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N_2O Production by Nitrifying Biomass Under Anoxic and Aerobic Conditions

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Abstract The capacity of nitrifying biomass, grown in biofilms or in suspension, to reduce NO_2^- and NO_3^- under anoxic conditions was tested in batch experiments. The estimated reduction rates were 5 and 25 mg N per gram volatile suspended solids (VSS) per day for nitrate and nitrite, respectively, in the case of the nitrifying biofilms. Activity tests carried out with successive feedings indicated that no acclimation of the biomass to the tested conditions occurred, as the obtained reduction rates remained almost constant. Another series of activity assays was carried out with nitrifying suspended biomass, and the reduction rates for nitrate and nitrite were 30.4 and 48.9 mg N per gram VSS per day, respectively. N_2O and N_2 were the final gaseous products, and their percentages depended on the source of nitrogen feed. The specific production of nitrous oxide during nitrification was investigated during continuous experiments in a biofilm airlift suspension reactor. Specific production rates up to 46 mg N_2O–N per gram VSS per day were measured. The percentage of N_2O produced represented up to 34.4% of the ammonia oxidized. Nitrite accumulation, low dissolved oxygen concentrations, and the presence of organic matter favored the production of nitrous oxide. N_2O gas was not detected during the oxidation of nitrite even when organic matter was present. To prevent N_2O gas production in nitrifying systems, the operation at low dissolved oxygen concentrations, nitrite presence, or organic matter content should be avoided.

Keywords Biofilm · Denitrification · Dissolved oxygen · Nitrification · Nitrous oxide

Introduction

Nitrous oxide (N_2O) is a gas present in nature in trace amounts, which contributes to the greenhouse effect and the destruction of the ozone layer [1]. Several studies about the

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production of nitrogen oxides have shown that the majority of these gases are produced by nature herself, a small proportion being due to human activity [2]. The biological nitrification and denitrification processes are extremely important in the emissions of N_2O into the atmosphere [3, 4]. Nevertheless, human activity may intensify these emissions, for example by the discharge of wastewater containing large organic matter concentrations that decreases the dissolved oxygen (DO) levels in water, and thus favors the N-oxides production [5].

N_2O gas is an intermediate or a sub-product of biological processes such as denitrification and nitrification, respectively [6–8].

It is formed as a final product during denitrification when denitrifying microorganisms lack N_2O reductase [9], the operational pH values are low [9], and toxic compounds [10] or low DO concentrations are present in the media [11].

Nitrifying bacteria are able to produce N_2O under aerobic [12–14] or anoxic [15–17] conditions. In anoxic conditions, both ammonia- and nitrite-oxidizing bacteria are able to produce this nitrogen oxide, while only ammonia-oxidizing bacteria are able to produce it in aerobic conditions. In the latter case, the production is stimulated by the presence of low DO concentrations [18, 19] and presence of nitrite [20, 21] or organic matter [22].

The objective of this work was to evaluate the production of N_2O by nitrifying biomass grown in biofilm or in suspension. Activity assays were carried out in anoxic and heterotrophic conditions to ascertain the reduction rates of nitrite and nitrate. Continuous assays were carried out in aerobic conditions to determine the production of nitrous oxide and the factors which favor it.

Materials and Methods

Experimental Setup

Denitrifying activity batch assays Biomass samples collected from the reactors were washed three times with phosphate buffer (1.43 g H_2KPO_4 per liter and 7.47 g HK_2PO_4 per liter) to guarantee the complete absence of substrates. Vials of a total volume of 500 ml were filled with 200 ml of a solution containing 1 g volatile suspended solids (VSS) per liter, 100 mg NO_x^-–N per liter, 1.43 g H_2KPO_4 liter, and 7.47 g HK_2PO_4 per liter. Eventually, acetate (as electron donor) was added to the liquid media, and acetylene (inhibitor of the reduction of N_2O to N_2) was injected in the headspace of the vial (15% *v*/*v*). The pH value was adjusted to 7.5 with NaOH 10 N, and the head space was purged with helium gas. Vials were placed in a shaker (200 rpm, 25 °C), and samples of the liquid and gaseous phases were taken every 2 h.

Two series of experiments were carried out with the nitrifying biofilm and suspended biomass, respectively.

Series I: Samples of nitrifying biofilms with a nitrifying specific activity of 0.18 g NH_4^+–N per gram VSS per·day were collected from a biofilm airlift suspended (BAS) reactor fed with an autotrophic medium. Denitrifying batch tests were performed to estimate the maximum specific reduction rates of nitrate and nitrite (Table 1). Assays were performed in sextuplicate. The acclimation of the biomass was also evaluated by adding three successive feedings with nitrate/nitrite and acetate every 24 h.

Table 1 Series of denitrifying activity batch assays.

Experiment	Group	Biomass	N source	e^- donor	Inhibitor
Series I	a	Biofilm	NO_3^-, 100 mg N/L	Acetate, 100 mg C/l	–
	a	Biofilm	NO_2^-, 100 mg N/L	Acetate, 100 mg C/l	–
Series II	a	Activated sludge	NO_3^-, 100 mg N/L	–	–
	a	Activated sludge	NO_2^-, 100 mg N/L	–	–
	b	Activated sludge	NO_3^-, 100 mg N/L	Acetate, 100 mg C/l	Acetylene, 15%[a]
	b	Activated sludge	NO_2^-, 100 mg N/L	Acetate, 100 mg C/l	Acetylene, 15%[a]
	c	Activated sludge	NO_3^-, 100 mg N/L	Acetate, 100 mg C/l	–
	c	Activated sludge	NO_2^-, 100 mg N/L	Acetate, 100 mg C/l	–

[a] % *v*/*v* in gas phase

Series II: Samples of a nitrifying suspended biomass with a specific activity of 1 g NH_4^+–N per gram VSS per·day were collected from a reactor fed with an autotrophic medium. To confirm if reduction of NO_x^- was due to a biological process, two controls were carried out: (a) without the presence of any external electron donor and (b) with both acetate, as electron donor, and acetylene to inhibit the activity of the enzyme N_2O reductase [23]. Denitrifying assays were done to determine the reduction rates of NO_x^- using acetate as electron donor (c). The experiments were done in duplicate for both substrates nitrite and nitrate.

Continuous assays A nitrifying BAS reactor of 2.6 l with a biomass concentration of 8 g VSS per liter was used. The reactor was operated at 23 °C and with pH controlled at 7.5.

Two sets of assays were carried out to quantify the effects of DO concentrations (A), nitrite, and organic matter concentrations (B) on the production of N_2O gas:

Experiments A: Biomass samples were collected from a BAS reactor fed with an autotrophic synthetic medium (Table 2, experiments A). This reactor treated an NH_4^+–N loading rate (ALR) of 2.4 g NH_4^+–N per liter per·day and operated at a hydraulic retention time (HRT) of 1 h with a DO concentration of 7.4 mg O_2 per liter. Short experiments of 3 h were performed consecutively to test five DO concentrations: 0.3, 0.5, 1.1, 3.0, and 7.4 mg O_2 per liter. To fix the required DO concentration, the reactor was gasified with a variable mixture of N_2 and O_2 at an inflow rate of 2 l/min to maintain a constant K_{La}. Pseudo-steady-state conditions for liquid and gas phases were reached for each DO concentration tested.

Experiments B: Biomass samples were collected from the previous reactor treating an ALR of 1.5 g NH_4^+–N per liter per·day and operated at a HRT of 8 h. Initially, the system was fed with a medium containing 500 mg NH_4^+–N per liter (Table 2, experiments B-I) and later with a medium containing 500 mg NO_2^-–N per liter (Table 2, experiments B-II). In each set of experiments (B-I and B-II), the reactor was operated at a fixed DO concentration during 1 week to achieve constant composition of the liquid phase (Table 3). Three different DO concentrations were tested in each case: 1, 2, and 5 mg O_2 per liter. After 1 week of operation, once

Table 2 Composition of the synthetic media used during the continuous assays.

Compound	Experiment A (mg/l)	Experiment B-I (mg/l)	Experiment B-II (mg/l)
NH_4Cl	382	956	–
$(NH_4)_2SO_4$	471	1,178	–
$NaNO_2$	–	–	2,482
KH_2PO_4	100	250	250
$MgSO_4$	24	60	60
NaCl	400	1,000	1,000
$NaCH_3COO$	140	0	0
Traces solution[a]	0.5 ml/l	0.5 ml/l	0.5 ml/l

[a] According to Vishniac and Santer [23]

steady-state conditions were reached at a certain DO concentration, quick changes (3–5 min in length) in the DO concentration were carried out, varying the air flow rate. DO concentrations tested in this quick change assays were 0.5, 1, 2 and 5 mg O_2 per liter. Periodical samples of the outlet gas were taken until verifying that a constant concentration of nitrous oxide was reached for each DO concentration during these quick changes. These experiments were repeated in the presence of ammonia or nitrite together with organic matter (100 mg acetate-C per liter). [24]

Analytical Methods

The pH value and dissolved oxygen (selective electrode Aqualitic OX-921), ammonia, and biomass concentrations were determined as proposed in the Standard Methods [25]. Nitrite and nitrate ions were analyzed by capillary electrophoresis using a Waters Quanta 4000 system with sodium sulfate as the electrolyte [26]. Acetate was measured as total organic carbon (TOC) with a TOC 500-Shimadzu. Gaseous compounds (N_2, CO_2, and N_2O) were analyzed by gaseous chromatography with a Hewlett Packard 5890 series II.

Results and Discussion

Denitrifying Activity Batch Assays

Experiments performed with nitrifying biofilms (series I) resulted in NO_x^- consumption rates practically constant around 4.9 and 27.6 mg NO_x–N per gram VSS per·day for nitrate

Table 3 Ammonia, nitrite, and nitrate concentrations achieved in the liquid phase operating the reactor at different DO values during 1 week.

Experiment	DO (mg O_2 per liter)	NH_4^+–N (mg N per liter)	NO_2^-–N (mg N per liter)	NO_3^-–N (mg N per liter)
B-I	1	124	170	170
	2	122	90	236
	5	9	3	451
B-II	1	–	35	454
	2	–	2	482
	5	–	1	487

and nitrite, respectively (Table 4). No lag phase was observed in the experiments. Obtained nitrate reduction rates were similar to those obtained by Tijhuis et al. [27] with nitrifying biofilms.

The composition of the gaseous nitrogen products obtained depended clearly on the fed nitrogen source. Nitrate was always fully converted to N_2, while nitrite produced N_2O and N_2 (≈55:45 *v*/*v*). This fact would be related to the different reduction rates of nitrate, nitrite, and nitrous oxide. The consumption rate of nitrite is faster than that of nitrate, and N_2O is produced only when nitrite is tested. Therefore, taking into account the nitrogen reduction sequence, it could be deduced that $(-rNO_2^-) > (-rN_2O) > (-rNO_3^-)$.

When the effect of successive feedings was tested, no changes in the reduction rates or in the percentages of final products were observed. These results showed that no acclimation of these microorganisms to anoxic conditions took place.

Experiments performed with nitrifying suspended biomass (series II) without external addition of organic matter resulted in nitrite and nitrate consumption rates almost negligible (Table 5, group a). Experiments with acetylene addition (Table 5, group b) produced N_2O as the final product in the gas phase. These assays confirm that the production of nitrous oxide N_2O was due to a biological process, as this gas might also be produced by chemical decomposition of nitrite [28]. Experiments performed with acetate addition (Table 5, group c) showed reduction rates of nitrate and nitrite of 30.6 and 45.5 mg NO_x^-–N per gram VSS per·day, respectively. The percentages of nitrogen gases obtained were around 75% for N_2 and 25% for N_2O in nitrite assays and 100% for N_2 in nitrate assays.

The reduction rates obtained for both nitrite and nitrate with the nitrifying suspended biomass (series II) were higher than those obtained with the nitrifying biofilm (series I). These results could be related to the higher nitrifying specific activity observed for the suspended biomass.

In both cases, the presence of such NO_x^- reduction activities cannot be attributed to the existence of an important population of denitrifying bacteria due to the fact that the biomass used to do these assays came from a nitrifying reactor fed with an autotrophic synthetic medium. No significant concentrations of organic matter, except those coming from the biomass dead, should be present in the reactor. Furthermore, these amounts did not justify the presence of such a great denitrifying population, and it might be reasonable to assert that nitrifying bacteria were involved in these processes. In fact, Freitag et al. [15] and Bock et al. [17] observed that nitrite-oxidizing bacteria were able to reduce nitrate and nitrite in anoxic conditions using organic matter as electron donor, as these microorganisms have both nitrite reductase and nitrite oxidoreductase enzymes. However, ammonia-oxidizing bacteria are only able to reduce nitrite, but not nitrate, in anoxic conditions because they have no nitrite oxidoreductase enzymes [29, 30]. Thus, the reduction of nitrate might be attributed exclusively to nitrite-oxidizing bacteria, while the reduction of nitrite might be attributed to both genera.

Table 4 Results of denitrifying activity batch tests with nitrifying biofilms (series I).

Nitrogen source	(r_N) (mg N per gram VSS·per day)	N_2–N (%)	N_2O–N (%)
NO_3^-	4.9±0.6	99.1±0.5	0.8±0.5
NO_2^-	27.6±3.0	45.5±2.5	54.4±2.4

Table 5 Denitrifying activity batch tests with activated sludge (series II).

N source	Group	e^- donor	Inhibitor	$(-r_N)$ (mg N /g VSS·d)	N_2-N (%)	N_2O-N (%)
NO_3^-	a	–	–	1.0±0.2	n.d.	n.d.
NO_2^-	a	–	–	1.2±0.1	n.d.	n.d.
NO_3^-	b	Acetate	Acetylene	n.m.	0	100
NO_2^-	b	Acetate	Acetylene	n.m.	0	100
NO_3^-	c	Acetate	–	30.6±2.1	100	0
NO_2^-	c	Acetate	–	45.5±3.5	75.4±0.5	25.1±0.9

n.d. not detectable

n.m. not measured

Continuous Assays

Experiments A: During these experiments, the DO level was changed stepwise each 3 h to analyze its influence on the production of the different nitrogen compounds in the liquid phase (NO_2^- and NO_3^-) and in the gaseous phase (N_2O). NO_2^- was produced in the liquid phase for all the DO concentrations tested (0.3, 0.5, 1.1, 3.0, and 7.4 mg O_2/l; Fig. 1). The nitrite accumulation increased with the DO concentration up to 3 mg O_2 per liter, while nitrate production increased with the increase of DO concentration. Significant N_2O production was measured in the gas phase at DO concentrations up to 3.0 mg O_2 per liter.

To check the production of other nitrogen compounds in the experiments, nitrogen balances were calculated by adding all the nitrogen amounts corresponding to the measured compounds. From the calculations, the obtained standard deviation was of 3% for each DO concentration tested, which might be attributable to an experimental error and not to the production of other nitrogen compounds (N_2, NO, NH_2OH, etc.).

In these experiments, the specific nitrous oxide production reached its maximum of 46 mg N_2O–N per gram VSS per·day at a DO level of 1.1 mg O_2 per liter, and its production ceased at a DO level of 7.4 mg O_2 per liter (Fig. 2). These values disagree with

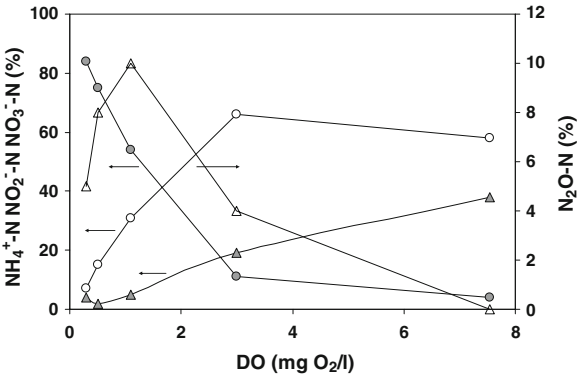

Fig. 1 Percentages of the nitrogen compounds obtained in the effluent at different DO concentrations in experiment A [NH_4^+–N (*filled circle*); NO_2^-–N (*open circle*); NO_3^-–N (*filled triangle*); N_2O–N (*open triangle*)]

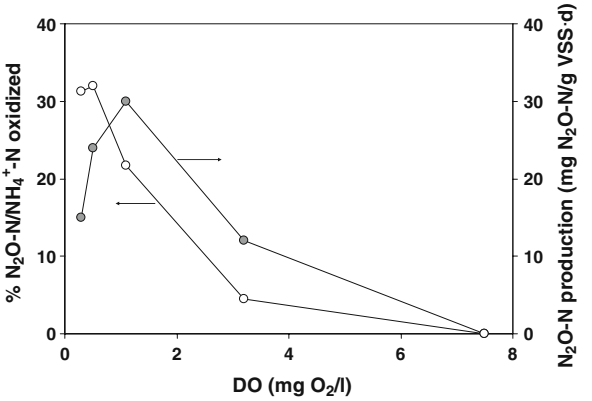

Fig. 2 N_2O–N production (*filled circle*) and yield of N_2O–N produced/NH_4^+-N oxidized (*open circle*) at different DO levels in experiment A

those obtained by Zheng et al. [31] who still observed production of nitrogen oxides up to DO values around 7 mg O_2 per liter with a maximum production at 0.2 mg O_2 per liter.

The maximum N_2O percentage produced with respect to ammonia oxidized was 28.8%. Garrido et al. [32] and Osada et al. [33] also obtained similar percentages when treating wastewater. In works performed with soil samples, the obtained percentages were lower, 0.02–1.7% [13, 34].

Experiments B: When the reactor was fed with ammonia (experiment B-I), concentrations of both ammonia and nitrite increased by decreasing the DO level, while when the reactor was fed with nitrite (experiment B-II), the main product was nitrate for every DO level tested.

Experiments B-I: During these experiments, the maximum percentage of N_2O produced with respect to the ammonia oxidized was 34.4%. All the curves of production of N_2O had a maximum value at a DO level of 1 mg O_2 per liter, the specific N_2O production being favored by the presence of nitrite. The presence of nitrite (Fig. 3) seemed to play an important role in the production of N_2O, as the results showed that this production was higher when concentrations of nitrite increased; the same was found by other authors [20, 21, 31, 34]. In fact, Kester et al. [35] observed that ammonia-oxidizing bacteria produced N_2O, while a mixture of ammonia-oxidizing bacteria and nitrite-oxidizing bacteria did not produce them, as the latter consumed the formed nitrite. Hwang et al. [36, 37] observed that the increase of nitrous oxide production was closely related to the accumulation of free ammonia. Nevertheless, during the present experiments, the concentrations of free ammonia were always lower than 2 mg NH_3–N per liter even during the operation at low DO concentrations, as the system operated at a pH value of 7.5.

The results also showed clearly that the presence of organic matter increased the production of N_2O except for the concentration of 0.5 mg O_2 per liter, probably due to the low N_2O production. This production was once more maximal at DO concentration of 1 mg O_2 per liter. Tortoso and Hutchinson [13] also detected an increase in the production of N_2O during the oxidation of ammonia when organic matter was added, but they proposed that this fact was due to the depletion of oxygen available for nitrifying bacteria.

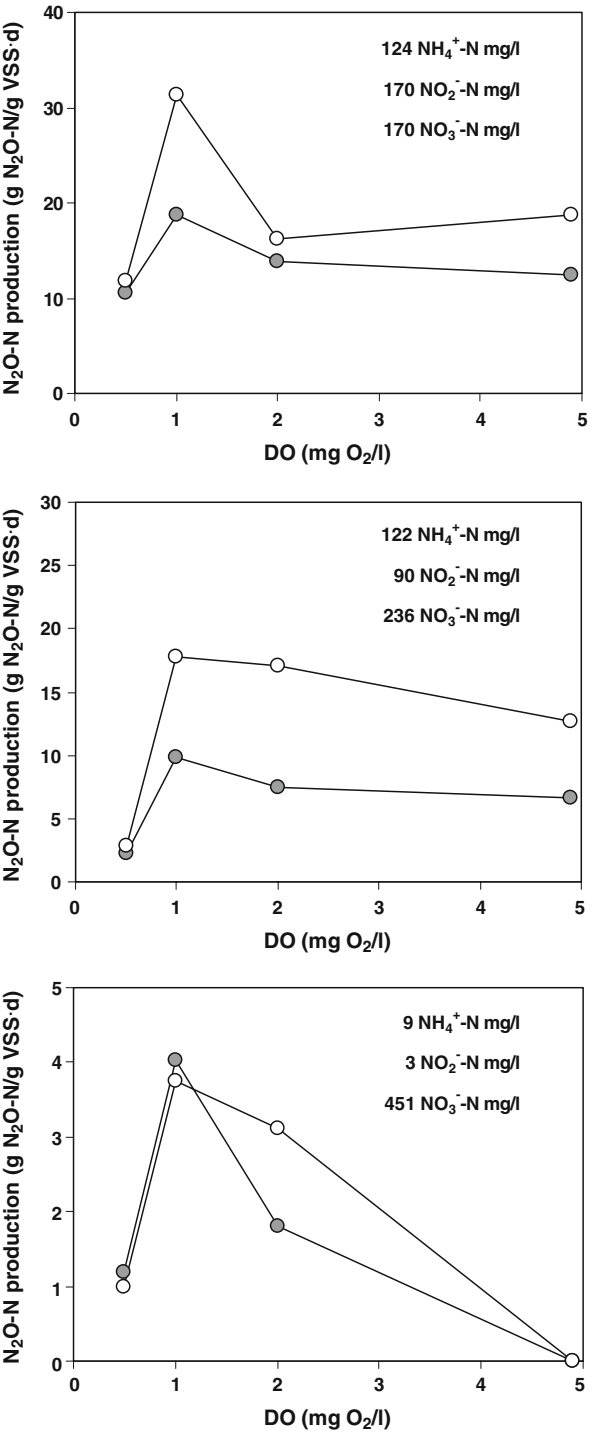

Fig. 3 Influence of DO and organic matter on production of N_2O–N for different compositions of the liquid phase in experiment B-I (*closed symbols*: without organic matter, *open symbols*: with organic matter)

Experiments B-II: Nitrous oxide was never detected even when nitrite was present. The balances of nitrogen confirmed that neither N_2 nor NO was produced. These results agree with those of Blackmer et al. [34] who observed that neither ammonia-oxidizing bacteria nor nitrite-oxidizing bacteria produced nitrous oxide from nitrite. These authors also found that ammonia-oxidizing bacteria do not reduce nitrite to N_2O in aerobic conditions in the absence of ammonia, as the enzymatic system of this reduction required the oxidation of ammonia. This fact might explain why N_2O was not produced in aerobic conditions without the presence of ammonia even when nitrite was accumulated. In this case, the addition of the organic matter did not cause the production of N_2O or N_2 by denitrification, as it was observed in the denitrifying activity batch assays; therefore, this process seemed to be very sensitive to DO.

The results obtained from denitrifying activity batch assays and continuous experiments showed that nitrous oxide might be produced by the reduction of nitrite in anoxic and heterotrophic conditions or by the oxidation of ammonia. The presence of organic matter was not necessary in the latter conditions. As nitrite was not reduced in aerobic conditions and specific productions of N_2O, obtained in continuous experiments, were larger than those obtained in batch assays with the biofilms, the nitrous oxide produced could be attributed to the oxidation of ammonia in normal operational conditions (high DO). The scarce organic matter present in the reactor fed with autotrophic medium would not explain the production of N_2O observed as a consequence of denitrification.

Several catabolic routes were proposed to explain the production of nitrous oxide in aerobic conditions. Stüven et al. [38] proposed that the accumulation of hydroxylamine might cause its own decomposition or reaction with nitrite, producing N_2O in both cases. On the contrary, Poth and Fotch [18] found that the reduction of nitrite was the cause of the production of nitrous oxide. The use of the nitrite as the electron acceptor at low dissolved oxygen conditions had three possible advantages: (1) it conserves the oxygen available, (2) removes nitrite, and (3) decreases the consumption of oxygen by the nitrite-oxidizing bacteria.

Conclusions

Both nitrifying biomasses assayed were able to denitrify nitrite or nitrate into N_2 in anoxic conditions and in the presence of organic matter. N_2O was only accumulated when nitrogen source was nitrite due to the fact that the reduction rate of NO_2^- was higher than that of nitrous oxide.

Nitrifying biofilms were also able to produce N_2O during aerobic conditions. This production was enhanced by low DO levels (maximum at 1 mg O_2 per liter), nitrite accumulation, and the presence of organic matter. N_2O was only detected during oxidation of ammonia, but not during oxidation of nitrite.

To minimize N_2O production, it is recommended to avoid the operation of nitrifying systems under low dissolved oxygen concentrations [31] and under anoxic conditions in the presence of organic matter [39].

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