Quantifying Potential N Turnover Rates in Hypersaline Microbial Mats by Using ^{15}N Tracer Techniques

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ABSTRACT Microbial mats, due to stratification of the redox zones, have the potential to include a complete N cycle; however, an attempt to evaluate a complete N cycle in these ecosystems has not been yet made. In this study, the occurrence and rates of major N cycle processes were evaluated in intact microbial mats from Elkhorn Slough, Monterey Bay, CA, USA, and Baja California Sur, Mexico, under oxic and anoxic conditions using ^{15}N -labeling techniques. All the major N transformation pathways, with the exception of anammox, were detected in both microbial mats. Nitrification rates were found to be low at both sites for both seasons investigated. The highest rates of ammonium assimilation were measured in Elkhorn Slough mats in April and corresponded to high *in situ* ammonium concentrations in the overlying water. Baja mats featured higher ammonification than ammonium assimilation rates, and this, along with their higher affinity for nitrate compared to ammonium and low dissimilatory nitrate reduction to ammonium rates, characterized their differences from Elkhorn Slough mats. Nitrogen fixation rates in Elkhorn Slough microbial mats were found to be low, implying that other processes, such as recycling and assimilation from water, are the main sources of N for these mats at the times sampled. Denitrification in all the mats was incomplete, with nitrous oxide as the end product and not dinitrogen. Our findings highlight N cycling features not previously quantified in microbial mats and indicate a need for further investigations of these microbial ecosystems.

IMPORTANCE Nitrogen is essential for life. The nitrogen cycle on Earth is mediated by microbial activity and has had a profound impact on both the atmosphere and the biosphere throughout geologic time. Microbial mats, present in many modern environments, have been regarded as living records of the organisms, genes, and phylogenies of microbes, as they are one of the most ancient ecosystems on Earth. While rates of major nitrogen metabolic pathways have been evaluated in a number of ecosystems, they remain elusive in microbial mats. In particular, it is unclear what factors affect nitrogen cycling in these ecosystems and how morphological differences between mats impact nitrogen transformations. In this study, we investigate nitrogen cycling in two microbial mats having morphological differences. Our findings provide insight for further understanding of biogeochemistry and microbial ecology of microbial mats.

KEYWORDS microbial mats, nitrogen, stable isotope labeling, incomplete denitrification, nitrous oxide

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Microbial mats are multilayered sheets of *Eukarya*, *Bacteria*, and *Archaea*, which under moist conditions are usually held together by extracellular polymeric

substances secreted by these microorganisms (1). Microbial mats have been found in extreme environments where competition from other microbial groups and predation by grazing organisms are limited, such as the benthic-planktonic interface of hot springs, deep-sea vents, hypersaline lakes, and marine estuaries (2), as well as in dry environments like deserts (3). Most, if not all, biogeochemical processes that exist in aquatic ecosystems are considered to occur in microbial mats, due to the presence of highly diverse physiological groups of microorganisms therein (4, 5). Microbial mats have layered redox stratification during the day, when photosynthesis is occurring, and are completely anoxic during the night (6). This makes them ideal communities for the study of selective pressures acting separately on anaerobic and aerobic metabolism of the inhabiting microbes.

Microorganisms have an important role in the global nitrogen (N) cycle. The N cycle has had a profound impact on both the atmosphere and the biosphere throughout geologic time. There is significant evidence that N availability limits production of biomass on Earth today and has over geologic time (7–11). Briefly, the major microbial transformations of N are as follows: N-fixing microbes combine gaseous N with hydrogen to produce ammonia, present in these environmental conditions in its protonated form, ammonium (NH_4^+), which is assimilated by microorganisms and further incorporated into organic compounds. Through ammonification, organic N can be converted back into NH_4^+ . This NH_4^+ is oxidized to nitrite (NO_2^-) and nitrate (NO_3^-) with oxygen (O_2) as the terminal electron acceptor in the process known as nitrification. The produced NO_3^- can further undergo denitrification, mostly under anaerobic conditions, which is the stepwise reduction of NO_3^- via NO_2^- to nitrous oxide (N_2O) and dinitrogen (N_2) using organic carbon, hydrogen, or reduced inorganic species (e.g., Fe and S) as electron donors. Nitrate can also be reduced back to NH_4^+ in the process known as dissimilatory nitrate reduction to ammonium (DNRA). Along with denitrification, anaerobic ammonium oxidation (anammox) is a N cycle transformation that contributes to net loss of fixed N in the environment. Here, NH_4^+ and NO_2^- are combined to form N_2 with a side production of NO_3^- (12).

While extensive research has been performed on N cycling processes in soils and marine ecosystems, N cycling in microbial mats has received limited study. Most studies of N cycling in microbial mats have focused on the process of N_2 fixation (e.g., studies by Omeregic et al. in 2004, Moisaner et al. in 2006, and Woebken et al. in 2015 [13–15]). This is likely due to both its importance as a source of reactive N in otherwise low-N environments and the relative ease with which it may be measured using acetylene reduction (16) and $^{15}\text{N}_2$ incorporation (17) techniques. Some studies have been focused on nitrification and denitrification in mat communities (3, 18–20). While nitrification rates have been quantified and a nitrifying community investigated in a few microbial mats (19, 21), the interactions between nitrification and other NH_4^+ -consuming processes, such as NH_4^+ assimilation in microbial mats, have not been evaluated. It has been shown that assimilation of NH_4^+ from the water column and ammonification from deeper layers can be the main sources of N for some microbial mats (22). Many other N transformations, like anammox and DNRA, have been overlooked in microbial mats. In the only study measuring anammox in hypersaline photosynthetic mats, this process was undetected (3). DNRA has been shown to occur in marine microbial mats (19) and along with ammonification and N_2 fixation can contribute to the pool of NH_4^+ in mats.

Our knowledge of N cycling in microbial mats is at a rudimentary state, and there is a need for comprehensive studies evaluating a complete N budget for different types of microbial mats. In this study, we address the following objectives: (i) to detect and quantify the major N pathways in two photosynthetic microbial mats, (ii) to assess if there are substantial differences in N transformations between two different types of photosynthetic mats, and (iii) to explore the effect of environmental conditions on N cycling in studied microbial mats. The mats studied here were chosen as they grow in very different environments. One of the two mats studied (from Baja California,

Mexico) is constantly submerged, whereas the other mat (from Elkhorn Slough [ES], Monterey Bay, CA, USA) grows in an intertidal environment (Fig. 1). Both mats experience turbulence from storm events, but the disturbance caused by these events is likely far greater at the Elkhorn Slough field site. Physical disturbances resulting from a combination of wind-driven water movement, desiccation impacts on the attachment of the mats to sediment, and even disturbance by fauna result in far greater disruption of physical structure and integrity in the ES mats than the Baja mats. Differences in some nitrogen cycling processes, such as assimilation and ammonification, between the Baja mats and a mat very similar to the ES mat have previously been reported (22). Here, we present a more comprehensive study of nitrogen cycling processes in mats located in very different physical environments, which we hypothesized have a profound effect on nitrogen cycling. We determined the occurrence and rates of major N cycling processes in order to assess a complete N budget for two different types of microbial mats and for two seasons in one of them. For consistency, all rates were measured using ^{15}N -labeling techniques.

RESULTS

Nitrification and nitrate assimilation/aerobic denitrification. The nitrification rate was determined as NO_3^- production in microbial mat incubations amended with $^{15}\text{NO}_3^-$ under light. In the same incubations, measured NO_3^- consumption reflected a NO_3^- assimilation/aerobic denitrification rate. In general, while the NO_3^- concentration decreased significantly over experimental time in all incubations, the ^{15}N dilutions of the NO_3^- pool were low for both types of microbial mats, ranging from 0.1 to 0.2 atom% for ES mats in October to 0.4 atom% for Baja mats in February (Fig. 2). These correspond to low NO_3^- production rates, which were calculated to be $3.1 \pm 1.7 \text{ mmol N m}^{-2} \text{ day}^{-1}$ in ES October mats, $5.6 \pm 3.2 \text{ mmol N m}^{-2} \text{ day}^{-1}$ in ES April mats, and $6.5 \pm 5.1 \text{ mmol N m}^{-2} \text{ day}^{-1}$ in Baja February mats. Gross NO_3^- consumption rates were higher than NO_3^- production rates: $28.1 \pm 5.1 \text{ mmol N m}^{-2} \text{ day}^{-1}$ in ES October mats, $23.9 \pm 16.2 \text{ mmol N m}^{-2} \text{ day}^{-1}$ in ES April mats, and $47.8 \pm 23.5 \text{ mmol N m}^{-2} \text{ day}^{-1}$ in Baja February mats. Net NO_3^- consumption rates were slightly lower than gross NO_3^- consumption rates and were $25.0 \pm 5.2 \text{ mmol N m}^{-2} \text{ day}^{-1}$ in the ES October mats, $18.3 \pm 13.1 \text{ mmol N m}^{-2} \text{ day}^{-1}$ in the ES April mats, and the highest in Baja mats, at $41.3 \pm 20.3 \text{ mmol N m}^{-2} \text{ day}^{-1}$. Means of all the estimated rates were somewhat higher in Baja mats than in ES mats, although the differences were not statistically significant ($P > 0.05$).

Ammonification and ammonia assimilation. Ammonification and ammonia assimilation rates were evaluated as NH_4^+ production and consumption, respectively, in microbial mat incubations amended with $^{15}\text{NH}_4^+$ and nitrapyrin for nitrification inhibition under light (Fig. 3). The results reveal strong gross NH_4^+ consumption by ES microbial mats in October ($14.9 \text{ mmol N m}^{-2} \text{ day}^{-1}$), but weak NH_4^+ production ($2.4 \text{ mmol N m}^{-2} \text{ day}^{-1}$), as indicated by a significant decrease in the NH_4^+ concentration and a weak decrease of ^{15}N signal. Data for ES April mats show a significant decrease of both the ^{15}N signal and NH_4^+ concentration, which reflects strong NH_4^+ production ($22.3 \pm 23.0 \text{ mmol N m}^{-2} \text{ day}^{-1}$) and gross NH_4^+ consumption ($85.1 \pm 66.5 \text{ mmol N m}^{-2} \text{ day}^{-1}$). For Baja February mats, a strong decrease in ^{15}N signal is observed, but no substantial decrease in NH_4^+ concentration, which means NH_4^+ production and gross NH_4^+ consumption are almost equal, resulting in a rather balanced N turnover (24.0 ± 2.9 and $17.8 \pm 3.7 \text{ mmol N m}^{-2} \text{ day}^{-1}$, respectively). In the case of the results for Baja February mats, a higher NH_4^+ production rate than a gross NH_4^+ consumption rate resulted in the mat being a net producer of NH_4^+ ($-6.3 \pm 6.6 \text{ mmol N m}^{-2} \text{ day}^{-1}$). The means of both net and gross consumption rates were somewhat higher in ES mats in April than in ES mats in October and Baja mats in February, although the differences were not statistically significant ($P > 0.05$).

Anammox and denitrification. For anammox rate evaluation, the nonrandom distribution approach for ^{15}N in N_2 was used based on the analyses of $^{29}\text{N}_2$ and $^{30}\text{N}_2$ after amendment of the microbial mat incubations in the dark with $^{15}\text{NO}_2^-$ and NH_4^+ . A ^{15}N enrichment in N_2 was, however, not detectable in any of the incubations, and the $^{29}\text{N}_2$

FIG 1 View of the Baja (A) and Elkhorn Slough (ES [C]) field sites and samples of microbial mat (inset) from each of the field sites. Cross-sectional views of the Baja (B) and ES (E) mats are shown with representative oxygen microelectrode profiles under lighted (yellow symbols) and dark (black symbols) conditions. The oxygen microelectrode profiles of the Baja mat (C) and ES mat (F) are plotted at the same spatial scale as the mat cross sections. Error bars in the microelectrode profiles represent the standard deviation of 3 profiles taken in the same location at steady state. The view of the ES field site in panel D shows a very heterogeneous distribution of mats, mats of apparently different thicknesses, and desiccation features resulting in mats wetted to different degrees. In contrast, the mats on the floor of the saltern where the Baja mats were collected (A) can be almost completely flat across tens of meters of horizontal distance. Photo credits: Skylar J. Laham (A), Leslie Bebout (B), Natalie Jones (E).

level was not higher than that of laboratory air (data not shown); therefore, anammox activity was not detected.

Denitrification was assessed in microbial mats as production of ^{15}N -labeled N_2 and N_2O gases after amendment of the microbial mat incubations in the dark with $^{15}\text{NO}_3^-$. Interestingly, ^{15}N enrichment in N_2 was lacking in all investigated mats and seasons. ^{15}N enrichment in N_2O , however, was found in all incubations, although each data set

FIG 2 Nitrate turnover for ES mats in October and April and Baja mats in February.

FIG 3 Ammonium turnover (without nitrification) for ES mats in October and April and Baja mats in February. (Due to loss of replicates, only one value is shown for ES October.)

represents different patterns. In the of Baja February mats, weak $^{15}\text{N}_2\text{O}$ enrichments were detected; however, all the N_2O concentrations were below the limit of quantification (<0.3 ppmv). This corresponds to a maximum N_2O production rate below $1.0 \pm 0.4 \mu\text{mol N}_2\text{O-N m}^{-2} \text{day}^{-1}$. A peak of enrichment of about 0.4 atom% was observed after a 1-h incubation time (Fig. 4).

ES mats in October produced a strong ^{15}N enrichment of N_2O at time step zero (less than 5 s of incubation) (Fig. 5a). ^{15}N enrichment of N_2O abundance increased from 75.2 ± 10.4 atom% at time zero to 86.9 ± 1.3 atom% at incubation time 3 h and subsequently decreased to 74.0 ± 9.5 atom% at the end of the experiment. As for N_2O production rates, N_2O production was found to be the highest at incubation time 1 h ($4.0 \pm 0.8 \text{ mmol N}_2\text{O-N m}^{-2} \text{day}^{-1}$), and the average rate was $2.3 \pm 1.7 \text{ mmol N}_2\text{O-N m}^{-2} \text{day}^{-1}$.

In contrast to ES October mat data set, the ^{15}N data for N_2O in ES April mats (Fig. 5b) indicated a consistent increase in ^{15}N abundance over time that reached 2.9 ± 1.0 atom% after 6 h of the experiment. N_2O production also increased over incubation time, with an average of $5.2 \pm 4.1 \mu\text{mol N}_2\text{O-N m}^{-2} \text{day}^{-1}$.

DNRA. DNRA rates were calculated from the isotopic enrichment of the NH_4^+ pool after addition of ^{15}N -labeled NO_3^- to microbial mats in the dark in the same incubations that were used for the denitrification rate measurement. ES microbial mats in April exhibited a higher DNRA rate than ES mats in October (1.8 ± 0.8 and $0.4 \pm 0.2 \text{ mmol N m}^{-2} \text{day}^{-1}$, respectively), and generally, ES mats exhibited higher DNRA rates than Baja mats in February ($0.2 \text{ mmol N m}^{-2} \text{day}^{-1}$) ($P = 0.022$) (Fig. 6).

FIG 4 ^{15}N abundance of nitrous oxide in Baja mats in February.

FIG 5 Nitrous oxide production rates (bars) and $^{15}\text{N}_2\text{O}$ (lines) in ES mats in October (a) and April (b). Units of the y axis of panels a and b differ by 3 orders of magnitude.

Nitrogen fixation. The nitrogen fixation rate was evaluated by two techniques: $^{15}\text{N}_2$ isotope labeling and acetylene reduction assay (ARA). Due to experimental failure with N_2 fixation rate measurement for Baja February and ES October mats, only data for ES April mats will be shown here. ES April microbial mats were preincubated under light prior to being placed in the dark for N_2 fixation rate determination during the night. The average rate of N_2 fixation as measured by the $^{15}\text{N}_2$ -labeling technique was found to be $8.1 \pm 3.1 \mu\text{mol N m}^{-2} \text{day}^{-1}$, and the average rate of ethylene production as measured by ARA was $555.4 \pm 257.5 \mu\text{mol C}_2\text{H}_4 \text{m}^{-2} \text{day}^{-1}$ (Fig. 7). The highest rates for ARA were observed after 1 h of incubation time and the lowest after 0.5 h ($P < 0.001$). As for N_2 fixation rates measured by the $^{15}\text{N}_2$ -labeling technique, the highest rate was found to be also after 1 h of incubation ($P = 0.003$); however, the rate at 0.5 h of incubation was not taken into account for the significant difference calculation due to very high variation between replicates.

DISCUSSION

Detection and quantification of the major N pathways in the ES and Baja microbial mats. In this study, N turnover rates were evaluated for ES microbial mats in October and April and for Baja microbial mats in February. Nitrate turnover was estimated under light (oxic) conditions such that NO_3^- consumption would reflect NO_3^- assimilation/aerobic denitrification and that NO_3^- production would be assigned to ni-

FIG 6 Ammonium production rate via DNRA for ES mats in October and April and Baja mats in February. (Due to loss of replicates, only one value is shown for Baja February.)

FIG 7 Nitrogen fixation rates in ES April measured as $^{15}\text{N}_2$ assimilation (a) and ARA (b).

trification. A key finding was that nitrification in both microbial mats occurred only at very low rates. All investigated samples of microbial mats were characterized by much higher NO_3^- consumption than NO_3^- production rates. This is evidenced by significant NO_3^- concentration decreases reflecting high NO_3^- consumption rates and weak ^{15}N dilution that corresponds to low NO_3^- production rates. However, the calculated gross NO_3^- consumption is also dependent on the expression of the pool dilution effect and therefore could be underestimated, considering that ^{15}N dilution was low, especially in ES October mat incubations. Nevertheless, considering the near equality of the calculated net and gross consumption rates and that net consumption rate calculation is independent of ^{15}N data, then estimated NO_3^- turnover is accurate. In other words, net turnover rates that are close to gross ones are another confirmation that nitrification rates are low at both sites for investigated seasons. This is additionally supported by the concentration ratio of NH_4^+ to NO_3^- at all sampling sites, which is always >1 (Table 1).

There are several possible explanations for the low nitrification rates. Likely, there is competition for NH_4^+ between ammonia oxidizers and cyanobacteria and diatoms, which are the main structural components of the photosynthetic microbial mats (23). As activity of ammonia-oxidizing bacteria is also inhibited by high salinity, this could be another possible explanation of low nitrification rates in studied hypersaline microbial mats (24). Additionally, photoinhibition of nitrifying microorganisms has been previously shown (25, 26) and could be partially responsible for quantified low nitrification rates.

High rates of NO_3^- consumption were observed in all investigated mats and seasons. Since it is not possible to differentiate between NO_3^- assimilation and aerobic denitrification (denitrification occurring in the mat under lighted conditions) in these experiments, the NO_3^- consumption reflects a total of these two processes. Some denitrification activity under anoxic conditions in the form of $^{15}\text{N}_2\text{O}$ production was indicated in all the investigated mat samples (see below), and therefore aerobic denitrification might occur as well in studied microbial mats. However, as we do not know about the extent of denitrification in the presence of O_2 in microbial mats, and whether or not denitrification is confined to layers below the upper oxic layers, it is not feasible to compare these two data sets. High rates of denitrification (e.g., 2 to 22 mmol N m^{-3} sediment h^{-1}) under oxic conditions ("aerobic denitrification") have been previously shown (27–29), and this could be a sink for NO_3^- , along with microbial assimilation in the investigated mats under light. In microbial mats, deeper layers (below 3 mm) are usually anoxic, and denitrification could occur there even under lighted conditions.

Ammonium is likely present in higher concentrations than other nitrogenous nutrients in these mats (22). Multiple pathways of NH_4^+ consumption are possible: i.e., microbial assimilation, nitrification, and anammox. However, anammox was found to

TABLE 1 Nutrient concentrations and environmental parameters in overlaying water at the time of microbial mat collections

Field site	Sample description	Concn, μM		Salinity, ‰	Surface water depth, m
		Ammonium	Nitrate		
Concentration Area 5, Exportadora de Sal S. A. de C.V., Guerrero Negro, Baja California Sur, Mexico (27° 41'15.22''N, 113° 55'17.92''W)	Baja February	1.6 ± 0.6	0.8 ± 0.3	98	1
Elkhorn Slough, Monterey Bay, CA, USA (36°48'46.61''N, 121°47'4.89''W)	ES October	1.4 ± 1.1	0.9 ± 0.9	55	0.1
	ES April	21.2 ± 1.6	1.2 ± 0.5	55	

be absent in the studied microbial mats. As nitrapyrin was added to inhibit nitrification in our incubations, all the NH_4^+ consumption should be assigned to microbial assimilation. It has been shown that photosynthetic microbial mats, in particular from the Baja location, are able to fulfill their N requirements from inorganic N present in the water column and via pore water through diffusive flux (22). In this study, while ES mats showed comparable rates of NH_4^+ and NO_3^- consumption, Baja mats had very low NH_4^+ assimilation rates relative to both NO_3^- consumption and NH_4^+ assimilation in ES mats. Such differences can be possibly explained by very high *in situ* water NH_4^+ concentrations in ES in April when also NH_4^+ assimilation was found to be the highest. Elevated NH_4^+ concentrations in ES may stimulate NH_4^+ assimilatory gene transcription rates necessary for acquiring these nutrients; that may not be the case for Baja mats when NH_4^+ concentrations remain low all the time. Still, as NH_4^+ and NO_3^- water concentrations are within the same range in Baja mats (Table 1), it remains unclear why rates of NO_3^- consumption were found to be higher in these mats and needs to be further investigated. Given that NH_4^+ is generally preferred over NO_3^- as a substrate due to the lower energy cost for NH_4^+ assimilation (30), high rates of NO_3^- consumption in Baja microbial mats are probably related to aerobic denitrification. As for NH_4^+ production, the possible pathways are ammonification and DNRA. Since the NH_4^+ turnover experiments were conducted under light and the resultant oxygen production via photosynthesis (6) and given that DNRA occurs optimally under anoxic conditions (31), we assume here that most of the NH_4^+ production was via ammonification. As the NH_4^+ production in the Baja mats was higher than its consumption, ammonification could fulfill the N requirements for these mats. Recycling via ammonification (along with assimilation from the water column and N_2 fixation) has previously been shown to be an important source of N in Baja microbial mats (22).

DNRA rates, measured in the dark, when microbial mats are anoxic (6), were the highest in ES microbial mats in April, when the NH_4^+ concentration and NH_4^+ assimilation rates were also highest. Baja mats exhibited a very low DNRA rate relative to those measured in ES mats. Factors causing the difference in DNRA rates between these mats should be investigated further.

Anammox was not observed in either of the mats studied. Anammox has been found to be absent in other microbial mats (21, 32), and to the best of our knowledge, no study has documented the occurrence of this process in these ecosystems. This might imply the absence of zones featuring favorable conditions for anammox in microbial mats. Although mats are exposed to anoxic environments below 2 mm when photosynthesis occurs, other factors, and not O_2 absence, may limit anammox in these zones. Risgaard-Petersen et al. (33) suggested that anammox is of very limited significance in environments that periodically experience N limitation, and this could be the case for microbial mats. Other studies have, however, measured high anammox

activities in marine suboxic zones with very low inorganic N concentrations (34, 35). Our finding of very high rates of NH_4^+ assimilation may imply that anammox bacteria in microbial mats are outcompeted by other microorganisms for NH_4^+ . Also, large amounts of organic matter in microbial mats (with C/N ratios in these mats of between 5.8 and 7.2 [data not shown]) would result in heterotrophic denitrification outcompeting anammox bacteria, which are slower growing. Fluctuating conditions are also known to be less beneficial for anammox microbes (36).

Amendment with $^{15}\text{NO}_3^-$ under anoxic (dark) conditions did not result in $^{15}\text{N}_2$ enrichment in any investigated microbial mat samples. However, these incubations resulted in very different patterns of $^{15}\text{N}_2\text{O}$ enrichment. Baja microbial mats in February revealed slight ^{15}N enrichment in N_2O ; however, all the N_2O concentrations were below the limit of quantification. This could be evidence of weak denitrifier activity in these mats. In the case of the ES October data set, a strong $^{15}\text{N}_2\text{O}$ labeling occurs at time step zero (only a few seconds of incubation), which would not be expected in case of a progressive microbial N_2O production by denitrification. The calculated evolution of labeled N_2O might indicate increasing N_2O production at the beginning (ca. for 1 h), followed by a decreasing contribution of labeled N_2O . However, over the experimental duration of 6 h, no changes in ^{15}N - N_2O or total N_2O concentration occurred (with the exception of the immediate labeling of N_2O at time zero). While this might invoke the presence of an abiotic mechanism of N_2O production using the added labeled source at the beginning of the experiment (37), consideration of the entire time course of the incubation provides no clear evidence of microbial N_2O production by denitrification (as would be expected from the N_2O concentration increasing over time). In these incubations, ZnCl_2 , which is routinely used as a preservative in ^{15}N tracer studies, was added to incubation bottles prior to sampling to stop microbial activity. Considering that these studies do not directly measure N_2O production via denitrification, it is unclear if the high concentrations and ^{15}N abundance of N_2O are an artifact of abiotic N_2O production in our study or if it is rather the case that ZnCl_2 does not inhibit activity of denitrifying enzymes, except for N_2O reductase. In either of these cases, precautions should be taken when using ZnCl_2 as a preservative in similar studies.

In the case of ES microbial mat incubations in April, where headspace was transferred directly without prior addition of ZnCl_2 , the ^{15}N composition of N_2O indicated increasing ^{15}N abundance over time. However, the analyzed N_2O concentrations were very low (most of them below <0.3 ppmv), and hence, while there is an indication for microbial N_2O production, the reliability of this data set is questionable. The differences between both data sets (ES October and ES April) are obvious, however, and clearly indicate that they behave differently with respect to the observed N turnover.

N_2O can be produced via several N transformation processes such as co-denitrification, heterotrophic nitrification, nitrifier denitrification, and incomplete denitrification to N_2O (38, 39). In the present work, N_2O production was determined based on the ^{15}N abundance of N_2O and the ^{15}N abundance of NO_3^- calculated via the mole fraction of $^{45}\text{N}_2\text{O}$ and $^{46}\text{N}_2\text{O}$, assuming denitrification as the only relevant source of microbial N_2O production. In fact, according to the ^{15}N approach of Spott and Stange (39), no hints could be found that a hybrid N_2O production by, e.g., co-denitrification took place. However, it should be taken into account that NO_3^- reduction to NO_2^- and further reduction of NO_2^- to N_2O can be separated. Many denitrifying microorganisms can reduce NO_3^- to NO_2^- and excrete NO_2^- into the environment, which can be further used by another functional group, such as nitrifiers. However, under the experimental conditions used here (anoxic), nitrifier denitrification could not have occurred.

Most of the denitrification rates in microbial mats reported by other studies were measured either using an acetylene inhibition technique or by isotope pairing technique evaluation that only quantifies $^{15}\text{N}_2$ and not N_2O production (18–21, 40). Using these techniques, it is impossible to evaluate whether incomplete denitrification to N_2O took place in the microbial mats. A direct comparison of incomplete denitrification

rates found in our study and other studies cannot, therefore, be made. However, there are reports of incomplete denitrification for similar ecosystems. For instance, Abed et al. (32) reported high rates of N_2O emissions from cyanobacterial soil crusts that were assigned to incomplete denitrification and reported to make up to 54 to 66% of the total produced gases during denitrification. In contrast, Molina et al. (41) have reported that microbial mats in evaporitic ponds in high-altitude wetlands in Chile may be sinks of N_2O . Further work is needed to understand the fluxes of N_2O between microbial mats and the atmosphere.

It is not clear why the microbial mats used in these studies exhibited incomplete (to N_2O , rather than to N_2) denitrification. Incomplete denitrification has been shown to occur under low O_2 concentrations as N_2O reductase is more sensitive to O_2 than the other denitrifying enzymes (42). However, considering that under natural conditions microbial mats are completely anoxic in the dark and that our experiments were carried out in dark and after flushing with N_2 , O_2 was not present during the incubations and would therefore not inhibit N_2O reductase. Incomplete denitrification has been shown to occur in agricultural lands and soils under carbon-limited conditions (i.e., a C/N ratio of <2) (43, 44). The microbial mats used for these studies exhibited C/N ratios of between 5.8 and 7.2 (data not shown) and so are outside the range of C/N ratios that are known to trigger incomplete denitrification. N_2O reduction might be inhibited by increased salinity (45) as N_2O reductase is a periplasmatic enzyme that is more sensitive to environmental stress than NO_3^- reductase, which is membrane bound (46). Another possible explanation for denitrification with N_2O as the end product instead of N_2 is copper limitation. Copper is a cofactor of the N_2O reductase, and its shortage has shown to limit the activity of this enzyme, causing N_2O accumulation in cultures of denitrifying bacteria (47). Sulfide has also been shown to inhibit N_2O reduction, resulting in production of N_2O and not N_2 as the major product of denitrification (48). Further research is needed on possible causes of incomplete denitrification in the studied microbial mats.

Nitrogen fixation was evaluated using both stable isotope and acetylene reduction assay techniques. It has been reported numerous times that a conversion factor from C_2H_4 production to N_2 fixation should be evaluated for an individual system (49). In our study, the $C_2H_4/^{15}N_2$ assimilation ratio was found to be 69 and so is on the higher end of reported values by other studies (50–52). Low solubility of N_2 gas when introduced as a gas bubble is one of reasons for underestimating N_2 fixation rates when using the $^{15}N_2$ tracer method (49, 53). Nevertheless, the obtained N_2 fixation rate for the ES April data set is 1 to 2 orders of magnitude lower than other estimated N turnover rates (i.e., NH_4^+/NO_3^- assimilation), and it is evidence that N fixation is probably an insignificant source of N in these mats in comparison with recycling/assimilation from a water column. Still, while assimilation of N from ammonification in deeper mat layers can supply the N demand of anabolism in the surface layers, N_2 fixation likely remains an important source for “new” (external to the mat) N necessary for the production of new mat biomass. Low N_2 fixation rates can be explained from a bioenergetic perspective, because energetically expensive N_2 fixation generally does not dominate in the presence NH_4^+ or NO_3^- . Bioenergetic considerations may be of greater relative importance in hypersaline environments, where more energy is required to overcome salt stress (54). A similar conclusion about N cycling in other hypersaline microbial mats was recently reported by investigators using completely different methods (55). Specifically, these investigators also saw evidence for an inhibition of nitrification, as well as suppression of denitrification and anammox, and observed the N isotopic signature of efficient NH_4^+ recycling.

Overview of N cycles of the ES and Baja microbial mats and differences between them. Analysis of the N transformations in the ES microbial mats revealed low rates of nitrification and high rates of NO_3^- consumption that may indicate both NO_3^- assimilation and aerobic denitrification (Fig. 8). There was a significant seasonal difference in NH_4^+ assimilation rates in these mats that may be explained by seasonal fluctuations of *in situ* water NH_4^+ concentrations (higher in spring compared to fall) and/or the availability of organic matter on top of, or below, the surface of the

FIG 8 Nitrogen turnover in ES mats in October and April and Baja mats in February. Concentrations of NO_3^- and NH_4^+ (in white squares) are expressed in μM , and rates (colored arrows) are expressed in $\text{mmol N m}^{-2} \text{ day}^{-1}$.

photosynthetic layers of the mats (see comments about the physical structure the ES mats below). Anammox was undetectable, DNRA rates were low in both measured seasons, and the N_2 fixation rate was found to be low in spring (not measured in fall). Incomplete denitrification to N_2O was observed; however, its rate was very low. We also detected low rates of N_2 fixation in ES mats in April (not measured in October). Overall, the N cycle in these mats was characterized by high rates of recycling of nutrients from the water column, with seasonal variations depending on the nutrient concentrations.

Baja microbial mats were also characterized by low rates of nitrification (Fig. 8). However, these mats were found to have a higher affinity for NO_3^- compared to the ES mats. Also, rates of NH_4^+ assimilation from the water were found to be lower than rates of NO_3^- consumption in these mats. This might indicate the presence of an additional sink for NO_3^- in the Baja microbial mats consistent with what would be expected from aerobic denitrification. Baja mats also had rates of ammonification far higher than those in the ES mats in October. Baja mats were similar to ES mats in having low DNRA rates and an apparent absence of the anammox process. Evidence for incomplete denitrification to N_2O was also found in these mats.

Macroscale constraints on microscale processes in microbial mats. While both mats exhibited a great capacity for consumption of both NH_4^+ and NO_3^- from the water column, somewhat higher mean rates of ammonification and indications of aerobic denitrification distinguish the Baja mats from the ES mats. We hypothesize that these differences in N cycling processes likely result from differences in morphological characteristics of the mats, which are, in turn, related to the environments in which they grow; Baja mats are constantly submerged, whereas ES mats grow in a highly dynamic intertidal environment. The degree to which the physical integrity of the mats is disrupted by turbulence and storm events likely changes the availability of regenerated nitrogen from lower layers to the active photosynthetic zone. While the upper 3 to 4 mm of each mat have very similar redox-related depth stratification (Fig. 1), the delivery of the substrates necessary for these transformations to the respective redox zones conducive to them is likely impacted over larger spatial scales.

Physical disturbances resulting from a combination of wind-driven water movement, desiccation impacts on the attachment of the mats to sediment, and even disturbance by fauna result in far greater disruption of physical structure and integrity in the ES mats than the Baja mats (Fig. 1). ES mats are commonly observed to be forming on the surface of older layers of mat and on top of deposits of filamentous green algae, which grow in abundance during some seasons of the year. The profound physical disturbance of the ES mat likely results in a highly variable flux of NH_4^+ from lower layers of mat, as well as the possibility of the lower layers of the mat coming into contact with oxygen from the water and even air during low tides. In contrast, the Baja mat is far more structurally cohesive, and the upper layers of the mat likely receive an uninterrupted and much higher flux of NH_4^+ diffusing from lower layers. These differences

in physical structure would explain the observed differences in measured rates of aerobic denitrification and ammonification and likely result in profound differences in depth-related microbial community compositions, even in the upper layers of the mat. The depth-dependent microbial community composition and nitrogen cycling functional genes in these mats are presently being investigated using iTag and omics techniques.

Conclusions. Respiratory N transforming pathways were dominated by incomplete denitrification to N_2O , a finding that revealed photosynthetic mats as being net sources of this important greenhouse gas. Low rates of N fixation in Elkhorn Slough microbial mats hint at a rather closed N transformation cycle, based on recycling and assimilation as the main sources of N in the studied mats at the sampling times investigated here. Differences in N consumption may in large part be explained by structural differences in the mats imparted by physical factors in their environments and by NH_4^+ concentrations in surrounding water. The underlying biological processes driving the observed activities should be investigated with improved depth resolution in future studies using omics techniques in order to reveal the responsible organisms and pathways.

MATERIALS AND METHODS

Study areas and microbial mat sampling. This study evaluated microbial mats collected from two distinct locations. The first study area is the salt works managed by Exportadora de Sal SA, and located near Guerrero Negro, Baja California Sur, Mexico (further referred to as “Baja February” [GPS coordinates in Table 1]). Microbial mats at this location are permanently covered with 0.5 to 1 m of water at 80 to 100‰ salinity and can grow up to 10 cm thick (6) (Fig. 1A to C). Sections (20 by 25 cm) of microbial mats were collected from the seawater concentration Area 4, on the dike separating Area 4 from Area 5 (Area 4 near 5), on 25 February 2016 and transported to NASA Ames Research Center (Moffett Field, CA) within 3 days. During the transportation, microbial mats were kept moist with the brine from the sampling site.

The second study area is located within the Elkhorn Slough (ES) estuary at Monterey Bay, CA, USA (GPS coordinates in Table 1). This mat (further referred to as “ES mat”) is up to 1 cm thick, and its areal extent varies with seasonal changes in water flow, tides, and nutrient inputs (56) (Fig. 1D to F). The average salinity of the field site water is 55‰. The mats were collected at this experimental site twice, on 19 October 2015 (further referred to as “ES October”) and on 5 April 2017 (further referred to as “ES April”). After collection, the samples were kept moist with ambient seawater and brought to the laboratory at NASA Ames Research Center (Moffett Field, CA) within 2 h.

Nutrient concentrations and environmental parameters of water from sampling sites at the time of microbial mat collections are given in Table 1. Upon arrival, all samples were immediately prepared for incubations for biogeochemical rate measurements.

Incubation setup. Incubations were prepared by cutting small subcores (11-mm diameter, 10-mm depth) from whole sections of intact microbial mat with plastic core tubes, and the cores, still contained within the plastic core tubes, were placed on the bottom of 10-ml serum vials for the N_2 fixation measurements and in 38-ml serum vials for the other experiments. A 1% agar solution made using water from respective sampling site was added to each bottle up to the level of the top of the core tube. The agar served to physically fix the plastic core into position in the bottom of the bottle and to ensure that the only contact between the cores and the overlying water was across the top of the mat (across the mat-water interface). Fifteen milliliters of filtered (0.2- μm pore size) seawater from a boat ramp neighboring the sampling location (for ES mats) and ambient filtered water (for Baja mats) was then added to each 38-ml bottle, and bottles were subsequently sealed with a rubber stopper. Two milliliters of water was added to the 10-ml serum vials that were used for N_2 fixation measurement. Three replicates were set for each sampling time and treatment. Prior to the injection of a labeled compound, all samples were preincubated for 16 h under light for day incubations and for 24 h in the dark for dark incubations. Samples for N_2 fixation rate measurements were preincubated under light for 24 h, although the experiment was performed in the dark. Day incubations were performed under irradiance provided by white LED lamps ($\sim 1,200 \mu\text{E m}^{-2} \text{s}^{-1}$), and night incubations were performed under dark conditions (covered by aluminum foil). Night incubations were purged with N_2 for 15 min prior to the start of the experiment. All the incubations were performed on a shaker table with a rotation speed of ~ 30 rpm and constant temperature of $+16^\circ\text{C}$. One milliliter of water sample for nutrient analysis was taken at times zero, 0.5, 1, 3, and 6 h, filtered with a 0.2- μm syringe filter, and stored at 20°C . Ammonium analysis was performed using a colorimetric method as described by Parsons et al. (57), and NO_3^- was analyzed using a microplate reader-based colorimetric method adapted from the protocol of Ringuelet et al. (58). Samples for ^{15}N analysis were taken and preserved as described in the next section.

Biogeochemical rate measurements. The rate of each specific N transformation process (nitrification, NO_3^- and NH_4^+ assimilation, ammonification, anammox, denitrification, DNRA, and N_2 fixation) was measured for two seasons (fall and spring) in ES mats and one season (winter) in Baja mats. All the rates were measured as potential rates (maximum rates under optimal conditions [i.e., anoxic in dark/oxic under light] without limitation by substrate availability and with diffusive solute transport aided through

stirring of the water on the shaker). In acknowledgment of the possibility of circadian rhythms, all incubations under light were done during daylight hours (between 9:00 a.m. and 3:00 p.m.), and all incubations in the dark were done close to and after sunset (6:30 p.m. to 6:30 a.m.). Rates are presented as amounts of N (mol) per surface area of microbial mat (m^2) per time (day).

Nitrification and nitrate assimilation/aerobic denitrification rate measurement. Nitrate production (nitrification) and consumption (NO_3^- assimilation and denitrification under aerobic conditions) were monitored using an isotope dilution approach. One hundred microliters of 10 atom% $\text{Na}^{15}\text{NO}_3$ was injected into the vials, such that the final concentration of $\text{Na}^{15}\text{NO}_3$ was $100\ \mu\text{M}$. Incubations were performed under light. As NH_4^+ was present in ambient water at each sampling site (Table 1), it was not added to the incubation vials. Water samples (14 ml) were taken destructively at times zero, 0.5, 1, 3, and 6 h, filtered with a $0.2\text{-}\mu\text{m}$ syringe filter, and stored at 20°C until further $^{15}\text{NO}_3^-$ analysis. Samples for ^{15}N in NO_3^- analysis were further prepared by bacterial denitrification assay (59) and analyzed on a Thermo Finnigan GasBench + PreCon trace gas concentration system interfaced to a Thermo Fisher Scientific (Bremen, Germany) DELTA V Plus isotope ratio mass spectrometer (IRMS) at the Stable Isotope Facility of UC Davis, Davis, CA, USA. The gross nitrification and NO_3^- consumption rates were calculated as previously described (27), and the net nitrification rates were calculated as NO_3^- concentration change per day as the difference between the gross nitrification and consumption rates.

Ammonification and ammonia assimilation rate measurement. Ammonium production (ammonification) and consumption (NH_4^+ assimilation) were monitored using an isotope dilution approach. In order to distinguish NH_4^+ assimilation from nitrification, nitrapyrin (a nitrification inhibitor) was added to all vials at a final concentration of $0.5\ \text{mg liter}^{-1}$ (60). Nitrapyrin was shown to effectively inhibit nitrification in liquid cultures at concentrations of 0.5 to $1.0\ \text{mg liter}^{-1}$ for up to several days (61, 62). One hundred microliters of 10 atom% $^{15}\text{NH}_4\text{Cl}$ was injected into the vials, such that the final concentration of $^{15}\text{NH}_4\text{Cl}$ was $100\ \mu\text{M}$. Incubations were run under light conditions. Incubations were stopped by removing all the water from incubation vials at times zero, 0.5, 1, 3, and 6 h. The water samples were then filtered with a $0.2\text{-}\mu\text{m}$ syringe filter and stored at 20°C until further $^{15}\text{NH}_4^+$ analysis. Samples for ^{15}N in NH_4^+ analysis were prepared by hypobromite-azide method as per Zhang and Altabet (63). The ^{15}N of the resultant N_2O was measured on a Thermo Fisher Scientific (Bremen, Germany) Finnigan DELTAplus XP IRMS at the University of Utrecht, Utrecht, Netherlands. The gross ammonification and NH_4^+ assimilation rates were calculated as previously described (27), and the net ammonification rates were calculated as NH_4^+ concentration change per hour as the difference between the gross ammonification and NH_4^+ assimilation rates.

Anammox rate measurement. To estimate the potential rate of anammox in studied microbial mats, the experimental setup related to the isotope pairing technique (IPT) was used. Fifty microliters of 99 atom% $^{15}\text{NH}_4\text{Cl}$ and $50\ \mu\text{l}$ of NaNO_2 were injected into the vials, such that the final concentration of $^{15}\text{NH}_4\text{Cl}$ was $50\ \mu\text{M}$ and the final concentration of NaNO_2 was $50\ \mu\text{M}$. Incubations were run in the dark. For the ES October samples, incubations were stopped at 0, 0.5, 1, 3, and 6 h by adding $200\ \mu\text{l}$ of a 7 M ZnCl_2 solution. Afterwards, the samples were stored at $+4^\circ\text{C}$ upside down for 3 months before the headspace was transferred to evacuated 12-ml vials (Exetainer; Labco, Ltd., United Kingdom). For the Baja February and ES April samples, headspace was directly transferred at 0, 0.5, 1, 3, and 6 h to evacuated 12-ml Exetainer vials. The ^{15}N in N_2 was analyzed using a Thermo Fisher Scientific GasBench + Precon gas concentration system interfaced to a Thermo Fisher Scientific DELTA V Plus IRMS at the Stable Isotope Facility of UC Davis, Davis, CA, USA. The rates of anammox were calculated from the mole fraction of $^{29}\text{N}_2$ and $^{30}\text{N}_2$ in the sampled vial atmosphere of samples using the equation for hybrid N gas formation of Spott and Stange (39).

Denitrification and DNRA rate measurement. In the ES October samples, denitrification rates were measured using the experimental setup related to the IPT after injection of $100\ \mu\text{l}$ of 99 atom% $\text{Na}^{15}\text{NO}_3$ into the vials such that the final concentration of $\text{Na}^{15}\text{NO}_3$ was $100\ \mu\text{M}$. Addition of 99% labeled $\text{Na}^{15}\text{NO}_3$ led to very high ^{15}N content in N_2O , and therefore for further mat incubations with the Baja February and ES April samples, 10 atom% $\text{Na}^{15}\text{NO}_3$ was used instead. Incubations were run in the dark. For the ES October samples, incubations were stopped at 0, 0.5, 1, 3, and 6 h by injecting $200\ \mu\text{l}$ of a 7 M ZnCl_2 solution. Afterwards, the samples were stored at $+4^\circ\text{C}$ upside down for 3 months before the headspace was transferred to evacuated 12-ml Exetainer vials. For the Baja February and ES April samples, incubations were stopped at 0, 0.5, 1, 3, and 6 h by direct transfer of headspace to evacuated 12-ml Exetainer vials. The ^{15}N in N_2 and N_2O was analyzed using a Thermo Fisher Scientific GasBench + Precon gas concentration system interfaced to a Thermo Fisher Scientific DELTA V Plus IRMS at the Stable Isotope Facility of UC Davis, Davis, CA, USA. The rates of denitrification and incomplete denitrification to N_2O were calculated from the mole fractions of $^{29}\text{N}_2$ and $^{30}\text{N}_2$ and of $^{44}\text{N}_2\text{O}$ and $^{45}\text{N}_2\text{O}$, respectively, in the vial atmosphere of samples using equations 1 and 2 of Spott and Stange (39).

DNRA rates were quantified in the same vials used for denitrification incubations. For ES October samples, additional incubations were set with 10 atom% $\text{Na}^{15}\text{NO}_3$ additions identical to ES April and Baja February denitrification incubations. After headspace was sampled for the denitrification rate estimation, 14 ml of water from the vial was filtered with a $0.2\text{-}\mu\text{m}$ syringe filter and stored at 20°C until further $^{15}\text{NH}_4^+$ analysis. Samples for ^{15}N in NH_4^+ analysis were prepared by the hypobromite-azide method as described by Zhang and Altabet (63). The ^{15}N of the resultant N_2O was measured on a Thermo Fisher Scientific (Bremen, Germany) Finnigan DELTAplus XP IRMS at the University of Utrecht, Utrecht, Netherlands. The rates of DNRA were calculated using the isotope mixing approach (64).

Nitrogen fixation rate measurement. Unlike other dark incubations, all the samples for N_2 fixation rate measurements were preincubated under light for 16 to 24 h before the beginning of the experiment that was run in the dark as the light period has been shown to be required for energy and

reductant generation in other photosynthetic microbial mats (65). One milliliter of headspace was replaced by 99 atom% $^{15}\text{N}_2$, resulting in the isotopic composition of $^{15}\text{N}_2$ of 18.3 atom%. Microbial mat biomass was transferred to scintillation vials at times zero, 0.5, 1, 3, and 6 h and frozen at -20°C . Afterwards, the biomass was dried, homogenized, weighed into tin capsules, and analyzed for ^{15}N content with IRMS using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon, Ltd., Cheshire, United Kingdom) at the Stable Isotope Facility of UC Davis, Davis, CA, USA. The rates of N_2 fixation were calculated as the relative contribution of the $^{15}\text{N}_2$ pool to the microbial mat biomass using the isotope mixing approach (64). The N_2 fixation rate as nitrogenase activity was also estimated with the acetylene reduction assay (ARA) as previously described (65). Five hundred microliters of water was exchanged for acetylene, which was injected through the stopper into the aqueous phase to start the incubation. One milliliter of headspace was transferred at 0, 0.5, 1, 3, and 6 h to the Exetainer vials containing a saturated solution of sodium chloride (NaCl) and inverted for storage (the stored gas thereby being trapped by the NaCl solution below it and glass above it). Ethylene concentrations were quantified using a Shimadzu GC-14A gas chromatograph with a flame ionization detector (FID) and a 2-m Porapak N column held at 80°C .

Statistical analysis. Statistical analyses for differences in biogeochemical process rates were performed using SigmaPlot software (v.13.0; Systat Software, Inc.). Normality of the data was checked using the Shapiro-Wilk test. A one-way analysis of variance (ANOVA) was performed to check for differences between the two types of microbial mats and seasons. When significant differences were found, treatment means were separated with a pairwise multiple comparison test (Tukey's, Dunn's, or Holm-Sidak) at a P value of 0.05. The rates presented are means of three replicates. Error bars represent ± 1 standard deviation. As replicates represented biological samples, standard deviations of the measured rates were found to be sometimes very high due to well-known (e.g., Dillon et al. [66]) heterogeneity of microbial mat samples.

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