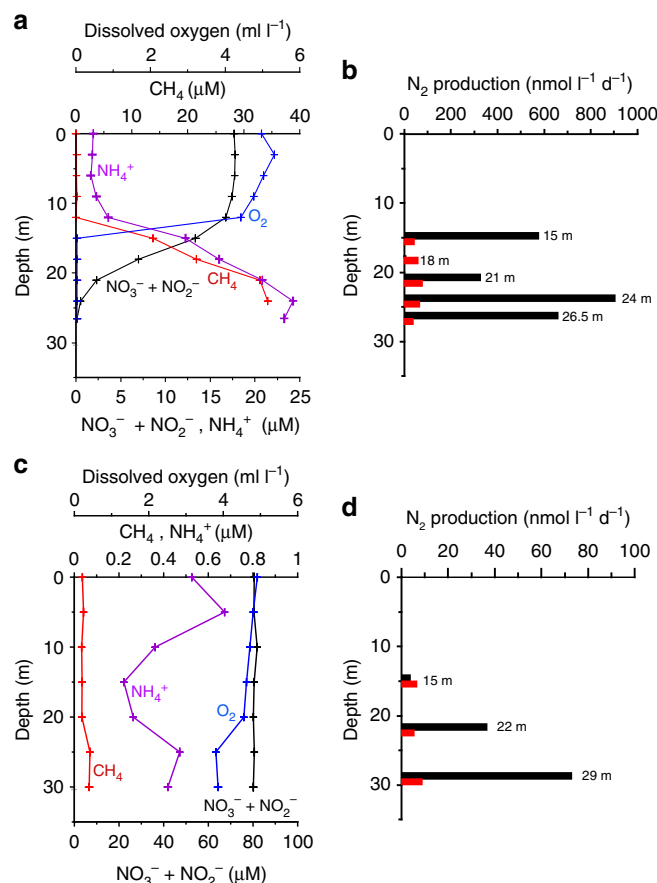


Fig. 3 Water characteristics and denitrification rate in Markandeya Reservoir. Depthwise variations in concentrations of dissolved oxygen, nitrate (+nitrite), ammonium and methane on 15.06.2011 (a), and 12.01.2012 (c). Total denitrification rate measured by isotope pairing experiments (black bars - with methane; red bars - without methane) on 15.06.2011 (b), and 12.01.2012 (d)

winter-early spring and rapid loss in late spring-early summer was invariably observed in the following years (data not shown). Since NO_3^- is often the dominant species of fixed nitrogen, the combined dissolved inorganic nitrogen ($\text{DIN} = \text{NO}_3^- + \text{NO}_2^- + \text{NH}_4^+$) concentration showed similar declining trend as NO_3^- through the spring and summer (Supplementary Fig. 6 for Markandeya Reservoir). However, bottom-water NH_4^+ concentrations in late summer could occasionally be quite high (up to $\sim 48 \mu\text{M}$ and $106 \mu\text{M}$ in Tillari and Markandeya, respectively) when the water turns mildly sulphidic.

A striking feature of nitrogen cycling in the reservoirs studied is the generally low concentrations of NO_2^- , as well as N_2O in anoxic waters, unlike typical OMZs in marine systems. For instance, NO_2^- in the Tillari Reservoir was below $0.1 \mu\text{M}$ in 327 out of 419 samples, exceeding $0.5 \mu\text{M}$ only in 2 samples with a maximum of $0.72 \mu\text{M}$ (Supplementary Fig. 7). Out of 815 measurements of NO_2^- made in all reservoirs, only 27 values exceeded $0.5 \mu\text{M}$ (maximum $1.35 \mu\text{M}$ —Fig. 4). Similarly, in Tillari Reservoir low N_2O concentrations ($< 10 \text{ nM}$) persisted throughout the summer in the presence of CH_4 at micromolar levels, rising in winter up to 53.1 nM when CH_4 content was three orders of magnitude lower (Fig. 1). Overall, N_2O content was $< 20 \text{ nM}$ in 681 samples (84% of all measurements, Fig. 4), and values in excess of 100 nM (maximum 357.2 nM) were recorded only in 34 samples (in 4% cases). None of the latter samples came from the epilimnion, and most were associated with $\text{O}_2 < 0.5 \text{ ml l}^{-1}$ and relatively elevated NO_2^- (Fig. 4). A majority of

these samples were from Markandeya (4 trips) with the remainder coming from Tillari (3 trips), Idukki and Koyna (1 trip each) over summer months. Thus, most of the reservoirs did not show significant N_2O accumulation despite the prevalence of low-oxygen conditions.

Anaerobic nitrogen transformation rates. To identify the pathway(s) of nitrogen loss, we performed ^{15}N -incubation experiments to determine rates of denitrification and anammox during periods when anoxic conditions prevailed in the following reservoirs: Koyna and Selaulim (once each); Idukki and Markandeya (three times each); and Tillari (five times) (Supplementary Table 3). Although the choice of these reservoirs was primarily dictated by logistic convenience, these reservoirs also differ considerably in terms of human impact. Surprisingly, only in one instance—Markandeya Reservoir in May 2007—was consistently high denitrification activity observed throughout the hypolimnion (Denitrification results in the production of $^{15}\text{N}^{15}\text{N}$, and also of $^{14}\text{N}^{15}\text{N}$ through isotope pairing of the ^{15}N -labelled NO_x^- with ambient non-labelled (i.e., ^{14}N) NO_3^- and/or NO_2^-). In this instance, $^{15}\text{N}^{15}\text{N}$ and $^{14}\text{N}^{15}\text{N}$ production rates from $^{15}\text{NO}_2^-$ incubations reached up to 832.1 and 111.4 $\text{nmol N}_2 \text{ l}^{-1} \text{ d}^{-1}$, respectively (Supplementary Fig. 8), with the mean (\pm standard deviation) total denitrification rate (TDR)²⁰ at $1371 \pm 368 \text{ nmol N}_2 \text{ l}^{-1} \text{ d}^{-1}$ ($n=5$). On other occasions, $^{15}\text{N}^{15}\text{N}$ and $^{14}\text{N}^{15}\text{N}$ production rates were generally low (Supplementary Table 4) with a mean TDR of $53.2 \pm 149.9 \text{ nmol N}_2 \text{ l}^{-1} \text{ d}^{-1}$ (Table 1). Moreover, despite high NH_4^+ concentrations

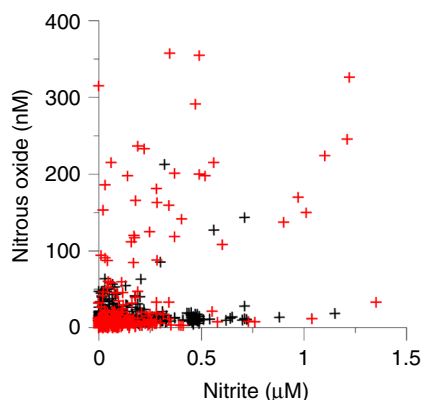


Fig. 4 Nitrous oxide versus nitrite in all reservoirs. Red and black symbols denote samples with dissolved $\text{O}_2 \leq$ and $>0.5 \text{ ml l}^{-1}$, respectively

(0.11–19.58 μM) in anoxic waters, the anammox rates determined from the production of $^{14}\text{N}^{15}\text{N}$ from $^{15}\text{NH}_4^+$ and $^{15}\text{NO}_2^-$ incubations were even lower (Supplementary Table 4), averaging $3.98 \pm 8.82 \text{ nmol N}_2 \text{ l}^{-1} \text{ d}^{-1}$ and $12.62 \pm 22.47 \text{ nmol N}_2 \text{ l}^{-1} \text{ d}^{-1}$ respectively (Table 1). The lower rates obtained from $^{15}\text{NH}_4^+$ incubations were likely due to the presence of ambient $^{14}\text{NH}_4^+$ in the samples. The low anammox activity was consistent with that detected via a combination of $^{15}\text{NO}_2^-$ and $^{14}\text{NH}_4^+$, thus ruling out low ambient NO_2^- levels ($0.071 \pm 0.076 \mu\text{M}$; $n=71$) being a major underlying reason here. The DNRA rates measured by production of labelled NH_4^+ from incubations involving $^{15}\text{NO}_3^-$ or $^{15}\text{NO}_2^-$ on nine trips to four reservoirs were also very low (Supplementary Table 4). DNRA was often not detectable, with the mean rates of 9.70 ± 11.16 and $8.71 \pm 13.44 \text{ nmol N l}^{-1} \text{ d}^{-1}$ obtained with $^{15}\text{NO}_3^-$ and $^{15}\text{NO}_2^-$ incubations, respectively, being quite similar (Table 1). The only exception was Selaulim Reservoir, for which consistently high rates (70.97–162.14 $\text{nmol N l}^{-1} \text{ d}^{-1}$ from $^{15}\text{NO}_2^-$) were measured throughout the anoxic hypolimnion during the one summer visit (Table 1).

Enhancement of N_2 production by CH_4 . The measured rates of nitrogen loss in the methane-free ^{15}N -incubations are much lower than expected from the in situ NO_x^- and N_2/Ar depth profiles. For example, in the Markandeya Reservoir sampled on 17.04.2015, the NO_3^- concentration decreased from 53.9 μM from the top of the anoxic hypolimnion (12 m) to 27.5 μM close to the bottom (28 m); the excess N_2 within this layer ranged between 8.21 and 12.83 μM (Supplementary Fig. 4). The loss of NO_3^- and accumulation of N_2 could only have occurred after the development of anoxic conditions, probably in less than a month, implying an average loss rate on the order of $1 \mu\text{mol NO}_3^- \text{ l}^{-1} \text{ d}^{-1}$. This is comparable to the average rate of nitrogen loss ($\sim 2 \mu\text{mol l}^{-1} \text{ d}^{-1}$) estimated by Deemer et al.²¹ for Lakamas Lake, a small eutrophic reservoir in the State of Washington (USA), from the observed accumulation of N_2 in the hypolimnion in early summer. Intrigued by low rates of canonical denitrification, we incubated samples spiked with $^{15}\text{NO}_2^-$ and $^{15}\text{NO}_3^- + ^{14}\text{NO}_2^-$, with and without CH_4 , on two trips to Tillari Reservoir and one trip to Markandeya Reservoir during periods of anoxia (in summer), and on one trip to Markandeya Reservoir in winter when the water column was well-oxygenated. The $^{15}\text{NO}_3^- + ^{14}\text{NO}_2^-$ amendment was designed to determine if NO_3^- reduction to NO_2^- was directly and efficiently coupled to the N_2 production process and its dependence on CH_4 . The results of both the $^{15}\text{NO}_2^-$ and $^{15}\text{NO}_3^- + ^{14}\text{NO}_2^-$ incubations showed that CH_4 amendments significantly enhanced nitrogen loss

Table 1 Rates of anaerobic nitrogen transformations

	Process rates ($\text{nmol N}_2 \text{ l}^{-1} \text{ d}^{-1}$)			Process rates ($\text{nmol N l}^{-1} \text{ d}^{-1}$)	
	Denitrification from $^{15}\text{NO}_2^-$	Anammox from $^{15}\text{NO}_2^-$	Anammox from $^{15}\text{NH}_4^+$	^b DNRA from $^{15}\text{NO}_3^-$	^c DNRA from $^{15}\text{NO}_2^-$
Range	N.D.–920.9 ^a	N.D.–101.73	N.D.–50.66	N.D.–32.07	N.D.–45.89
Mean \pm SD	53.2 ± 149.9	12.62 ± 22.47	3.98 ± 8.82	9.70 ± 11.16	8.71 ± 13.44
Median	13.0	3.47	0.26	6.34	2.27
N	70 ^a	52	57	10	28
Remarks on rates	$>100 \text{ nM d}^{-1} = 6x$ $>50 \text{ nM d}^{-1} = 14x$ $<10 \text{ nM d}^{-1} = 30x$	$>100 \text{ nM d}^{-1} = 1x$ $>50 \text{ nM d}^{-1} = 5x$ $>10 \text{ nM d}^{-1} = 16x$ N.D. = 14x	$>10 \text{ nM d}^{-1} = 7x$ $>5 \text{ nM d}^{-1} = 12x$ N.D. = 26x	$>10 \text{ nM d}^{-1} = 3x$	$>10 \text{ nM d}^{-1} = 8x$

N.D. = not detectable
^a Excluding 5 values (1024–1886, mean $1371 \pm 368 \text{ nmol N}_2 \text{ l}^{-1}$) from Markandeya on 3 May 2007 (See Supplementary Fig. 8)
^b Excluding 4 values (2.94–118.45, mean $69.66 \pm 50.01 \text{ nmol N l}^{-1}$) from Selaulim
^c Excluding 5 samples (70.97–162.14, mean $116.70 \pm 37.83 \text{ nmol N l}^{-1} \text{ d}^{-1}$) from Selaulim

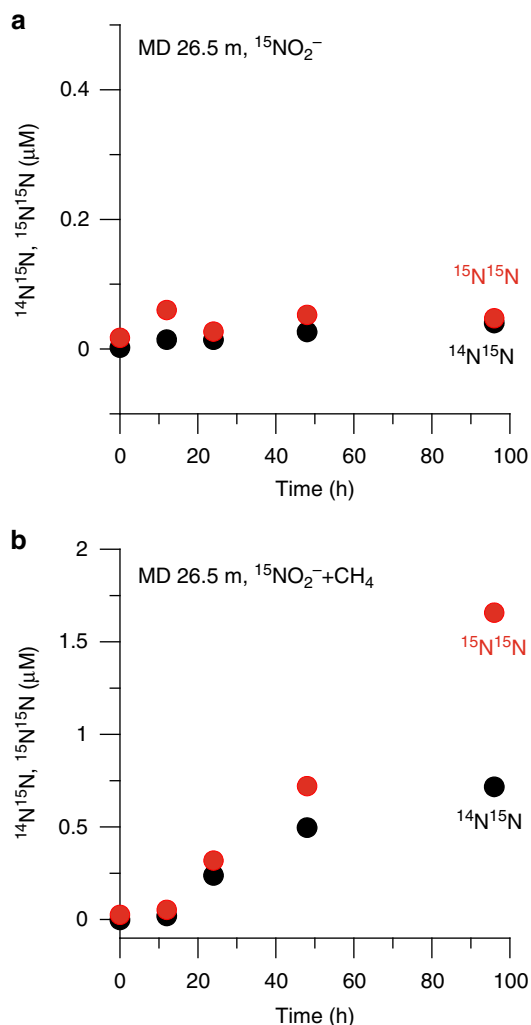


Fig. 5 Stimulation of molecular nitrogen production by methane. Changes in concentrations of $^{14}\text{N}^{15}\text{N}$ and $^{15}\text{N}^{15}\text{N}$ versus time in a water sample collected from 26.5 m depth in Markandeya Reservoir on 15.06.2011 and incubated with ^{15}N -labelled nitrite in the absence (a) and presence (b) of methane

through denitrification relative to experiments without CH_4 (Figs. 2 and 3, Supplementary Fig. 9). The nearly linear increase in $^{15}\text{N}^{15}\text{N}$ from $^{15}\text{NO}_2^-$ incubations (Fig. 5)—in all cases except one (Markandeya Reservoir, 18 m, 15.06.2011)—plus the linear increase in $^{15}\text{N}^{14}\text{N}$ from $^{15}\text{NO}_3^- + ^{14}\text{NO}_2^-$ incubations showed that NO_2^- was the key oxidant in N_2 production, and that nitrate reduction was closely coupled to this process. Even when denitrification was detectable in the absence of CH_4 ($>10 \text{ nmol N}_2 \text{ l}^{-1} \text{ d}^{-1}$; 10 out of 14 cases), denitrification rates increased by a factor of over 12 on average (mean 13.4; median 12.4) in CH_4 -enriched samples. It should be noted that almost all of these samples originally contained significant amounts of CH_4 (up to $\sim 34 \mu\text{M}$), comparable to CH_4 introduced in the amendment experiments ($\sim 44 \mu\text{M}$). However, measurements of N_2 production rates necessitate sparging of samples with helium before all incubation experiments in order to lower N_2 background prior to ^{15}N -label additions, thus also removing dissolved CH_4 originally present in samples. Interestingly, during winter in the Markandeya Reservoir when the water column was well-oxygenated, potential CH_4 -enhancement of N_2 production was also detected, albeit to a lesser extent (Fig. 3d). These samples were incubated under anoxic conditions with the dissolved oxygen removed

through helium-sparging. For these four trips to Tillari and Markandeya, DNRA rates measured from the same $^{15}\text{NO}_2^-$ incubations remained consistently low or undetectable (Supplementary Fig. 10), as did the anammox rates computed from these $^{15}\text{NO}_2^-$ incubations ($0\text{--}12.24$, mean $4.22 \pm 4.52 \text{ nmol l}^{-1} \text{ d}^{-1}$). With such negligible anammox activity, exemplified by lack of significant $^{14}\text{N}^{15}\text{N}$ production in the absence of CH_4 in Markandeya Reservoir on 15.06.2011 (Fig. 5) despite high ammonium concentrations in bottom waters (Fig. 3a), the enhancement of $^{14}\text{N}^{15}\text{N}$ production in the presence of CH_4 must then be due to denitrification.

Methanotrophic community structure. Analyses of summer microbial community structure in the Markandeya Reservoir, based on amplicon sequencing of 16S rRNA genes, revealed the presence of the NC10 bacteria capable of N-DAMO in the hypolimnion, albeit at low relative abundance (0.003–0.022%). In comparison, the conventionally known aerobic methanotrophs were prevalent not only in the oxic but also in the anoxic layer, accounting for up to almost 14% of total community at the oxycline (12 m) (Fig. 6). Among the methanotrophs, all Type I, Type II, and Type III methanotrophs were present. Type I *Methylococcaceae* usually predominated (0.97–12.23%), of which *Methylocaldum* and *Methylomonas* were frequently identified though the majority remained unresolved at the genus level (Supplementary Table 5). Type II methanotrophs, including *Methylocystaceae* and *Methylobacteriaceae*, particularly the former, gained importance at the anoxic deeper depths, whereas Type III *Methylacidiphilales* superseded *Methylococcaceae* at the upper oxic depth (Supplementary Fig. 11). The presence of these methanotrophs, especially *Methylomonas*, *Methylobacter* and *Methylocaldum* were further corroborated by phylogenetic analyses of the biomarker gene *pmoA* that encodes particulate monooxygenase gene subunit A (Supplementary Fig. 12), though the primers used would have missed Type III methanotrophs as observed. The *pmoA* sequences directly related to NC10 bacteria were however not detected in samples collected in 2011 even using NC10-specific primers⁸, most likely due to their low abundance. In addition, highly diverse community of potential denitrifiers were detected via phylogenetic analyses of biomarker gene encoding the *cd1*-containing nitrite-reductase (NirS). Most sequences obtained were closely affiliated with sequences from methane-rich settings—rice-paddy soils and freshwater sediments. Notably, one of the Markandeya NirS cluster is associated with the Type I methanotroph *Methylomonas* sp. 16a (Supplementary Fig. 13). Though not as abundant as methanotrophs, organisms feeding on C-1 compounds known as methylotrophs also made a noticeable contribution ($\leq 2.62\%$) at the same depths, especially *Methylophilaceae*. Moreover, small numbers of 16S rDNA sequences of methane-producing archaea were also retrieved especially at the lower anoxic depths based on 16S amplicon sequencing, with *Methanobacteriaceae*, *Methanocellaceae* and *Methanospirillaceae* being the most prominent (Supplementary Fig. 11, Supplementary Table 5). However, their abundances should not be treated as quantitative, as the primers used were meant for bacteria and not archaea.

Discussion

With this much expanded database, our study lends strong support to the previously reported¹⁹ absence of strong buildup of reactive nitrogen in Indian freshwater reservoirs, especially during summer when anoxic conditions develop in the hypolimnion of most reservoirs. The very low concentrations of NO_x^- in the anoxic hypolimnion can only result from dissimilatory NO_x^-

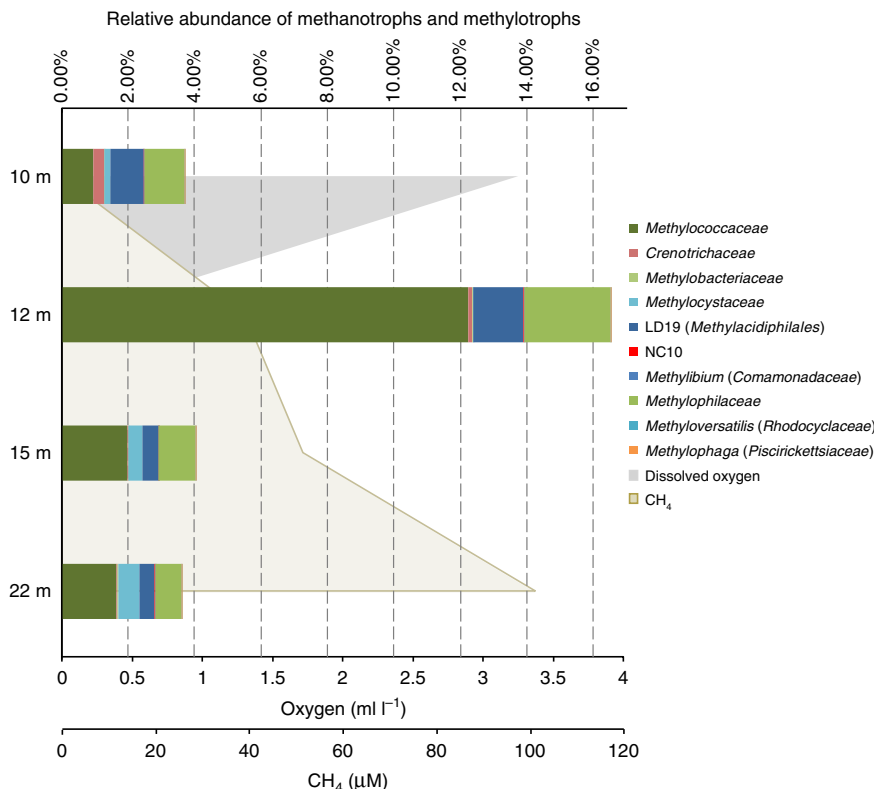


Fig. 6 Relative percentage of known methanotrophs and methylotrophs based on amplicon sequencing of 16S rRNA genes in Markandeya Reservoir. Sampling was done on 17.06.2014. All methanotrophs (shown in bold) are at the family level except for the phylum NC10, while the methylotrophs are shown at genus level except for the family *Methylophilaceae*. Dissolved oxygen and methane (CH_4) levels at corresponding depths are shown as grey and light brown shaded areas, respectively. Dissolved oxygen was undetectable at or below 15 m

reduction to N_2 through denitrification and/or anammox, or to NH_4^+ through DNRA. Their occurrences would be consistent with the accumulation of both N_2 and NH_4^+ in the anoxic hypolimnia. Nonetheless, the measured rates of denitrification, anammox and DNRA in CH_4 -free incubations—the first from any freshwater systems in South Asia—are surprisingly low (Table 1). In contrast, N_2 -production rates are high when CH_4 is added to the $^{15}\text{NO}_x^-$ incubations. Most of this N_2 -production can be attributed to denitrification, while anammox is only a minor nitrogen sink in the investigated Indian reservoirs. Our combined results provide the first direct evidence that CH_4 , which is present throughout the anoxic hypolimnia, significantly enhances denitrification rates (over 12 times) in anoxic Indian lake waters.

It is highly unlikely that the above observations resulted from methodological artefacts, because the same method yielded consistently high denitrification rates in Markandeya Reservoir in May 2007 (Supplementary Fig. 8), as well as over the western continental shelf of India during seasonal anoxia in September 2011, where additional CH_4 -enhanced N_2 production was not observed (Supplementary Fig. 14). Based on these results, we hypothesise that the stimulation of denitrification by CH_4 is restricted only to those aquatic environments where CH_4 builds up to sufficiently high levels to support the development of denitrifying methanotrophs. Unlike most marine systems, CH_4 can accumulate to very high levels (tens to hundreds of μM) in freshwater lakes and reservoirs, where methanogens face little competition from sulphate reducers for acetate^{22,23}. This is consistent with the accumulation of CH_4 and the presence of methanotrophs observed in the hypolimnion during summer stratification period.

Consequently, diverse methanotrophic communities, both aerobic and anaerobic, benefitted from this carbon source. The denitrifying methanotrophs NC10 inhabited particularly in the lower anoxic depths (Fig. 6, Supplementary Fig. 11). Although they were not exactly abundant ($\leq 0.023\%$), their low numbers are consistent with those reported in the few water-column studies to date, both marine^{17,18} and freshwater¹¹. Meanwhile, increasing evidence has demonstrated active occurrences of the traditionally considered aerobic methanotrophs and methylotrophs in hypoxic to anoxic conditions, including freshwater lakes, marine OMZs and anoxic fjords^{24–28}. Even when NC10 bacteria were present alongside, these aerobic methanotrophs were usually at least an order of magnitude more abundant, most notably being the Type I *Methylococcaceae*, as has also been observed here in Markandeya Reservoir. In fact, some studies have even reported occurrences of *Methylobacter* (*Methylococcaceae*) only in anoxic but not oxic layers in some stratified lakes^{27,28}, indicating likely alternative anaerobic lifestyles.

Recent whole genome and environmental metagenome analyses have revealed that various assimilatory and dissimilatory nitrogen reduction genes, such as those encoding nitrate, nitrite, and nitric oxide- reductases, are relatively widespread among all Types I–III methanotrophs, possibly through horizontal gene transfers²⁵. While denitrifying microbial communities are undeniably diverse and no primer sets to date have been adequate to capture the full spectrum of denitrifiers by targeting the biomarker gene *nirS*²⁹, our limited denitrifier screening has already revealed a potential relative to a denitrifying methanotroph *Methylomonas* sp., and *Methylomonas* was one of the more abundant genera found in Markandeya. Using cultured strains, nitrate reduction activity has been demonstrated with

*Methylomonas denitrificans*³⁰, while the Type II *Methylocystis* sp. SC2 has been shown capable of N₂ production from nitrate with methanol as a carbon source under anoxic conditions³¹. Genome analyses and anaerobic experiments with enrichment cultures indicate that the filamentous methanotroph *Crenothrix*, also present in Markandeya Reservoir (of *Crenotrichaceae*, Fig. 6, Supplementary Table 5), can reduce nitrate to N₂O³², just like the methylotroph *Methylotenera*³³. In other words, while the NC10 bacteria present were likely conducting N-DAMO, the much more prevalent conventional methanotrophs and methylotrophs could potentially be more important contributors to nitrogen reduction steps linked to methanotrophy.

It should be noted, however, that most of the genomes of conventional methanotrophs analysed to date still lack the nitrous oxide reductase gene (*nos*) responsible for the conversion of N₂O to N₂, which seems to be at odds with our observed general lack of N₂O accumulation in the reservoirs and CH₄-enhanced production of ¹⁵N-labelled N₂ in incubated samples. Nevertheless, the genetic capability to oxygenically dismutate nitric oxide (NO) to produce N₂ that bypasses N₂O—the pathway used by NC10 bacteria⁵—has recently been found to be more widespread in bacteria other than NC10: The *nod* gene encoding putative NO dismutase has been detected in the alkane-oxidising proteobacterium Hdn1, and in a number of samples from contaminated aquifers and wastewater treatment systems³⁴. A further search for this gene in public databases (NCBI and EMBL), including available whole genomes and environmental metagenomes, however, did not yield any reliable positive hits (below 36% amino acids identity) apart from those already reported³⁴, and none so far in conventional aerobic γ -/ α -proteobacterial methanotrophs. Therefore, while environmental metagenomics studies of such systems have been sparse and the ability to dismutate NO remains to be fully explored within the methanotrophic community residing in the hypolimnia, our combined data thus far do not support N-DAMO by NC10 being the major contributor to our observed methane-induced denitrification.

It has been reported that ANME-2d archaea related to '*Candidatus* Methanoperedens nitroreducens' oxidise CH₄ with NO₃⁻ under anaerobic conditions, but *M. nitroreducens* only reduces NO₃⁻ to NO₂⁻, which may then be converted to N₂ by either anammox or NC10 bacteria³⁵. Despite the measured anammox rates being generally low and the abundance of anammox bacteria ($\leq 0.013\%$) in the sequencing data being about half that of NC10 bacteria, it cannot be completely ruled out that the anammox bacteria were also involved in some N₂ production. Although several studies have reported co-occurrence of NC10 bacteria and ANME-2d archaea^{36–38}, the NC10 bacteria, or for that matter any other microbes mediating the production of N₂ from NO₂⁻, do not have to rely only on *M. nitroreducens* for NO₂⁻ supply, given multiple NO₂⁻ sources, including the denitrifying methanotrophic bacteria. Significantly, in incubation experiments with ¹⁵NO₃⁻ and ¹⁴NO₂⁻ combined, we observed linear production of ¹⁵N-labelled N₂ (largely ¹⁴N¹⁵N due to high ¹⁴NO₂⁻ levels) in the presence of CH₄ but not in its absence (Supplementary Fig. 9). This clearly shows the production of NO₂⁻ from NO₃⁻, which is rapidly converted to N₂, only in the presence of CH₄.

Hence, the CH₄-dependent production of N₂ from NO_x⁻ is best explained by the concerted activity of a diverse microbial community comprising NC10 bacteria, canonical denitrifiers, anammox bacteria and aerobic methanotrophic α - and γ -proteobacteria. Considering the low abundance of NC10 bacteria, methanotrophic α - and γ -proteobacteria perhaps account for a large part of the observed effect. They could switch from respiring oxygen to nitrate, and might either produce N₂ themselves or N-compounds of intermediate redox states that are further reduced to N₂ by canonical denitrifiers or anammox

bacteria. Further assessment of single-cell activity and (meta)genomics/transcriptomics analyses are necessary to pinpoint the exact nature (single-organism versus syntrophic modes) of the observed denitrifying methanotrophy. The removal of NO₃⁻ and NO₂⁻ in the presence of CH₄ may explain the lack of large NO₂⁻ accumulation observed in reservoirs as compared to several other marine (e.g., up to 23 μ M for Peru³⁹, 16 μ M off western India⁴⁰) and freshwater (e.g., 18 μ M in Lake Kinneret⁴¹) systems.

The data from Markandeya Reservoir on 03.05.2007 show that canonical denitrification could occasionally be an important nitrogen loss pathway. What determines the relative importance of canonical and CH₄-dependent denitrification is, however, unclear. Oxygen concentrations of a few 100's nanomolar, such as determined by the highly sensitive STOX (Switchable Trace amount Oxygen) sensors, can be sufficient to inhibit denitrification in seawater^{42,43}. Thus, despite experimental evidence for aerobic denitrification in intertidal sediments⁴⁴, water-columns in regions like the Bay of Bengal and the Gulf of California do not exhibit pronounced nitrogen losses as dissolved oxygen, though vanishingly low, remains detectable within their OMZs^{45–47}. Analogous to these observations, we propose that since the anoxic hypolimnia are located very close to the surface, canonical denitrification pathway may be hampered by frequent incursions of oxygen into the anoxic zones of these reservoirs. The sensitivity of denitrifying methanotrophs to oxygen at the very low range is poorly known, as the only study that assessed oxygen inhibition on NC10 bacteria utilised relatively high oxygen concentration (>2%)⁴⁸. However, since these bacteria can themselves produce oxygen, they are expected to tolerate higher oxygen levels than canonical denitrifiers. Even though the latter are facultative anaerobes, they still require almost complete anoxia to switch from aerobic to nitrate/nitrite respiration⁴³. The conventional methanotrophs, also postulated to be involved in our observed denitrifying methanotrophy, are known to be normally aerobes themselves. Therefore, given the availability of sufficient CH₄, denitrifying methanotrophs may out-compete canonical denitrifiers for NO₂⁻. However, canonical denitrification is expected to become important if and when truly anoxic conditions persist long enough for the facultative bacteria to activate their denitrification enzymes, such as during calm weather conditions and strong stratification over extended periods, as presumably experienced during the May 2007 visit to Markandeya Reservoir.

In spite of the heavy anthropogenic impacts on Markandeya Reservoir, the nitrogen inventory therein decreased over the winter/spring mixing period when the water-column was well-oxygenated (from post-monsoon to winter to early spring; note that the only input of nitrogen to the reservoirs occurs during the monsoon). The reason for this decrease is unclear, but we speculate that it is due to the nitrogen uptake by phytoplankton followed by sedimentation of organic matter to the bottom. It is not known what fraction of nitrogen is permanently buried in the sediment, as opposed to how much is regenerated to NH₄⁺ in the water column through aerobic respiration and how much is lost via benthic denitrification to N₂/N₂O. Benthic respiration is expected to be very important in shallow reservoirs²¹. It is likely that a significant amount of NH₄⁺ in the water column originates from the sediment through degradation of organic matter via sulphate reduction. Smaller contribution could also come from DNRA both in the water column and sediments. The loss of N₂ may occur throughout the year through coupling between denitrification and methanotrophy within the sediment. This is supported by the appreciable N₂ production observed in the near-bottom samples of Markandeya in January 2012, when the oxygen-stripped samples were incubated with ¹⁵NO₂⁻ and CH₄ (Fig. 3d).

Generally low nitrate concentrations in CH₄-bearing hypolimnia combined with reduced CH₄ concentrations in the absence of measurable oxygen are consistent with the proposed CH₄ involvement in nitrogen loss (Figs. 2a, c, 3a, Supplementary Fig. 2). This phenomenon may in part be responsible for the observed lower-than-expected² runoff of reactive nitrogen by rivers in South Asia⁴⁹, as well as the moderate build-up of CH₄ in the reservoirs¹⁹. Moreover, our data on N₂O in Indian reservoirs, one of the largest of their kind from the freshwater systems in the world, indicate that a very small fraction of nitrogen loss is in the form of N₂O. As a by-product of nitrification and an intermediate of denitrification, N₂O in oxygenated waters is mostly attributed to nitrification^{50–52}. Although N₂O production has been observed to be linearly related to oxygen consumption in some systems (e.g., in Lake Kizaki, Japan⁵⁰), such a relationship is not universal⁵². Nitrification rates were not measured in the present study. However, going by the relationship reported from Lake Kizaki⁵⁰, a water sample having a temperature of 23 °C (typical of bottom waters in Markandeya Reservoir) is expected to contain ~74.4 nM N₂O (8.3 nM saturation value plus 66.1 nM produced through nitrification) when dissolved oxygen has been fully consumed. Since ~84% of our measured N₂O concentrations were below 20 nM, nitrification is most likely responsible for most of the N₂O in the reservoirs sampled. The yield of N₂O during denitrification in various environments is highly variable^{53,54}. In the relatively well-studied lentic freshwater and marine ecosystems, it generally varies between 0.1 and 1.0%, although values as high as 6% have been reported⁵⁴. Accordingly, N₂O concentrations observed in anoxic freshwaters vary from below detection to several micromolar^{21,52,55,56}. This is because while N₂O is produced as well as consumed during denitrification, the highest (micromolar) N₂O concentrations observed in both marine²² and freshwater^{52,55,56} ecosystems (a maximum of 88,400% N₂O saturation in the anoxic hypolimnion of the Brookville Lake⁵⁶) have been ascribed to this process. The moderately high peak N₂O concentration measured in this study (357.2 nM, or 4,544% saturation) at 45 m in Tillari Reservoir on 03.05.2012, when the water column was very strongly stratified, is similarly attributed to CH₄-independent denitrification. This, however, was only a rare occurrence, and denitrification generally does not seem to be a major contributor to N₂O production in the Indian freshwater reservoirs.

There exist striking similarities between conditions prevailing in the freshwater systems examined here and those found in semi-enclosed marine basins, such as the Black Sea, Baltic Sea, Cariaco Basin and the Saanich Inlet, where CH₄ accumulates in high concentrations in anoxic waters below the sill depth²². Concentrations of both NO₂⁻ and N₂O have been found to be consistently low above the oxic–anoxic interface (within the so called suboxic zone) in these basins as compared to the open ocean OMZs²². Hence, it is likely that the coupling between denitrification and methanotrophy may be more widespread and of much larger geochemical and environmental significance than realised thus far.

Methods

Sampling sites and measurements made. Fifteen dam-reservoirs (see Supplementary Fig. 1 for locations) were chosen for sampling in the present study that extended over a period of 9 years (2006–2015). Of these reservoirs, some were visited only once during the summer season, but a few others were sampled repeatedly, covering different seasons (Supplementary Table 1). Observations made included a number of biogeochemical parameters like temperature, dissolved oxygen, NO₃⁻, NO₂⁻, NH₄⁺, N₂O, CH₄ and N₂/Ar, as well as various nitrogen transformation rates determined by ¹⁵N-labelling experiments (Supplementary Tables 1 and 2). However, not all measurements could be made at the same time and in all reservoirs for logistic reasons. The two most frequently sampled reservoirs (Tillari and Markandeya) were chosen for more detailed studies because of logistic convenience, as they are within easy reach from the CSIR-National Institute

of Oceanography (NIO), Goa. They also represent two contrasting extents of anthropogenic impacts, the Tillari being more pristine.

Sample collection and chemical analysis. Sampling was carried out using an inflatable boat. Niskin samplers (5 litre) fitted with reversing thermometers and mounted on nylon ropes were used for the collection of water samples. Only a single sub-sample was taken from each depth for each measurement, including ¹⁵N-labelling experiments. Subsamples for dissolved oxygen were fixed immediately and analysed the same day following the Winkler procedure (precision ±0.03 ml l⁻¹). Subsamples for nutrients were stored in an ice box and frozen on arrival at the laboratory until analysis, usually carried out the next day following standard procedures⁵⁷ using a SKALAR analyzer. Separate subsamples were taken in ground-glass stoppered bottles from each sampling depth, one each for N₂O and CH₄, and preserved with HgCl₂ (500 µl saturated solution/100 ml sample). The analyses were performed within a few days of collection by head-space extraction⁵⁸ with helium followed by injection into gas chromatographs equipped with electron capture detector (for N₂O) and flame ionisation detector (for CH₄) with precisions of ~4% and 7%, respectively. Samples for N₂/Ar measurements were collected from four reservoirs in 60-ml serum bottles. Samples were preserved with 300 µl of saturated HgCl₂ solution and analysed three months later using a Hidden Quadrupole Membrane Inlet Mass Spectrometer. Preparation of standards and calibration were done following Hartnett and Seitzinger⁵⁹ and Charoenpong et al.⁶⁰. Excess N₂ was computed from the N₂/Ar ratio from N₂ solubility⁶¹ at in-situ temperature.

Labelling experiments. For rate determination of denitrification, anammox and DNRA, ¹⁵N-labelling experiments were performed as per the procedures described in Holtappels et al.⁶². All sample processing and ¹⁵N-substrate additions were carried out at the sites, except for Tillari, samples from where were brought to NIO and processed on the same day. Briefly, 250 ml of sample was purged with ultra-pure helium for 10 min. Substrates were then added to get the following final concentrations: 8 µM ¹⁵NO₂⁻ (denitrification and anammox), 8 µM ¹⁵NH₄⁺ (anammox), 8 µM ¹⁵NO₂⁻ + 8 µM ¹⁴NH₄⁺ (anammox + denitrification) and 16 or 20 µM ¹⁵NO₃⁻ (DNRA). The sample was purged with helium for 5 more minutes and then dispensed into 12-ml Exetainer glass vials (Labco) by applying a slight helium overpressure and allowing sufficient overflow. Incubations were conducted at near in situ temperatures in the dark, and were terminated at pre-determined time intervals by introducing a 2-ml helium headspace and adding 100 µl of saturated HgCl₂ solution. Inverted Exetainer vials were transferred as soon as possible to Max-Planck Institute for Marine Microbiology, Bremen, where ¹⁴N¹⁵N:¹⁴N¹⁴N and ¹⁵N¹⁵N:¹⁴N¹⁴N ratios in He-headspaces were determined by gas chromatography/isotope ratio mass spectrometry (Fisons VG Optima). The analysis was completed within a few weeks of sampling.

After analysis of labelled N₂ in the headspace, samples incubated with ¹⁵NO₂⁻ and ¹⁵NO₃⁻ were used to determine DNRA rates. NH₄⁺ was oxidised to N₂ using sodium hypobromite (NaOBr) for measuring its isotopic composition. Briefly, 5 µM ¹⁴NH₄⁺ and 200 µl of 4 M NaOBr were added to 5-ml of He-flushed sample in 6-ml Exetainers containing 1-ml He headspace. Isotopic ratios in N₂ in headspace were measured as described above.

For the experiments involving CH₄, deionised water filtered through 0.2 µm was saturated with CH₄. 10-ml of this water was added to samples (250 ml) previously sparged with helium and the incubations were carried out as for other samples after spiking with 20 µM ¹⁵NO₂⁻ or 20 µM ¹⁴NO₂⁻ + 20 µM ¹⁵NO₃⁻. Random analysis of CH₄-enriched samples (*n* = 5) yielded an average CH₄ concentration of ~44 µM, which is comparable to the observed in situ CH₄ concentrations in the samples (Figs. 2a, c, 3a).

Detection of functional and 16S rRNA genes. For the detection of biomarker functional genes, water samples were collected from Markandeya Reservoir on 15.06.2011 and filtered on to polycarbonate membrane filters or Sterivex cartridge filters (both of 0.22 µm pore size, Millipore). These filters were stored frozen until nucleic acids extractions. DNA was extracted from filter samples collected from 10 m, 12.5 m, 15 m, 17.5 m, 24 m, and a CH₄-amended incubation sample at 26.5 m. In addition, on a subsequent trip to the reservoir on 16.08.2011 when the water column became fully oxygenated and quite turbid following runoff during the monsoon, water sample was collected close to the bottom (30 m). This sample was filtered first through a 0.45 µm pore-size filter and then through a 0.2 µm filter, and analyses were performed on both filters. The *pmoA* and *nirS* genes were amplified with the primers A186-A689⁶³ and nirS1F-nirS6R⁶⁴. The *pmoA* of NC10 bacteria were also specially targeted by both nested and non-nested PCR, as previously described⁸, but no true *pmoA* sequences were yielded. PCR products of correct sizes, as checked with 2% agarose gel electrophoresis and visualised with SYBR Green staining, were gel-purified with the QIAquick PCR Purification Kit (Qiagen). Clone libraries were constructed for samples from 15 m, 26.5 m (CH₄-amended), 30 m (0.45 µm filtered) and 30 m (≥45 µm particulates) by using the TOPO TA Cloning Kit for Sequencing (pCR4 vector, Invitrogen). Ninety-six colonies were randomly picked from each clone library and PCR-screened with the primers M13F-M13R. Amplicons of 48 randomly chosen clone inserts were then submitted to GATC Biotech (Konstanz, Germany) for DNA sequencing. Phylogenetic

relationships were deduced based on amino acids sequences via neighbour-joining, maximum parsimony and maximum likelihood algorithms, with topologies further verified by bootstrapped resampling ($\times 1000$ for neighbour joining and $\times 100$ for maximum parsimony and likelihood methods), by using the ARB software package⁶⁵.

Water samples from 0, 10, 12, 15, and 22 m depths (2 litre each) were collected again on 17.06.2014 from Markandeya Reservoir. These were filtered on site through 0.22 μm Sterivex cartridge filters (Millipore) using a peristaltic pump. The filters were preserved with DNA storage buffer until nucleic acid extraction. DNA was extracted from the filters using Mo Bio Power Water DNA isolation kit (Cat # 14900-50-NF) as per the manufacturer's protocol. From each of the samples, 1 μg of clean genomic DNA was used for further analysis. The bacterial community present in the sample was evaluated using Illumina MiSeq Platform by sequencing the V3 and V6 regions of the 16S rDNA (Genotypic Technology Pvt. Ltd., Bangalore, India). Briefly, the primers V3F (5' CCAGACTCCTACGGGAGGCGAC 3' and V3R (5' CGTATTACCGCGGCTGCTG 3') targeting V3 hypervariable regions and V6F (5' TCGATGCAACGCGAAGAA 3') and V6R (5' ACATTCACAACACGAGCTGACGA 3') targeting V6 hypervariable regions of 16S rDNA genes were selected. Both forward and reverse primers were tagged with adaptor, pad and linker sequences. The PCR amplification was carried out and amplicons purified. The purified mixture was further processed as per MiSeq Reagent Kit Preparation Guide (Illumina, USA) and loaded on Illumina MiSeq for sequencing. After sequencing, the Illumina paired end raw reads was quality checked using SeqQC v2.1. The quality of demultiplexed raw read pairs was checked with FastQC (v 0.11.4; Babraham Bioinformatics). Forward and reverse reads with a maximum read length of 500 bp and a minimum Phred33 quality score of 0.8 were merged with the PANDAseq assembler software (v 2.8)⁶⁶. The merged sequences were quality-checked again with the FastQC software. Open reference OTU clustering was subsequently performed under QIIME (MacQIIME v 1.9.1_20150604; Caporaso et al.⁶⁷), using the UCLUST algorithm with a minimum sequence identity of 97% and the 16S rRNA Silva database (v 128)⁶⁸. Singleton OTUs and sequences affiliated with chloroplastic rDNA were removed from the data set. Due to the short read lengths of V6 sequences that yield much fewer OTUs, only V3 results are presented here.

Data availability. The data used in the study will be archived at National Institute of Oceanography Data Centre (did.nio.org) and made available on request. Nucleotide sequences obtained from this study have been deposited in the GenBank, under the accession numbers MG992020 to MG992185 for *nirS* genes, MG992186 to MG992311 for *pmoA* genes, and SRX3744137 to SRX3744145 for 16S rRNA genes.

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Author contributions

S.W.A.N., G.L., and M.M.M.K. designed the experiments. S.W.A.N., G.N., A.S., H.N., A.P., D.M.S., M.G., S.K., and S.D. participated in the field work. S.W.A.N., G.N., A.S., and H.N. performed ¹⁵N-labelling experiments. S.W.A.N., P.L., G.L., A.S., A.P., and S.D. analysed samples and processed data. M.D. assisted in processing of molecular data. S.W. A.N. and P.L. wrote the manuscript with input from all co-authors.

Additional information

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