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Anaerobic ammonium oxidation in marine environments: Contribution to biogeochemical cycles and biotechnological developments for wastewater treatment

Tesis que presenta

Esther Emilia Rios Del Toro

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Director de la Tesis:

Dr. Francisco Javier Cervantes Carrillo

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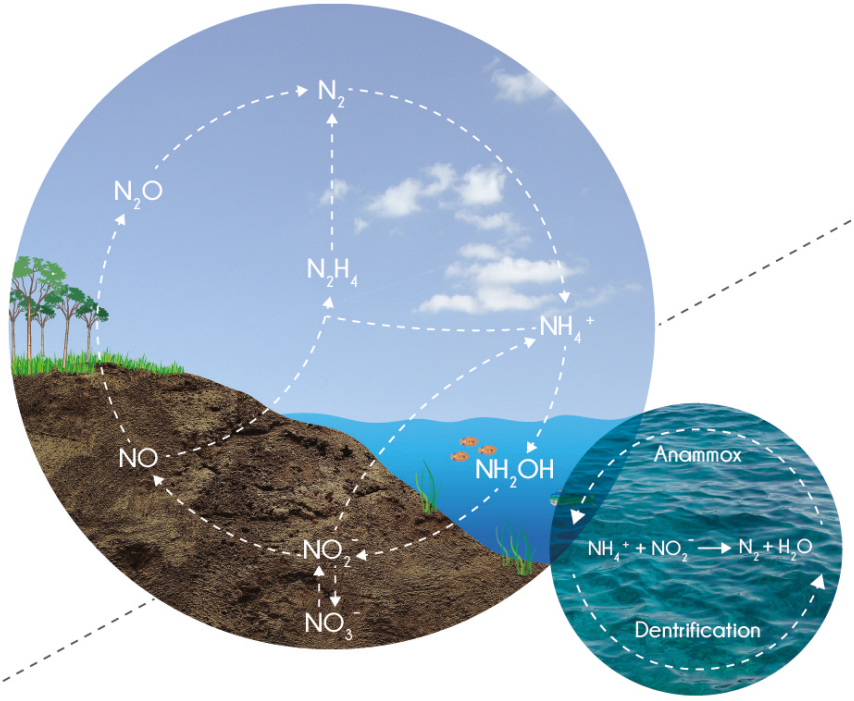
ANAEROBIC AMMONIUM OXIDATION IN MARINE ENVIRONMENTS: CONTRIBUTION TO BIOGEOCHEMICAL CYCLES AND BIOTECHNOLOGICAL DEVELOPMENTS FOR WASTEWATER TREATMENT



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Published results from this thesis

1. Rios-Del Toro, E. E. & Cervantes, F. J.

   Coupling between anammox and autotrophic denitrification for simultaneous removal of ammonium and sulfide by enriched marine sediments.

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2. Rios-Del Toro, E. E., López-Lozano, N. E. & Cervantes, F. J.

   Up-flow anaerobic sediment trapped (UAST) reactor as a new configuration for the enrichment of anammox bacteria from marine sediments.

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3. Rios-Del Toro, E. E., Cortés-Martínez, M. Guadalupe, Sánchez-Rodríguez Miguel A., Calvario-Martínez, Omar, Sánchez-Carrillo, Salvador & Cervantes, F. J.

   Anaerobic ammonium oxidation linked to sulfate and ferric iron reduction fuels nitrogen loss in marine sediments

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Conference papers

Rios-Del Toro E. Emilia and Cervantes, F. J. (2015). **Acoplamiento de los procesos anammox y desnitrificación autotrófica para la eliminación simultanea de amonio y sulfuro por un sedimento marino**. *XVI National Congress of Biotechnology and Bioengineering*, Guadalajara, Mexico, June 21-27, 2015.

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Abstract

"Anaerobic ammonium oxidation in marine environments: Contribution to biogeochemical cycles and biotechnological developments for wastewater treatment". Doctoral Thesis, IPICYT, San Luis Potosí, Mexico

Key words: Anammox, Feammox, Sulfammox, nitrogen removal, wastewater

Microbial processes govern almost entirely the nitrogen cycle in the oceans by driving key reactions, which determine the fluxes of this crucial nutrient in these environments. This dissertation focuses on the role of anaerobic ammonium oxidation (anammox) in marine biogeochemical processes and underlines the important contribution of microbial processes driving this activity in the development of wastewater treatment systems for the removal of nitrogen from saline effluents. Besides the conventional anammox process (the anaerobic ammonium oxidation with nitrite as electron acceptor), anaerobic ammonium oxidation coupled to the microbial reduction of alternative electron acceptors, such as sulfate (sulfammox) and ferric iron (feammox), is also described in the context of global marine biogeochemical cycles. Also, the complex interactions among the oceanic biogeochemical cycles of N, S, and Fe are discussed at the light of the results obtained in this Doctoral thesis. Anammox enrichments were carried out in batch experiments, inoculated with marine sediments collected from different sites of the Mexican littoral. Similarly, experiments with alternative electron acceptors (feammox and sulfammox processes) were also carried out in batch experiments.

Obtained results demonstrated the coupling between the anammox and sulfide-dependent denitrification in two distinct marine sediments. Mass balances revealed that there was more oxidation (up to 38 %) of the electron donors available (ammonium and sulfide) than that expected from stoichiometry. The extra oxidation of sulfide was proposed to be due to the recycling of nitrite, from nitrate produced through anammox, while the extra oxidation of ammonium by the sulfammox reaction.

To our knowledge, the obtained results demonstrated for the first time the prevalence of anaerobic ammonium oxidation with sulfate as electron acceptor in marine environments. Tracer analysis with ^{15}N-ammonium revealed that this microbial process, accounted for up

to 5 μg $^{30}N_2$ produced g $^{-1}$ day^{-1} in marine sediments. Likewise, the occurrence of the feammox process in marine environments accounted for up to 2 μg $^{30}N_2$ produced g $^{-1}$ day^{-1}. These results suggest that these novel nitrogen sinks may significantly fuel nitrogen loss in marine environments, making the interconnections among the oceanic biogeochemical cycles of N, S, and Fe even more complex.

Until now, different reactor configurations for the enrichment of anammox bacteria have been explored. The most popular are the sequential batch reactor (SBR) and UASB (up-flow anaerobic sludge blanket) reactor. However, these reactor configurations depend on sludge characteristics and granulation of biomass. Hence, due to the sediments characteristics, in this work a novel reactor configuration was implemented. UAST configuration (up-flow anaerobic sediment trapped) allowed to maintain the marine sediments inside the bioreactors thanks to the traps installed at different depths to prevent the wash-out of sediments. Three (UAST) reactors were set up (α, β and ω supplied with 50, 150 and 300 mg Ca^{2+}/L, respectively). Obtained results demonstrated that the UAST reactor allowed to reach nitrogen removal rates of up to 3.5 g N/L-d and removal efficiencies >95%. Besides, calcium enhanced biomass production as evidenced by increased volatile suspended solids and extracellular polymeric substances. Deep examination of biomass with massive sequentiation (Illumina Miseq) demonstrated that after the long-term operation, the dominant families detected were *Rhodobacteracea, Flavobacteracea,* and *Alteromonadacea*, while the main anammox genera detected in the three reactors were *Candidatus Kuenenia* and *Candidatus Anammoximicrobium*. The UAST reactor is proposed as suitable technology for the enrichment of anammox bacteria applicable for the treatment of saline industrial wastewaters with high nitrogen content.

Resumen

"Oxidación anaerobia de amonio en ambientes marinos: Contribución a los ciclos biogeoquímicos y desarrollos biotecnológicos para el tratamiento de aguas residuales". Tesis Doctoral, IPICYT, San Luis Potosí, México

Palabras clave: Anammox, Feammox, Sulfammox, eliminación de nitrógeno, aguas residuales

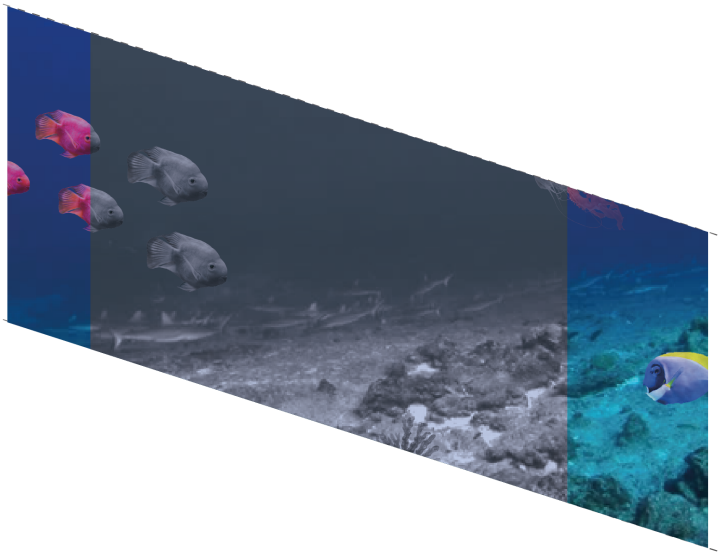
Los procesos microbianos gobiernan casi por completo el ciclo del nitrógeno en los océanos, ya que llevan a cabo reacciones clave que determinan los flujos de este nutriente crucial en estos ambientes. Esta Tesis de Doctorado se centra en el papel que juega la oxidación anaerobia de amonio (anammox) en los procesos biogeoquímicos marinos y subraya la importante contribución de los procesos microbianos que impulsan esta actividad en el desarrollo de sistemas de tratamiento de aguas residuales para la eliminación de nitrógeno de efluentes salinos. Además del proceso convencional anammox (oxidación anaerobia de amonio con nitrito como aceptor de electrones), también se describe, en el contexto de los ciclos biogeoquímicos marinos globales, la oxidación anaerobia de amonio acoplada a la reducción microbiana de aceptores de electrones alternos, como el sulfato (sulfammox) y el hierro férrico (feammox). Además, las complejas interacciones entre los ciclos biogeoquímicos oceánicos de N, S y Fe se discuten a la luz de los resultados obtenidos en esta Tesis Doctoral.

Los enriquecimientos de bacterias anammox se llevaron a cabo en experimentos en lote. Los cultivos fueron inoculados con sedimentos marinos recolectados de diferentes sitios provenientes del litoral mexicano. Los experimentos con aceptores de electrones alternos (procesos feammox y sulfammox) se llevaron a cabo de manera similar.

Los resultados obtenidos demostraron el acoplamiento entre la anammox y la desnitrificación dependiente de sulfuro. Los balances de masa revelaron que había más oxidación (hasta el 38%) de los donadores de electrones disponibles (amonio y sulfuro) de lo esperado por la estequiometria. Se propone que la oxidación extra de sulfuro se debió al reciclaje de nitrito, a partir del nitrato producido a través de anammox, mientras que la oxidación extra de amonio, al proceso sulfammox.

Los resultados obtenidos en esta Tesis demostraron, por primera vez, la prevalencia de la oxidación anaerobia de amonio con sulfato como aceptor de electrones en ambientes marinos. Los análisis llevados a cabo con amonio isotópico (^{15}N) revelaron que este proceso microbiano produce hasta 5 μg $^{30}N_2$ g^{-1} dia^{-1} en los sedimentos estudiados. Así mismo, la ocurrencia del proceso feammox en ambientes marinos representaron hasta 2 μg $^{30}N_2$ producidos g^{-1} dia^{-1}. Los resultados obtenidos sugieren que estos nuevos sumideros de nitrógeno pueden contribuir significativamente a la pérdida de nitrógeno en ambientes marinos, haciendo aún más complejas las interconexiones entre los ciclos biogeoquímicos oceánicos del N, S y Fe.

Hasta ahora, se han utilizado diferentes configuraciones de reactor para los enriquecimientos de bacterias anammox. Los más populares son el reactor secuencial por lotes secuenciados (SBR) y el reactor UASB (lecho anaerobio de flujo ascendente). Sin embargo, estas configuraciones de reactor dependen de las características del lodo y la granulación de la biomasa. Por lo tanto, debido a las características de los sedimentos, en este trabajo se implementó una nueva configuración de reactor. La configuración UAST (sedimento atrapado anaerobio de flujo ascendente) permitió mantener los sedimentos marinos dentro de los biorreactores gracias a la instalación de trampas a diferentes profundidades para evitar el lavado de los sedimentos. Se instalaron tres reactores (UAST) (α, β y ω suministrados con 50, 150 y 300 mg de Ca^{2+}/L, respectivamente). Los resultados obtenidos demostraron que el reactor UAST permitió alcanzar velocidades de eliminación de nitrógeno de hasta 3.5 g N/ L-d y eficiencias de eliminación > 95%. Además, la adición de calcio fomentó la producción de biomasa, que se evidenció por el aumento en los sólidos suspendidos volátiles y las sustancias poliméricas extracelulares. El análisis profundo de la biomasa con secuenciación masiva (Illumina Miseq) demostró que, después de la operación a largo plazo, las familias dominantes detectadas fueron *Rhodobacteracea*, *Flavobacteracea* y *Alteromonadacea*, mientras que los principales géneros de anammox detectados en los tres reactores fueron *Candidatus Kuenenia* y *Candidatus Anammoximicrobium*. El reactor UAST se propone como una tecnología adecuada para el enriquecimiento de bacterias anammox para el tratamiento de aguas residuales industriales con alto contenido de nitrógeno.



CHAPTER 1

General background

1.1 Introduction

Humans are altering the global cycle of nitrogen via excessive production of nitrogen fertilizers and combustion of fossil fuels, as well as intensive aquaculture activities, cultivation of nitrogen-fixing legumes, and other unrestrained actions[1]. The large acceleration of the nitrogen cycle caused by these anthropogenic activities has certainly satisfied the essential demand to sustain an increasing global population, such as providing enough food. However, the nitrogen cycle has shifted from how to promote food production to a realization that intensification of human activities damages environmental systems[2]. Indeed, the overall magnitude of anthropogenic relative to natural sources of fixed nitrogen (210 Tg N yr^{-1} anthropogenic and 203 Tg N yr^{-1} natural) is so large that it has doubled the global cycling of nitrogen over the last century. As nitrogen is a major nutrient, changes in its supply influence the productivity of ecosystems and change the competition between species and biological diversity. Nitrogen compounds as precursors of tropospheric ozone and atmospheric particulate material also degrade air quality[3]. Their effects include increases in human mortality, effects on terrestrial and aquatic ecosystems and contribute to the radiative forcing of global and regional climate[4,5]. Therefore, there are important consequences of the human modification of the global nitrogen cycle, with benefits in food production and costs due to impacts on human health, biodiversity loss and climate[6].

Marine ecosystems have been challenged to overcome intensive pollution caused by nitrogen-rich wastewaters, such as those derived from aquaculture farms. Aquaculture, including the freshwater and marine aquaculture, is a rapidly growing primary production sector implemented to feed the increasing human population. Since 1970, aquaculture has grown at an average rate of 8.9% per year, and the percentage contribution of aquaculture to the total world fisheries has grown from 17.0 to 31.7 % from 1993 to 2003.

Due to the worldwide decline of ocean fisheries and the continuous expansion of the human population, aquaculture will continue to grow greatly to satisfy the minimum protein requirement for human nutrition[7].

Nitrogen fluxes in marine environments are almost entirely governed by microbial processes, which tightly balance nitrogen fixation versus nitrogen loss in these ecosystems. Diazotrophic microorganisms, on one hand, are responsible of nitrogen fixation in the oceans, creating the nitrogen reserve demanded for primary productivity, which ultimately has enormous impact on global climate change due to photoautotrophic carbon dioxide assimilation. On the other hand, until the end of the last century, combination between nitrification and denitrification was considered as the main nitrogen sink in the oceans releasing dinitrogen gas (N_2) back to the atmosphere. More recently, anaerobic ammonium oxidation, the anammox reaction involving the oxidation of ammonium with nitrite as electron acceptor, has also been identified as a major nitrogen sink in marine environments. The aim of this chapter is to provide deep analysis of the literature to underline the importance of the anammox process and alternative microbial metabolisms driving anaerobic ammonium oxidation in marine environments, with special emphasis on their role in biogeochemical cycles.

1.2 Anammox

Anaerobic ammonium oxidation (anammox) is a biological process in which ammonium is oxidized under anaerobic conditions, using nitrite as the electron acceptor, to dinitrogen gas according to the stoichiometry described by Strous and collaborators[8] in 1998:

$$NH_4^+ + NO_2^- \quad N_2 + H_2O \qquad 1.1$$

The whole stoichiometric reaction should be as follows[9]:

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \longrightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \qquad \Delta G^{0'} = -358 \text{ kJ mol}^{-1} \qquad 1.2$$

In fact, as early as 1965, oceanographers already noticed that the amount of ammonium accumulating in an anoxic fjord was far less than that expected for inert ammonium under anoxic conditions[10], suggesting that ammonium could be oxidized in the absence of oxygen. Later, Broda[11] in 1977 described the potential existence of chemolithotrophic bacteria able to oxidize ammonia to N_2 with nitrate as oxidant. These predictions were based on thermodynamic calculations, but the existence of the microorganisms still had not been demonstrated. It was not until 1995 when the anammox process was experimentally discovered and documented in a denitrifying pilot plant at Gist-Brocades, Delft, The Netherlands[12]. Therefore, denitrification could no longer be considered as the only significant metabolic pathway of N_2 production, useful for nitrogen removal.

At the beginning, anammox metabolism was thought to be performed by nitrifying bacteria which were able to manage under anaerobic conditions, using nitrate instead of oxygen as electron acceptor[13]. Later, the bacterium responsible of the anammox reaction was identified by molecular techniques, branching off deep in the order *Planctomycetales*, and named as *Ca. Brocadia anammoxidans*. Physically separated cells of *Ca. B. anammoxidans* were shown to oxidize ammonium with nitrite to N_2 under strictly anaerobic conditions[14]. However, it has been impossible up to date to achieve a pure culture of anammox bacteria. Until now, five anammox "*Candidatus*" genera have been described, with 16S rRNA gene sequence identities of the species ranging between 87 and 99%[15].

These species are: "*Candidatus Kuenenia*", "*Candidatus Brocadia*", "*Candidatus Anammoxoglobus*", and "*Candidatus Jettenia*" enriched from activated sludge and "*Candidatus Scalindua*", enriched from natural habitats, especially from marine sediments and oxygen minimum zones[16–20].

Anammox bacteria are slowly-growing microorganisms with typical doubling times reported of 15-30 days[8,21]. However, there have been some reports on fast growing anammox bacteria. Lotti et al.,[22] in 2014 established a doubling time value of 3.3 days through kinetic characterization in a suspended cell anammox culture.

1.2.1 Anammox cells

Schematic drawing of the anammox cell internal structure is presented in Figure 1.1. Anammox bacteria belongs to the order of *Planctomycetales*, and as them, present an internal compartmentalization based on membrane systems. Specifically, the anammox cell presents three different compartments. The most external region of the cytoplasm, related with the cell wall and the cytoplasmic membrane, constitutes the paryphoplasm. The riboplasm, where the nucleoid is located, is placed in between the intracytoplasmic membrane and the anammoxosome membrane[23]. Finally, the anammoxosome is the third compartment, occupying most of the central part of the cell, being an unique structure for anammox bacteria[24]. All membranes in the anammox cells are composed exclusively of unique ladderane lipids that have 3-5 linearly concatenated cyclobutane rings, a structure unique in nature[25]. Anammox lipids contain a combination of ester-linked (typical of Bacteria and Eukarya) and ether- linked (typical Archaea) fatty acids[23]. It was proposed that anammox bacteria have evolved ladderane lipids as the major component of their cellular membranes to avoid the diffusion of toxic metabolic intermediates (such as hydrazine).

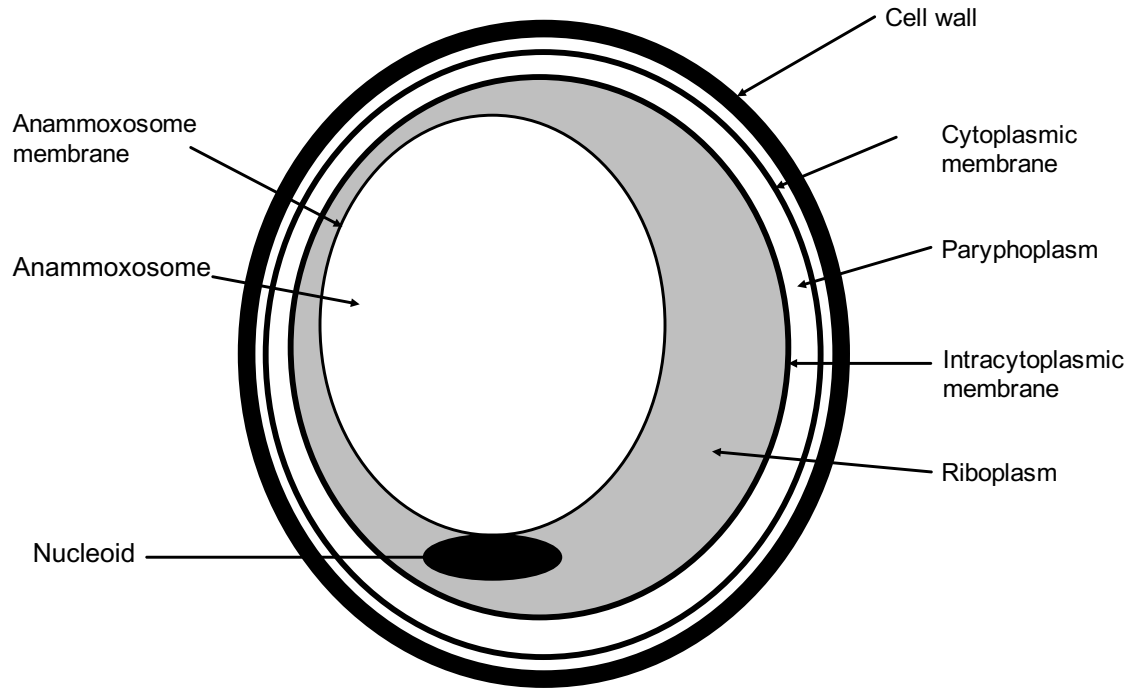


Figure 1.1 Schematic drawing of the anammox cell internal structure.

These lipids provide an unusual density (higher than a conventional membrane) and impermeability to the membrane because of their structural rigidity and size. These unique lipids are used as biomarkers for detecting anammox bacteria in environmental samples[23].

1.2.2 Growth and catabolism

The first metagenome of an anammox bacterium came from an enrichment culture of '*Candidatus Kuenenia stuttgartiensis*' in 2006[26]. In silico analysis of this genome assembly led to the postulation of three redox reactions essential for anammox catabolism.

These three are:

(1) reduction of nitrite to nitric oxide by a cd_1 nitrite reductase (NirS) (equation 1.3),

(2) condensation of ammonium and nitric oxide into hydrazine by a hydrazine synthase (HZS) (equation 1.4), and

(3) oxidation of hydrazine into dinitrogen gas by a hydrazine oxidoreductase (HZO) (equation 1.5) (Figure1.2).

$NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O$ (E_0' = +0.38 V) 1.3

$NO + NH_4^+ + 3e^- + 2H^+ \rightarrow N_2H_4 + H_2O$ (E_0' = +0.06 V) 1.4

$N_2H_4 \rightarrow N_2 + 4H^+ + 4e-$ (E_0' = -0.75 V) 1.5

Later, Kartal and collaborators[27] (2011) verified experimentally with *K. stuttgartiensis* single cells, the anammox metabolic pathway from metagenomic information. Also, some in vivo tests were carried out to confirm the NO importance and specify the role of the anammoxosome in the processes (Figure 1.3). Nitrite and ammonium would diffuse through the anammoxosome membrane and nitrite would be reduced inside the anammoxosome by the nitrite reductase (NirS) to the very reactive free-radical NO.

Loosely membrane associated hydrazine synthase would convert ammonium and NO to hydrazine. Afterwards, hydrazine would be oxidized to dinitrogen gas, which will diffuse through the anammoxosome membrane. Anammox catabolism and energy growth could be attributed to ATPase bounded in this membrane. The *bc1* complex would be involved in the electron transport from hydrazine oxidation to both nitrite and NO reduction and in the flow of protons to the external side of the anammoxosome.

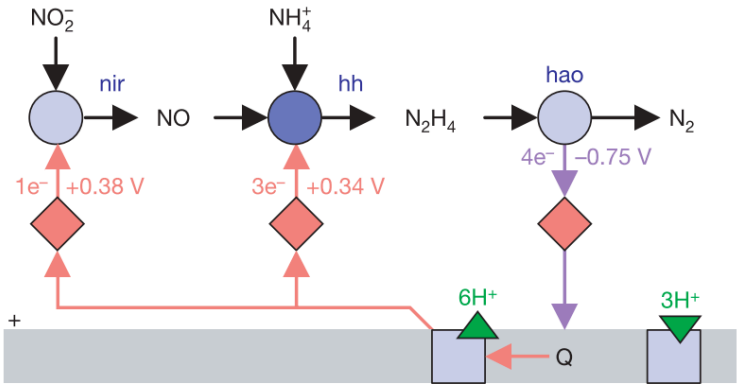


Figure 1.2 Metabolic pathways of K. stuttgartiensis. Anammox central catabolism with nitric oxide as intermediate, electron transport and energy conservation. Red diamonds, cytochromes; red arrows, reductions; purple arrows, oxidations. nir = nitrite reductase hh = hydrazine hydrolase, hao = hydroxylamine-oxidoreductase (Taken from reference[26]).

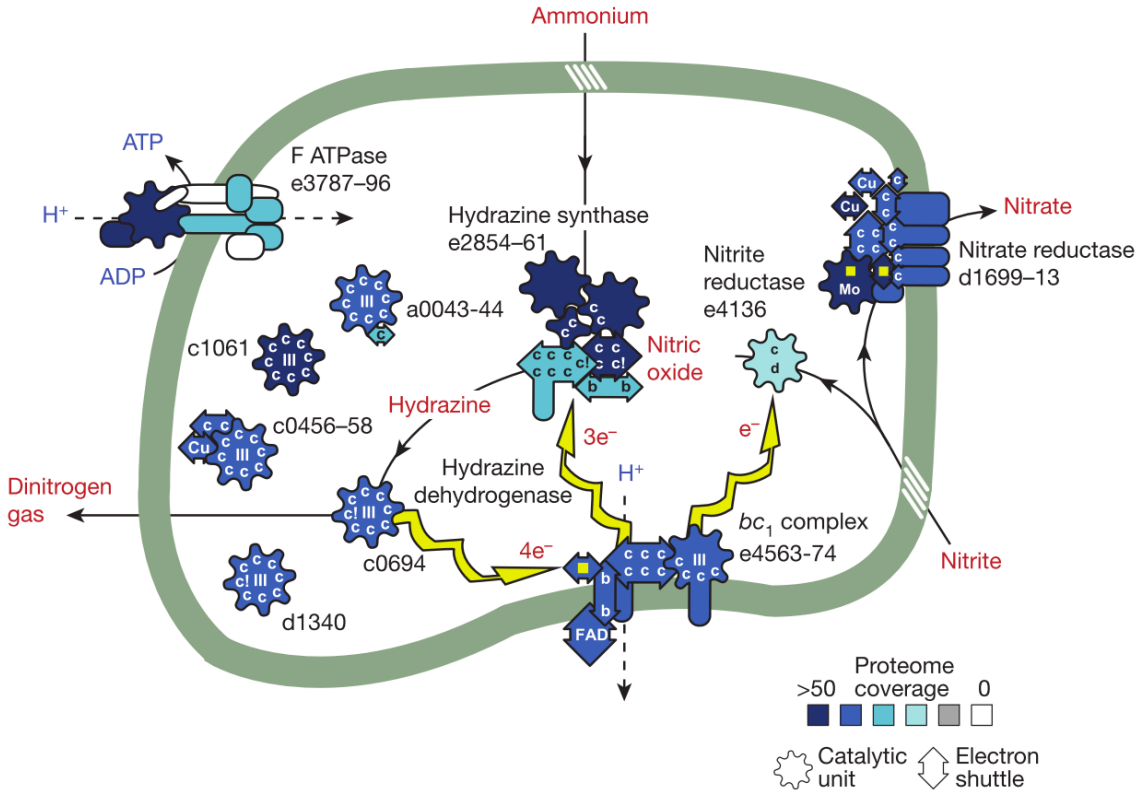


Figure 1.3 Biochemical pathway and enzymatic machinery of *K. stuttgartiensis*. Yellow arrows, electron flow; yellow square, iron– sulfur clusters; b, haem b; c, haem c; d haem d; Mo, molybdopterin. (Extracted from reference[27]).

Shortly after, van de Vossenberg et al.,[28] in 2013 reported the metagenome of the marine anammox bacterium '*Candidatus Scalindua profunda*' (Figure 1.4).

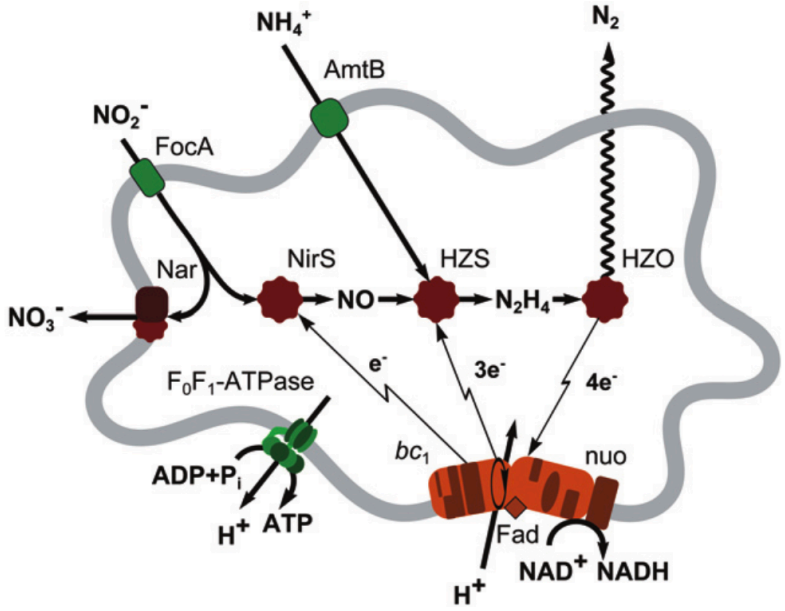


Figure 1.4 Overview of anammox metabolism in '*Candidatus Scalindua profunda*'. Nar/nxr, nitrite::nitrate oxidoreductase; NirS, nitrite reductase; HZS, hydrazine synthase; HZO, hydrazine oxidoreductase; FocA, nitrite transport protein; amtB, ammonium transport protein; nuo, NADH ubiquinone oxidoreductase (complex I).

They constructed a genome assembly, which was subsequently used to analyze the most abundant gene transcripts and proteins. In the *S. profunda* assembly, 4756 genes were annotated, and only about half of them showed the highest identity to the freshwater '*Candidatus Kuenenia stuttgartiensis*' studied by Kartal and collaborators[27]. Hence, they observed pronounced differences in the gene organization and expression of important anammox enzymes, such as hydrazine synthase (HzsAB), nitrite reductase (NirS) and inorganic nitrogen transport proteins. They postulated that adaptations of *Scalindua* to the substrate limitation of the ocean may include highly expressed ammonium, nitrite and

oligopeptide transport systems and pathways for the transport, oxidation, and assimilation of small organic compounds that may allow a more versatile lifestyle contributing to the competitive fitness of *Scalindua* in the marine realm.

Recent findings revealed metabolic diversity of anammox bacteria to produce N_2. Certainly, Oshiki et al. (2016) reported a novel biochemical pathway in *Candidatus Brocadia sinica*. *Candidatus Brocadia* lacks the genes that encode canonical NO-forming nitrite reductases (NirS or NirK) in its genome, which is different from *Candidatus K. stuttgartiensis*. ^{15}N-tracer experiments demonstrated that *Candidatus B. sinica* could reduce nitrite to hydroxylamine (NH_2OH), instead of NO, with as yet unidentified nitrite reductase(s). Further incubations with intact cells and with hydrazine synthase extracted from *Candidatus B. sinica* confirmed concomitant consumption of NH_2OH and NH_4^+, but not NO and NH_4^+, for N_2H_4 synthesis and further production of N_2[29]. Therefore, there are at least two different biochemical pathways in anammox to produce N_2, one NO-dependent and the other NH_2OH-dependent.

Finally, as a summary to date, draft genome sequences have been determined from the members of '*Ca. Kuenenia*'[26], '*Ca. Brocadia*'[29,30], '*Ca. Jettenia*' [31] and '*Ca. Scalindua*' [28,32]. When the genomes of the '*Ca. Kuenenia stuttgartiensis*', '*Ca. Scalindua profunda*' and '*Ca. Jettenia caeni*' were compared; one third of genes located on the genomes were commonly shared as can be seen in Figure 1.5. The shared genes include indispensable genes for anammox processes such as hydrazine synthase (hzs), hydrazine dehydrogenase/hydroxylamine dehydrogenase (hdt/hao), nitrite/nitrate oxidoreductase (nxrAB) and acetyl CoA synthase (acsABCD)[33].

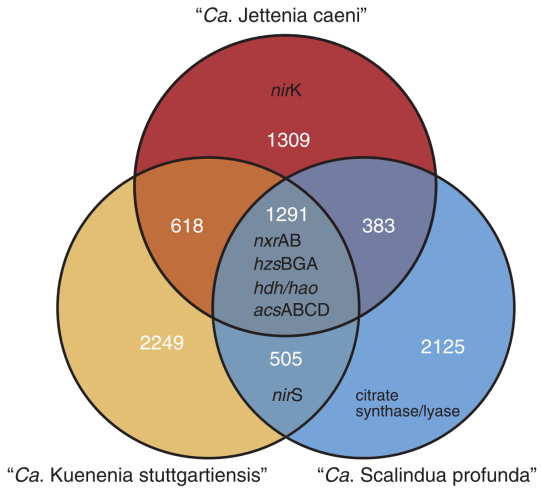


Figure 1.5 Venn diagram showing shared genes among three anammox bacterial genomes. (Reproduced from[33]).

1.2.3 Ecological distribution of anammox bacteria

At the beginning of the discovery of the anammox process in a wastewater treatment plant, it was unknown with certainty, the occurrence of anammox bacteria in different ecological niches. Nowadays, almost all anammox species have been detected in nature and also we know that they are distributed around the world[34–37]. However, little is known about the factors that control their spatial and temporal distribution. Some studies have examined different parameters, such as nitrate availability, organic content and salinity[38,39], but they have been inconclusive. The general conviction is that *Ca. Brocadia*, *Ca. Kuenenia* and *Ca. Anammoxoglobus* are commonly found in non-saline environments and man-made systems[40] while *Ca. Scalindua* species are mostly present in natural saline ecosystems[41].

1.3 Anammox in marine environments

Marine microbes, which are responsible for approximately half of the Earth's primary production, play an enormous role in global nutrient cycling [42].

The first clear evidence of anammox activity in marine environments was in 2002 by Thamdrup and Dalsgaard[43]. They used ^{15}N-nitrogen compounds and detected anammox activity in Danish coastal regions. Later, Marcel Kuypers and colleagues in 2003[18] estimated that anammox process contributes at least with 30% of the total nitrogen turnover in the Black Sea. At the same time, Dalsgaard and colleagues also detected high anammox activity in a coastal bay in Costa Rica, and they also observed that the contribution of the anammox process to the total turnover of nitrogen in the water column was as much as 35% of the total nitrogen turnover. They therefore predicted that anammox activity could be responsible for a major part of global nitrogen turnover[44].

Several other studies, conducted mainly in estuaries, have quantified the fraction of total N_2 production attributable to anammox[45–47]. Among those studies, the fraction of total N_2 production by anammox ranged from 0% to 79%, with several reporting that anammox was irrelevant. Nevertheless, typical contribution of anammox to N_2 production in marine environments was found between 10 and 40% [48].

Only bacteria from the genus *Ca. Scalindua* have been identified in tropical, temperate, and arctic anoxic marine ecosystems. *Ca. S. brodae/sorokinii* or close 16S rRNA gene sequences have also been detected in several locations, such as Black Sea[18], Benguela upwelling system[49], Peruvian oxygen minimum zone (OMZ)[50], and other marine sampling sites around the world[15].

In marine environments, the anammox diversity is rather low in regards to the rest of ecotypes and even comparing to marine ammonium oxidizing bacteria and nitrite oxidizing bacteria, bacterial diversity.

1.4 Anaerobic ammonium oxidation with alternative electron acceptors

The discovery of anaerobic ammonium oxidation process by Mulder 22 years ago[12], changed the global concept of nitrogen cycle, and allowed scientists to widen inside the nitrogen biogeochemical cycle. In addition, it was discovered that anammox bacteria also show metabolic versatility. Strous et al., [26] investigated the iron and manganese respiration by *K. stuttgartiensis*. Surprisingly, both iron and manganese oxides were respired with formate as electron donor by *K. stuttgartiensis*. Later, Van De Vossenberg et al., [51] discovered the nitrate-dependent use of formate, acetate and propionate, as well as formate-dependent reduction of nitrate, Fe(III) and Mn(IV) by "*Candidatus Scalindua*" *spp*. Also, anammox bacteria can oxidize ferrous iron, methylamine, and hydrogen for respiratory ammonification, even though the specific activities of NO_3^- reduction are in general an order of magnitude lower than anammox activities[52,53].

Furthermore, there exist alternative electron acceptors to nitrite for the anammox process. One of them is ferric iron, which has led to a curious name of the anaerobic ammonium oxidation coupled to ferric iron reduction, namely feammox. An alternative road for anaerobic ammonium oxidation is linked to sulfate reduction, called SR-anammox for sulfate-reducing anammox. Both processes (feammox and SR-anammox) will be briefly discussed in the following sections.

1.4.1 Feammox

Anaerobic ammonium oxidation coupled with Fe(III) reduction was reported for the first time by Clément et al.[54] in wetland soils. They observed during incubations of soil slurries, under strictly anaerobic conditions, an unexpected production of nitrite and Fe(II). Afterwards, anaerobic ammonium oxidation coupled to ferric iron reduction was termed

feammox by Sawayama,[55] who reported that Fe(III)-reducing microbes could oxidize ammonium to nitrite using chelated Fe(III) (with EDTA) as terminal electron acceptor. After the above mentioned studies, the feammox process has been reported in different ecosystems, such as tropical forest soils[56], paddy soils[57], and in intertidal wetland sediments[58].

Feammox process to N_2 is energetically more favorable than feammox to NO_2^- or NO_3^-. Marine sediments with amorphous forms of iron oxides, have the potential to support the feammox process. Feammox using ferrihydrite is carried out according to the following equations.

$$3Fe(OH)_3 + 5H^+ + NH_4^+ \longrightarrow 3Fe^{2+} + 9H_2O + 0.5N_2 \quad \Delta G = -245 \text{ kJ mol}^{-1} \quad 1.6$$

$$6Fe(OH)_3 + 10H^+ + NH_4^+ \longrightarrow 6Fe^{2+} + 16H_2O + NO_2^- \quad \Delta G = -164 \text{ kJ mol}^{-1} \quad 1.7$$

$$8Fe(OH)_3 + 14H^+ + NH_4^+ \longrightarrow 8Fe^{2+} + 21H_2O + NO_3^- \quad \Delta G = -207 \text{ kJ mol}^{-1} \quad 1.8$$

Little is known about the occurrence of feammox in marine environments. Also, due to the widespread occurrence of anammox bacteria in marine environments, there may exist alternate routes that combine the nitrogen and iron cycles. Therefore, the feammox process may involve several complex interactions within the biogeochemical cycles of iron and nitrogen.

1.4.2 Sulfate-reducing anammox (SR-anammox)

The first report of anaerobic ammonium oxidation coupled to sulfate reduction was by Fdz-Polanco in 2001[59]. Similar to the discovery of anammox, they observed an anomalous behavior in a fluidized-bed reactor in terms of unusual losses of high concentrations of N_2 gas. They suggested a new anaerobic removal process of ammonia and sulfate.

$$SO_4^{2-} + 2NH_4^+ \longrightarrow S + N_2 + 4H_2O \quad \Delta G_0 = -47.8 \text{ kJ mol}^{-1} \quad 1.9$$

Later Yang et al., [60] reported the simultaneous removal of ammonium and sulfate (SRAS) in an anaerobic up-flow bioreactor. The ratio of ammonium to nitrite consumption was approximately 1:1.15 in the reactor after 160 days, in the stationary phase of anammox process, which is much higher than 1:1.32 in the traditional anammox process. They hypothesized that the extra electron acceptor fueling ammonium oxidation was sulfate. They postulated that nitrite could be an intermediate product during the SRAS process. Also, they claimed that some denitrification process could be operated by the reduction of nitrite to nitrogen accompanied by the oxidation of sulfide by autotrophic denitrifiers according to the following reactions:

$$3SO_4^{2-} + 4NH_4^+ \longrightarrow 4NO_2^- + 3S^{2-} + 4H_2O + H^+ \qquad 1.10$$

$$3S^{2-} + 2NO_2^- + 8H^+ \longrightarrow N_2 + 3S + 4H_2O \qquad 1.11$$

At the same time, Schrum et al.[61] evaluated the thermodynamics of sulfate-reducing ammonium oxidation in a sediment from subseafloor in the Bay of Bengal. They used pore water chemical profiles to provide evidence that the process occurs in nature. However, they based their results mainly in fluxes of ammonium and sulfate and Gibbs free energy calculations, but the evidence provided was not compelling.

Recently, Rikmann et al.,[62] performed a comparison between sulfate-reducing and conventional anammox in UASB reactors. They found that nitrogen could be removed both from real wastewater and synthetic wastewater using either NO_2^- (more efficiently) or SO_4^{2-} (less efficiently) as an electron acceptor.

Until now, most studies involving sulfate-reducing anammox, have been carried out in bioreactors studying mainly the contribution of this process in biotechnological applications for the simultaneous removal of ammonium and sulfate from industrial wastewater.

Environmental implications of having SR-anammox and feammox in marine sediments is scantily researched. Until now there are no reports about the coexistence of SR-anammox and feammox process in marine environments to widen the approach between the biogeochemical cycles of nitrogen, sulfur and iron.

1.5 Contribution of anaerobic ammonium oxidation to marine biogeochemical cycles

Many natural and chemical substances circulate through the environment, which are important to the chemistry and biology of the Earth. The circulation of a particular element is termed "biogeochemical cycle". Biogeochemical cycles vary in time and space scale. The long-term circulation of Earth materials represents one extreme in which materials are transported through the atmosphere and geosphere (lithosphere and hydrosphere), which interacted profoundly with the biosphere. In the oceans, stream-borne solids, and some originally dissolved substances, now part of solids, sink and become sea-floor sediments, while some substances are returned to the atmosphere[63]. When we contemplate a specific biogeochemical cycle, we focus on a particular element, and how that element participates in chemical reactions, moving through various molecular configurations. The principal elements that are essential for life are: carbon, hydrogen, oxygen, and nitrogen. However, other elements are also certainly important, such as: phosphorus and sulfur. Some "non-essential" elements participate in biogeochemical cycles, entering organism tissues due to chemical similarity to essential elements. The present dissertation was focused on processes involved in the anaerobic ammonium oxidation and how they are interconnected within the nitrogen, iron and sulfur biogeochemical cycles. These three cycles will be briefly discussed.

Biogeochemical cycles are mainly driven by electron transfer processes and mediated thermodynamically by microorganisms, changing the chemistry of the Earth's surface profoundly. The fluxes of electrons can be combined with major elements to construct a global metabolic map for the Earth[64]. Each step in the element cycles is catalyzed by a specific group of microorganisms.

These may or may not be evolutionarily related, but they share a similar lifestyle and so form an 'ecological guild'. Thermodynamic calculations show that most of these guilds have already been discovered[65] and there are others which have not been detected yet. Figure 1.6 describes the reactions inside different biogeochemical cycles that are known along with the reactions studied in the present thesis.

Despite the Earth's surface is mostly oxic, anoxic environments are vastly distributed. These anoxic environments include deep sea, freshwater, soils, and sediments, providing habitats for diverse microorganisms. In these environments, anaerobic microorganisms perform complex redox reactions, driving the coupling of several elements[66]. The processes studied here are mainly carried out under anoxic environments.

In the following sections, the biogeochemical cycles of nitrogen, iron, and sulfur will be described and how they are involved in the processes studied in this doctoral dissertation to have an enhanced picture of the biological processes that will be examined afterwards in detail.

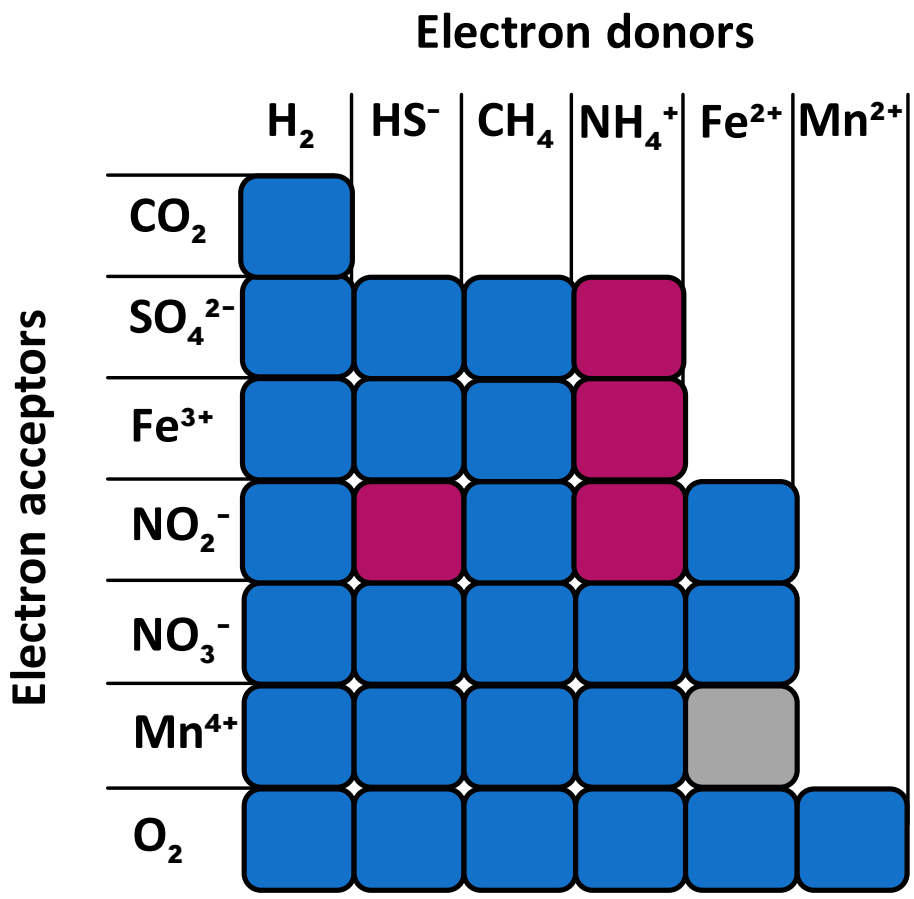


Figure 1.6 Contributions of microorganisms to biogeochemical cycles. Each step in the elements cycles is a redox reaction defined by an electron-donor and acceptor pair catalyzed by a specific ecological guild of microorganisms. The figure indicates the steps in the element cycles that are known (blue) and those that are still unknown but thermodynamically possible (grey). The reactions studied in the present work are indicated in pink and are a possible links among the nitrogen, iron and sulfur cycles. (Adapted from reference[65]).

1.5.1 Nitrogen cycle

The nitrogen cycle is the most complex of Earth's biogeochemical cycles, constituted by an unusually diverse set of transformations, many of which are carried out only by distinct groups of specialized microorganisms (Figure 1.7). Furthermore, it involves reservoirs of different forms of nitrogen in the atmosphere, oceans, soils and sediments, the crust, and

biota. Most of the major transformations were discovered more than a century ago. Nonetheless, our understanding of the functional relationships within the nitrogen cycle has changed substantially during the past 10–20 years. Important discoveries include new types of organisms that are involved in the well-known processes as well as those that convey new types of processes.

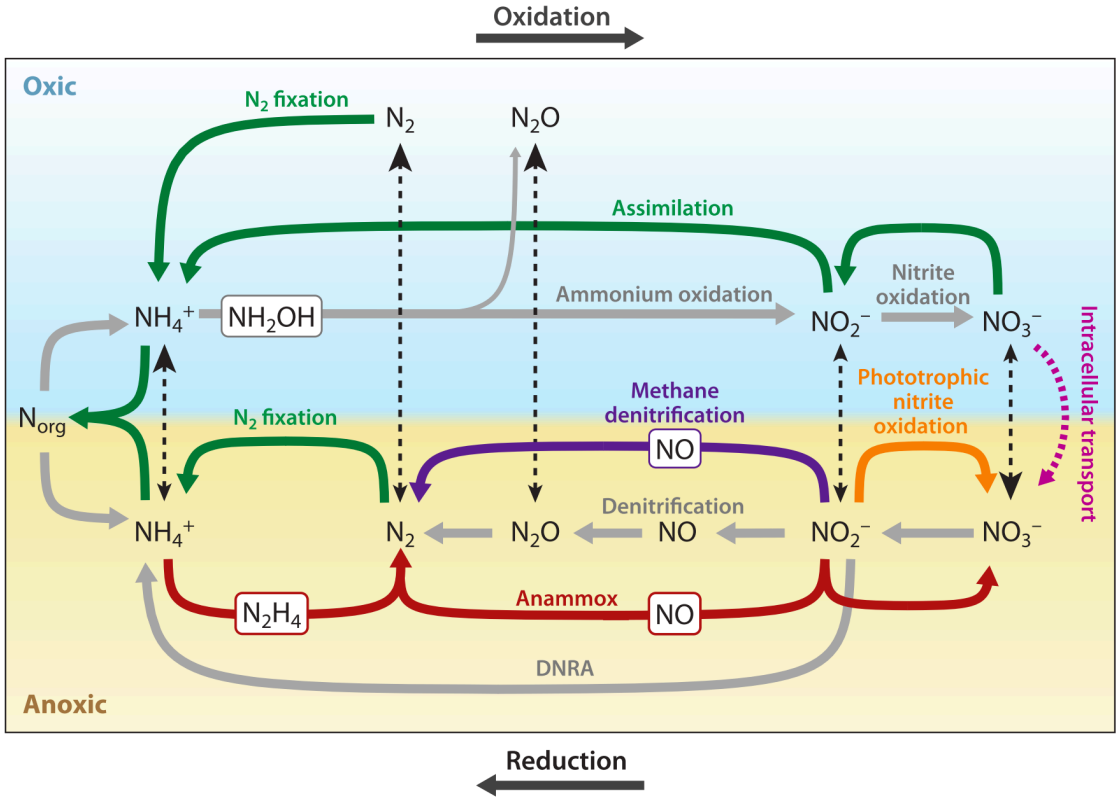


Figure 1.7 Schematic representation of the nitrogen cycle. Metabolic transformations are shown as thick arrows. Also shown are the classical processes of assimilation (green) and dissimilation (gray). Aerobic and anaerobic processes are separated, and dashed vertical arrows indicate exchange or transport between oxic and anoxic environments, with the relative size of arrowheads indicating the dominant direction of transport. DNRA: dissimilatory nitrate reduction to ammonium. (Taken from reference [67]).

Nitrogen is an essential element required in large amounts by all living organisms, mainly for the synthesis of amino acids and nucleotides, and it takes part in several different types of respiratory energy metabolism in which nitrogen compounds may serve as either electron acceptor or electron donor. On a global scale, the availability of fixed nitrogen is controlled by the balance between nitrogen fixation and the recycling of fixed nitrogen to N_2 through dissimilatory transformations[67] (Figure 1.7). The anammox process (equation 1.1 and 1.2) is carried out inside the nitrogen cycle presented in Figure 1.7, it is clear that it occurs in anoxic environment and that it is closely related to denitrification and DNRA (dissimilatory nitrate reduction to ammonium).

Hence, the nitrogen cycle is closely coupled to the carbon and sulfur cycles, and its processes and their regulation are of fundamental importance in both modern and ancient ecosystems. At the moment, the discovery of the anammox process (discussed above) as a completely autotrophic nitrogen removal process have focalized the researchers attention, not only as a powerful tool to achieve nitrogen removal from wastewater[68], but also as the explanation for nitrogen losses in natural systems and as an important contributor to the nitrogen cycle in marine systems[20,56].

1.5.2 Iron cycle

Iron (Fe) is the most abundant element on Earth and the fourth most abundant element in the crust. During the early evolution of the Earth, most iron deposits, along with nickel and sulfur, were incorporated into the core, but significant amounts remained in the crust, such that iron comprises approximately 5% by mass of the present-day crust. Iron can possess variable valence states, and energy involved in the transformation from one valence state to another has been utilized widely in biological systems. Iron can exist in a range of oxidation

states, from –2 to +6, but the most common oxidation states are Fe(II) (ferrous iron) and Fe(III) (ferric iron). Elemental Fe (with an oxidation state of zero) is rare at the Earth's surface as it is readily oxidized to Fe(III) (oxyhydr-) oxides (the components of "rust"). This oxidation variability state produce a wide range of geological, environmental, and economical important iron minerals[69] (see Table 1.1).

The redox transformation of Fe(II) to Fe(III), and vice versa, is of major importance to a number of biological and element-cycling processes. Figure 1.8 describes chemical and biological reactions than can occur in processes involving the iron cycle and their interaction with others in the sulfur cycle. Many of these interactions are promoted by microbes, the real workhorses in driving biogeochemical cycles.

1.5.2.1 Fe(II) oxidation

Biological Fe(II) oxidation could be carried out under aerobic and anaerobic conditions as can be seen in detail in Figure 1.9. Under anaerobic conditions, ferrous iron is relatively stable, but the biological oxidation of Fe(II) may still occur with nitrate as the electron acceptor via the following reaction:

$$10Fe^{2+} + 2NO_3^- + 24H_2O \longrightarrow 10Fe(OH)_3 + N_2 + 18H^+ \qquad 1.12$$

The tolerance of both autotrophic nitrate-reducing and photosynthetic Fe(II)-oxidizers to low levels of oxygen allows them to harvest substrate and energy along, and partly across, the oxic–anoxic interface where competition with aerobic Fe(II) oxidation could occur.

Table 1.1 Common iron minerals present at, or near, the Earth's surface. (Taken from reference[69].

Mineral Class	Name	Formula
Native or metal form	**Native iron**	**Fe**
Oxides/oxyhydroxides	Ferrihydrite	$Fe^{3+}_{4\text{-}5}(OH,O)_{12}$
	Goethite	$FeO(OH)$
	Lepidocrocite	$Fe^{3+}O(OH)$
	Hematite	Fe_2O_3
	Maghemite	$Fe_{2.67}O_4$
	Magnetite	Fe_3O_4
	Green rusts	$Fe^{(2+\text{-}3+)}$ hydroxysalts
		General formula
		$[Fe^{2+}_{(1\text{-}x)}Fe^{3+}_{x}(OH)_2]^{x+\cdot}$
		$[(x/n)A^{n-}\cdot(m/n)H_2O]x^-$,
		where x is the ratio Fe^{3+}/Fe_{tot}
Carbonates	Siderite	$FeCO_3$
	Ankerite	$Ca(Fe,Mg,Mn)(CO_3)_2$
Sulfides	Pyrite	FeS_2
	Marcasite	FeS_2
	Pyrrhotite	$Fe_{1\text{-}x}S$
	Mackinawite	FeS
	Greigite	Fe_3S_4
	Sphalerite	$(Zn,Fe)S$
Phosphates	Vivianite	$Fe_3(PO_4)2\cdot8H_2O$
	Strengite	$FePO_4\cdot2H_2O$
Silicates	Berthierine	$(Fe^{2+},Fe^{3+},Al)_3(Si,Al)_2O_5(OH)_4$
	Chamosite	$(Fe^{2+},Mg,Al,Fe^{3+})_6(Si,Al)_4O_{10}(OH,O)_8$
	"Glauconite"*	$KMg(FeAl)(SiO_3)_6\cdot3H_2O$
	Greenalite	$(Fe^{2+},Fe^{3+})_{2\text{-}3}Si_2O_5(OH)_4$
	Odinite	$(Fe^{3+},Mg,Al,Fe^{2+})_{2.5}(Si,Al)_2O_5(OH)_4$

* Glauconite is a series name rather than a mineral but is included here because of its common occurrence in many sedimentary rocks.

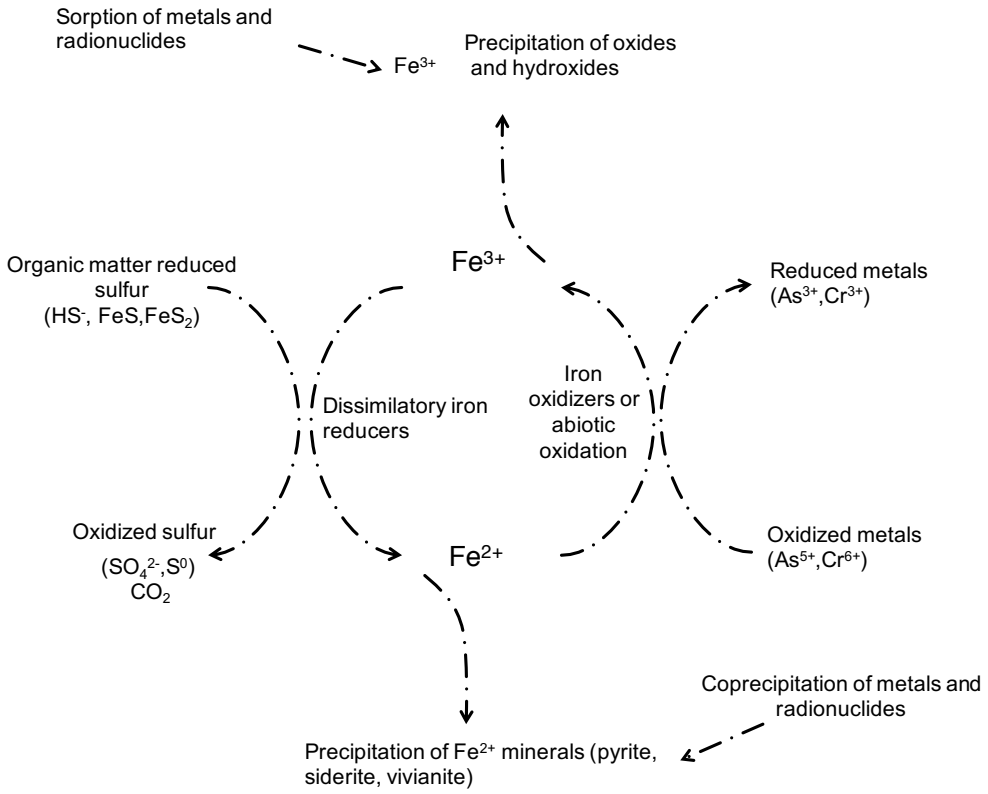


Figure 1.8 Interactions between the iron redox cycle and other elemental cycles. (Reproduced from reference[69]).

One interesting aspect of nitrate-dependent Fe(II) oxidation is that a variety of different Fe(III) minerals (including magnetite, ferrihydrite, goethite, lepidocrocite, and green rusts) are formed depending on the geochemical conditions[70].

1.5.2.2 Fe(III) reduction

Fe(III) biological reduction is a very interesting process, it occurs in nature below the zone of manganese reduction. The decomposition of freshly deposited organic material in sediments proceeds in a continuous sequence of redox reactions, with the most electropositive oxidants, such as O_2 and NO_3^-, being consumed at or near the surface and

progressively poorer oxidants, like Mn(IV), Fe(III), SO_4^{2-}, and CO_2, being consumed at depth. Decomposition continues until the labile organic fraction is exhausted and the deeper sediments are left with a composition very different from that of the sediments originally deposited.

The amorphous to poorly ordered iron (oxyhydr)oxides, such as ferrihydrite, are the preferred sources of solid-phase ferric iron for Fe(III)-reducing bacteria[71]:

$$CH_3COO^- + 8Fe(OH)_3 \longrightarrow 8Fe^{2+} + 2HCO_3^- + 15OH^- + 5H_2O \qquad 1.13$$

More-crystalline Fe(III) oxides (e.g. hematite and magnetite) and Fe(III)-rich clay minerals are also microbially reducible, and some experimental observations suggest that these minerals may provide energy for cellular growth comparable to that derived from the poorly crystal-line phases. The variations in reductive rates are related to a number of factors, including the amount of surface area exposure, crystal morphology, particle aggregation, the composition of the aqueous solution in which the microorganisms grow, and the amount of Fe^{2+} sorbed to the oxide surface. Importantly, with such wide variations in reactivity towards microbial reduction, it is not surprising that ferric iron can represent a long-term electron acceptor for organic matter oxidation, even at depths where other anaerobic respiratory processes are thermodynamically predicted to dominate[72].

Feammox process recently discovered in soils, links the nitrogen and iron cycles via ferric iron reduction coupled to ammonium oxidation[56] (Figure 1.9). The responsible microorganisms have not yet been identified. The end product of this anaerobic feammox reaction can be NO_3^-, NO_2^- or N_2. As N_2 is a gaseous N species, this reaction could lead to a substantial loss of nitrogen in environments that are rich in Fe(III) oxyhydroxides.

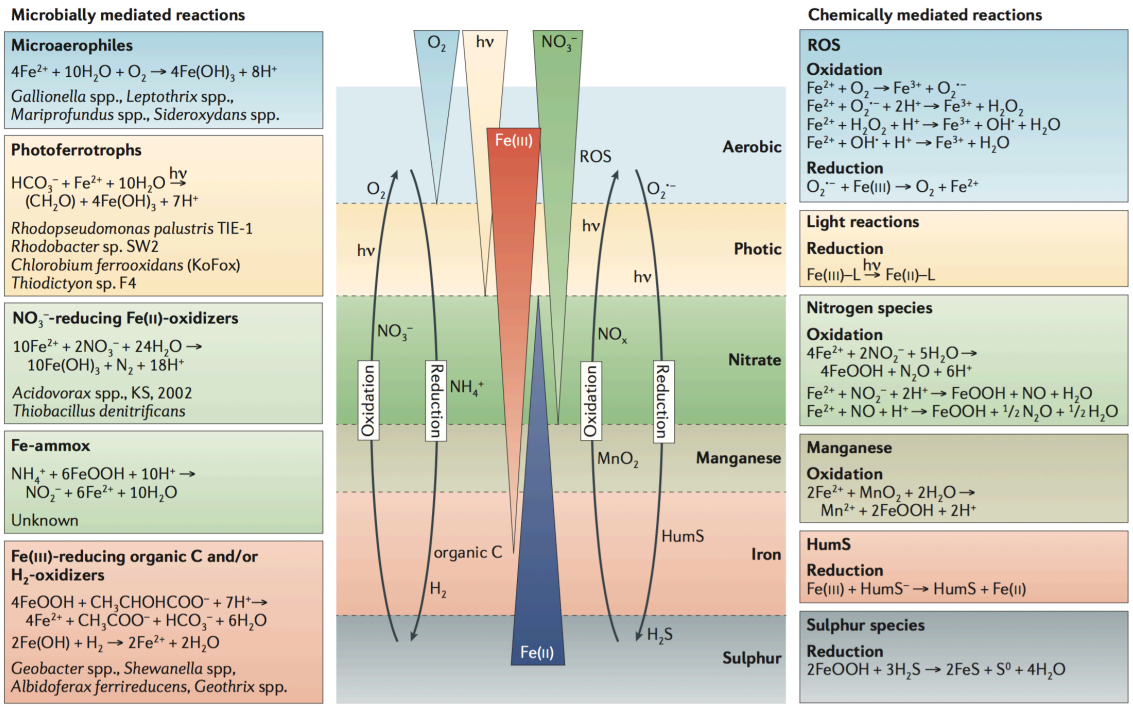


Figure 1.9 Microbially and chemically mediated reactions that form the biogeochemical Fe cycle. Microbially mediated iron (Fe) redox reactions are shown on the left-hand side and abiotic Fe redox transformations are shown on the right-hand side, listed in a thermodynamic order (although some of these reactions may overlap in the natural environment). Within the oxic zone, microaerophilic Fe(II) oxidizers oxidize ferrous Fe (Fe(II)) using oxygen (O_2). Reactive oxygen species (ROS) can oxidize Fe(II), and superoxide (O_2^-) can abiotically reduce ferric iron (Fe(III)). Within the photic zone, phototrophic microorganisms oxidize Fe(II) and photochemical reactions reduce Fe(III) that is bound to organic ligands (L). Mixotrophic and autotrophic nitrate (NO_3^-) dependent Fe(II) oxidation is restricted to anoxic conditions in the denitrification zone. NO_3^-- reducing Fe(II)-oxidizing bacteria use Fe(II) as an electron donor. Feammox bacteria couple the oxidation of ammonium (NH_4^+) to Fe(III) reduction. Fe(II) can be chemically oxidized via chemodenitrification by reactive nitrogen (N) species. Fe(II) is abiotically oxidized by manganese (Mn) via surface-catalyzed reactions. Fe(III)-reducing microorganisms can reduce Fe(III) coupled to the oxidation of various electron donors such as organic carbon (C) and H_2. Electron-rich (that is, reduced) humic substances (HumS) abiotically reduce Fe(III) to Fe(II). Fe(III) is chemically reduced by hydrogen sulfide (H_2S) to ferrous sulfide (FeS) species. (Taken from reference[73]).

Until now, if you have not asked, how iron-reducing microorganisms access iron? It is time to consider it. In fact, there is not a simple answer, as several processes seem to be involved. First, many Fe-reducing organisms require direct contact with the Fe (oxyhydr)oxide in order to conduct Fe reduction[74] (Figure 1.10). This contact is required because Fe (oxyhydr)oxides are quite insoluble[75], and redox-active proteins involved in electron transfer during Fe reduction are often located at the outer cell membrane. Not all electron transfer, however, proceeds through surface-bound proteins. *Geobacter sulfurreducens* can transfer electrons to Fe (oxyhydr)oxide surfaces with electron-conductive pili, so-called nanowires.

Thus, electrons may be transported between organisms through nanowires. Other strategies are also used by Fe reducers to access Fe which require no direct contact with the Fe (oxyhydr)oxides. Some Fe reducers secrete chelators, so-called siderophores, which can liberate the Fe(III) from Fe (oxyhydr)oxides and bring it into solution for utilization by Fe-reducing organisms. Another strategy used by Fe reducers is the production of redox-sensitive compounds (quinones for example) that can accept electrons from the bacteria and deliver them extracellularly to the Fe (oxyhydr)oxide surface, reducing the Fe(III) to Fe(II)[73]. All in all, a fascinating number of strategies are used by microbes to access Fe(III) for reduction. This demonstrates the extreme ingenuity of the microbial world in conducting their livelihoods and the ability of evolution to explore all corners for possible Fe acquisition strategies. Finally, iron has been established as a key elemental resource that shapes the magnitude and dynamics of primary production in the global ocean[76]. Taken together with biogeochemical cycles of, nitrogen and sulfur, iron cycle give us new insights of how these connections operates in the ocean.

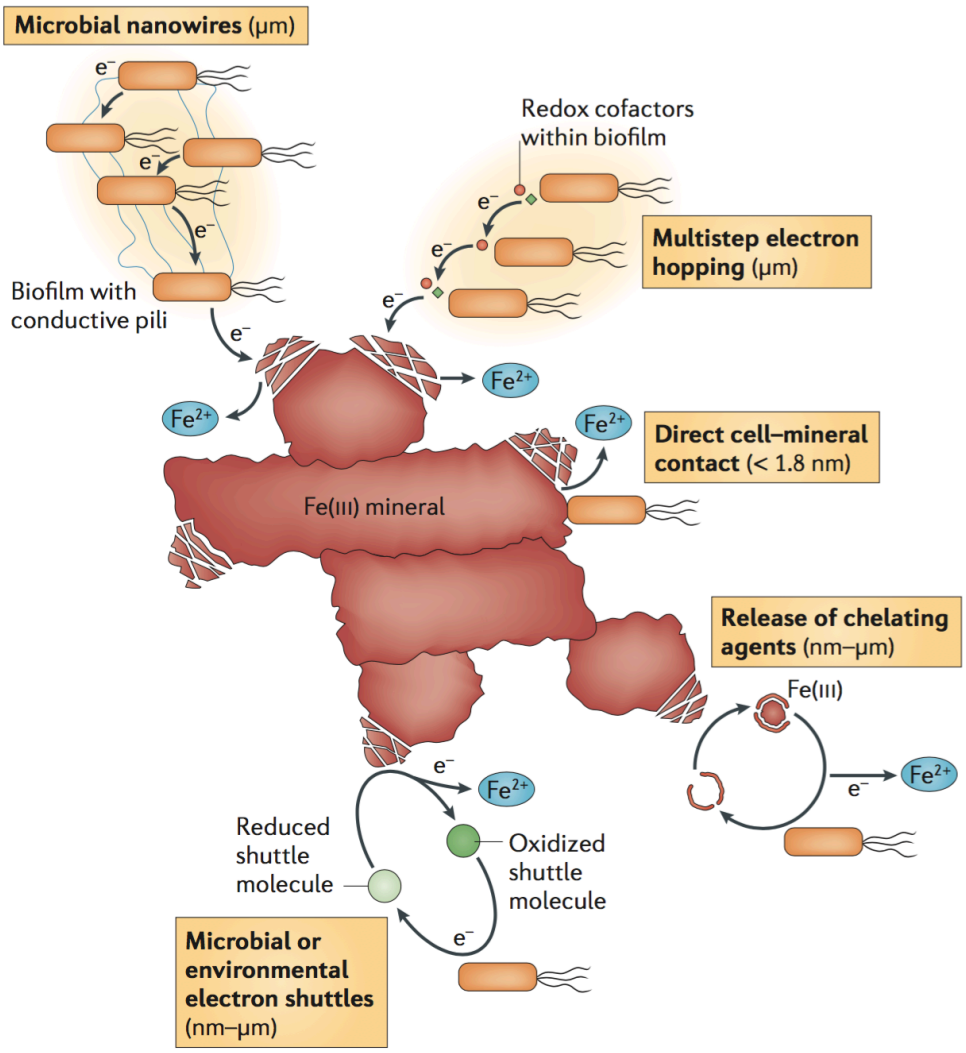


Figure 1.10 Mechanisms of electron transfer from microorganisms to Fe(III) minerals. Schematic representation of metabolic strategies by microbial Fe(III) reducers to reduce Fe(III) minerals. Direct contact between the bacterial cell and Fe(III) minerals facilitates Fe(III) reduction over short distances. Bacteria secrete chelating agents or exploit microbial or environmental redox-active electron shuttles (such as flavins or dissolved and solid-state humic substances, respectively) to facilitate electron transfer over short (nm) and long (μm) distances. Electrically conductive pili and multistep electron hopping via redox cofactors that are present in biofilms have been implicated in long-distance extracellular electron transfer. (Taken from reference[73]).

1.5.3 Sulfur cycle

Sulfur is among the most abundant elements on Earth. It is mainly present as pyrite (FeS_2) or gypsum ($CaSO_4$) in rocks and sediments and as sulfate in seawater. The sulfur cycle (Figure 1.11) is complex, because sulfur has a broad range of oxidation states, from –2 (completely reduced) to +6 (completely oxidized), and can be transformed both chemically and biologically. In addition, the sulfur cycle is closely linked to other element cycles, such as the carbon and nitrogen cycles. Microorganisms play an important part in sulfur transformations. Sulfate is taken up as a nutrient and reduced to sulfide, which is then incorporated into sulfur containing amino acids and enzymes. Oxidation and reduction reactions for the generation of metabolic energy are also important, such as sulfide oxidation by chemolithotropic sulfur bacteria and dissimilatory sulfate reduction by sulfate-reducing bacteria[77].

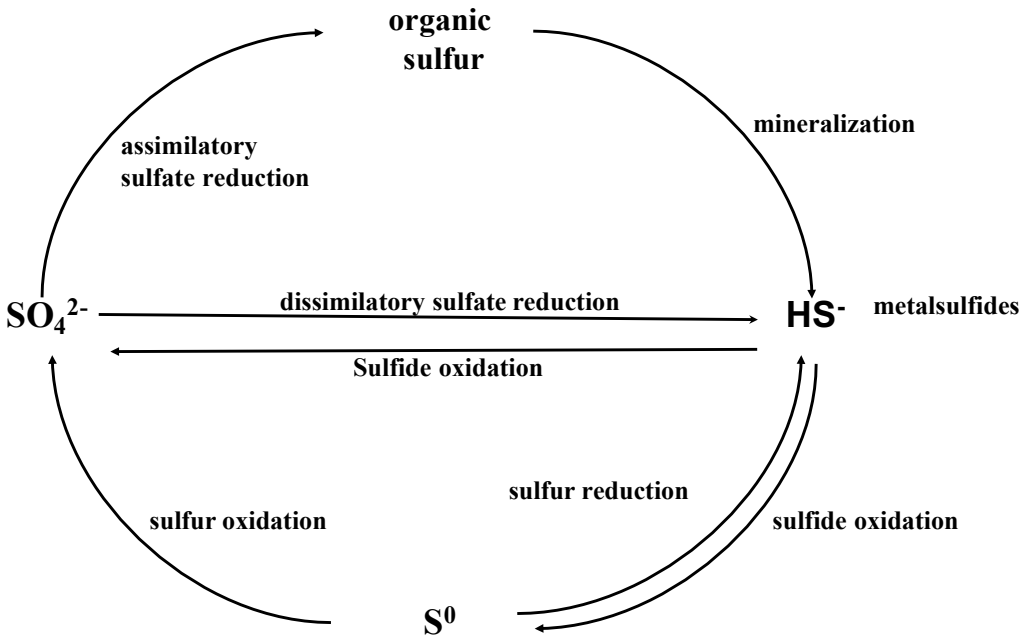


Figure 1.11 The biological sulfur cycle. Modified from reference[78].

Sulfate-reducing bacteria are anaerobic microorganisms that are widespread in anoxic habitats, where they use sulfate as a terminal electron acceptor for the degradation of organic compounds, resulting in the production of sulfide. Subsequently, sulfide can be oxidized under oxic conditions by chemolithotrophic sulfur bacteria or under anoxic conditions by phototrophic sulfur bacteria. It has been estimated that sulfate reduction can account for more than 50% of the organic carbon mineralization in marine sediments, which indicates the importance of sulfate reducers in both the sulfur and carbon cycles and, consequently, why sulfate-reducing bacteria have been extensively studied.

Oxidation of sulfide by autotrophic denitrifiers (autotrophic denitrification) accompanied by nitrite/nitrate reduction is also carried out inside the sulfur cycle (Equation 1.11). Autotrophic denitrification coupled to nitrite reduction represents a link between the sulfur and nitrogen cycles (process to be studied in detail in Chapter 2). Sulfate-reducing anammox, is a theoretically feasible reaction, which also connects the biogeochemical cycles of sulfur and nitrogen (Equations 1.9-1.10 and discussed in detail in Chapter 3).

1.6 Scope of dissertation

Even today, much remains to be discovered in the study of biogeochemical cycles and biological reactions around them. The discovery of the anammox process was undoubtedly one of those findings within the nitrogen cycle. This changed the scenario and the way of seeing the global nitrogen cycle, and even changed paradigms about life due to the peculiar cell composition and characteristics of anammox bacteria. Currently, many reactions within biogeochemical cycles, which are thermodynamically feasible, have not yet been identified in natural environments or laboratories. The discovery of these natural reactions offers the opportunity, on one hand, to increase the knowledge about how these reactions interact and

their contribution in global biogeochemical cycles; and on the other hand, to emulate on a large scale these processes with the purpose of using them in biotechnological applications.

In the present dissertation, the capacity of marine sediments obtained from different sites of the Mexican coasts to carry out the anammox process *ex situ* was evaluated. Also, it was evaluated the ability of these sediments to carry out the coupling between anammox and sulfide-dependent autotrophic denitrification. The present dissertation also considers to study the anaerobic ammonium oxidation coupled to ferric iron reduction (feammox process) and the anaerobic ammonium oxidation linked to sulfate reduction (SR-anammox), hypothesized thermodynamically by many authors. Finally, the enrichment of anammox biomass from marine sediments was carried out in a novel bioreactor for the treatment of synthetic wastewater similar to effluent from aquaculture.

The prospective of the anammox process in several marine sediments and the coupling between anammox and sulfide-dependent autotrophic denitrification are discussed in detail in Chapter 2. In Chapter 3 the capability of marine sediments from the Mexican littoral to perform the anaerobic ammonium oxidation with sulfate and ferric iron as terminal electron acceptors is reported. Chapter 4 describes the development of the novel reactor, up-flow anaerobic sediment trapped (UAST) reactor, for the enrichment of anammox bacteria from marine sediments. Finally, the results obtained in this research are globally discussed in Chapter 5.

1.7 Hypotheses and objectives

Hypotheses

- Microorganisms present in marine sediments from different sites of the Mexican littoral are capable of achieving anammox and sulfide-dependent denitrification simultaneously to remove ammonium and sulfide linked to nitrite reduction.
- Based on thermodynamics, it is postulated that the microbial community present in marine sediments from different sites of the Mexican littoral is able to perform the anaerobic ammonium oxidation coupled to the reduction of sulfate and ferric iron.
- Enrichment of anammox bacteria from marine sediments will be possible in a novel reactor configuration, referred to as up-flow anaerobic sediment trapped (UAST) reactor, designed for the biomass retention. Besides, the addition of calcium will promote the growth and granulation of biomass.

Objectives

- To test the capacity of enrichment cultures derived from marine sediments collected from different sites of the Mexican littoral to achieve anammox and sulfide-dependent denitrification and to study the coupling between these two respiratory processes to simultaneously remove ammonium and sulfide linked to nitrite reduction.
- To test that the sulfammox and feammox processes prevail in different marine sediments freshly collected from the eastern tropical North Pacific coast, and quantify the rates at which their microbial communities perform these processes.

- To test the capacity of a novel reactor configuration, referred to as up-flow anaerobic sediment trapped (UAST) reactor, to enrich and retain anammox biomass from marine sediments. In addition, the effect of calcium on the performance of the anammox process was also studied as well as the taxonomic characterization of the anammox enrichments.

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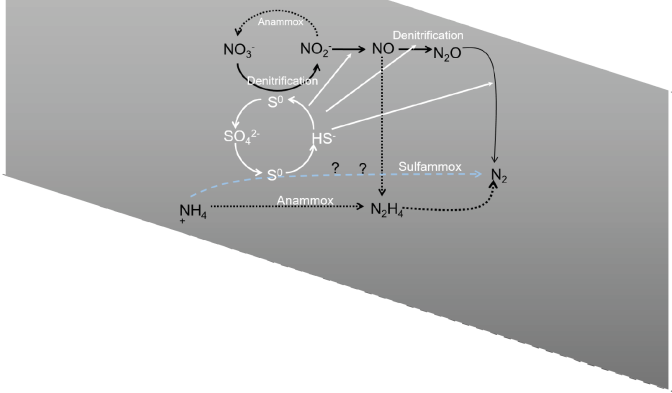

C H A P T E R

2

Coupling between anammox and autotrophic denitrification for simultaneous removal of ammonium and sulfide by enriched marine sediments

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Coupling between anammox and autotrophic denitrification for simultaneous removal of ammonium and sulfide by enriched marine sediments

2.1 Abstract

In the present study, the capacity of enrichments derived from marine sediments collected from different sites of the Mexican littoral to perform anaerobic ammonium oxidation (anammox) coupled to sulfide-dependent denitrification for simultaneous removal of ammonium and sulfide linked to nitrite reduction was evaluated. Sulfide-dependent denitrification out-competed anammox during the simultaneous oxidation of sulfide and ammonium. Significant accumulation of elemental sulfur (ca. 14–30 % of added sulfide) occurred during the coupling between the two respiratory processes, while ammonium was partly oxidized (31–47 %) due to nitrite limitation imposed in sediment incubations. Nevertheless, mass balances revealed up to 38 % more oxidation of the electron donors available (ammonium and sulfide) than that expected from stoichiometry. Recycling of nitrite, from nitrate produced through anammox, is proposed to contribute to extra oxidation of sulfide, while additional ammonium oxidation is suggested by sulfate-reducing anammox (SR-anammox). The complex interaction between nitrogenous and sulfurous compounds occurring through the concomitant presence of autotrophic denitrification, conventional anammox and SR-anammox may significantly drive the nitrogen and sulfur fluxes in marine environments.

2.2 Introduction

Excess nitrogen discharged from industrial wastewaters represents a threat to water reservoirs as it promotes eutrophication, blooms of toxic algae, acidification and direct

toxicity to aquatic species[1]. In addition, nitrogen pollution could induce adverse effects on human health[2]. Many countries have strictly regulated the discharge of nitrogen-rich wastewaters into aquatic ecosystems to prevent these environmental and public health concerns. For wastewater treatment plants, more stringent discharge standards demand an update on nitrogen removal technologies to replace the conventional ones (nitrification/denitrification), which cost high investment and energy. Anaerobic oxidation of ammonium (anammox) is a very promising technology and provides an attractive option for nitrogen removal from wastewaters[3,4]. The anammox process is catalyzed by chemolithoautotrophic bacteria of the phylum Planctomycetes, which can directly oxidize ammonium under anoxic conditions using nitrite as terminal electron acceptor, converting ammonium to dinitrogen gas[5]:

$$NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \longrightarrow$$

$$1.02\ N_2 + 0.26\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2.03\ H_2O$$

$$\Delta G^{0'} = -358\ kJ/mol \qquad 2.1$$

Application of anammox technology saves costs by requiring less aeration since only half of NH_4^+ Needs to be oxidized to NO_2^-, no exogenous electron donor is needed and sludge generation is minimum (0.06 mol C/mol NH_4^+)[6]. However, the use of anammox bacteria has two important disadvantages, which limit their widespread application. On one hand, they are quite sensitive to high concentrations of compounds, such as sulfide, nitrite, ammonium, heavy metals and antibiotics, which are commonly present in different industrial wastewaters and could significantly affect the process stability and performance[3,7,8]. Industrial effluents in which these contaminants coexist include livestock[9], antibiotics manufacturing factories[10],

hoggery and meat processing companies[11]. Secondly, due to the slow growth of anammox bacteria (with typical doubling time between 10 and 12 days), the start-up period of anammox reactors is very long. Industrial wastewaters, such as those originated from aquaculture and fish processing factories, contain a complex composition with high ammonium concentration, high salinity level and antibiotics[12]. These industrial sectors require suitable anammox inocula adapted to the conditions prevailing in these scenarios. Some efforts have been made to develop anammox biomass adapted to high salinity starting from anammox consortia derived from freshwater ecosystems or wastewater treatment facilities[13]. Adaptation of anammox biomass to high salinity conditions is costly and time-consuming; however, marine ecosystems represent a suitable alternative for anammox inocula to start-up bioreactors to treat saline wastewaters, which have not previously been explored despite the prevalence of anammox activity in marine environments[14–17]. Several studies have documented the coupling between anammox and denitrification to achieve simultaneous removal of nitrogen and organic matter from wastewaters[18–21].

Nevertheless, the coupling between anammox and sulfide-dependent denitrification has been poorly studied and it may play a significant role in environments where sulfur and nitrogen cycles are active[22,23]. Furthermore, assessing the coupling/competition between these two nitrogen removing processes in marine environments is required not only to better understand the microbial processes responsible for the fluxes of S and N in these ecosystems, but also to project biotechnological processes to integrate anammox and autotrophic denitrification to achieve the removal of sulfurous and nitrogenous pollutants from saline wastewaters. The aim of the present study was to test the capacity of enrichment cultures derived from marine sediments collected from different sites of the Mexican littoral to achieve anammox and

sulfide-dependent denitrification and to study the coupling between these two respiratory processes to simultaneously remove ammonium and sulfide linked to nitrite reduction.

2.3 Materials and methods

2.3.1 Sediments

Marine sediments were collected from five different sites of the Mexican littoral; four of them were located in the Pacific Ocean and one in the Gulf of Mexico. The collected sediments are referred to as the sites from where they originated: Baja California (BC), Oaxaca (OAX), Sinaloa (SIN), Sonora (SON) and Tabasco (TAB). Location and characteristics of collected marine sediments are shown in Table 2.1. Sampling sites were strategically chosen because these bays are exposed to wastewater discharges containing high concentrations of nitrogenous compounds. All sediments were collected at a depth of 1 m below the sea floor.

2.3.2 Basal medium

Basal medium used for sediments incubations was prepared with the following composition: (g/L): NaCl, 30; Na_2SO_4, 4; KCl, 0.70; $NaHCO_3$, 0.17; KBr, 0.1; $B(OH)_3$0.025; NaF, 0.003; $KHCO_3$, 1.25; $NaH_2PO_4 \cdot 2H_2O$, 0.029; $CaCl_2 \cdot 2H_2O$, 0.3; $MgSO_4 \cdot 7H_2O$, 0.2; EDTA, 0.00625; $FeSO_4$, 0.00625 and 1 mL/L trace element solution. The trace element solution was composed of: (g/L) EDTA, 1.5; H_3BO_4, 0.014; $MnCl_2 \cdot 4H_2O$, 1; $CuSO_4 \cdot 5H_2O$, 0.25; $ZnSO_4 \cdot 7H_2O$, 0.43; $NiCl_2 \cdot 6H_2O$, 0.19; $CoCl_2 \cdot 6H_2O$, 0.24; $NaMoO_4 \cdot 2H_2O$, 0.22; $NaSeO_4 \bullet 10H_2O$, 0.21 and $NaWO_4 \cdot 2H_2O$, 0.05. Ammonium and nitrite were added in the form of $NaNO_2$ and NH_4Cl as needed.

Table 2.1 Characteristics and location of collected sediments.

	BC	SIN	OAX	SON	TAB
TOC (mg/g)	6.5 ± 0.027	11.79 ± 1.25	BDL	1.24 ± 0.43	5.09 ± 0.34
VSS (%)	3.63 ± 0.9	8.50 ± 0.60	0.48 ± 0.32	2.31 ± 0.43	2.63 ± 0.33
NH_4^+ (µg N/g)	1.4 ± 0.2	12.8 ± 0.8	ND	ND	ND
pH	8.0 ± 0.1	8.1 ± 0.1	ND	ND	ND
Fe (mg Fe/g)	3.19 ± 0.39	9.14 ± 2	1.67 ± 0.16	4.34 ± 0.04	0.11 ± 0.02
Mn (mg Mn/g)	0.044 ± 0.00	0.118 ± 0.05	0.026 ± 0.01	0.458 ± 0.00	0.003 ± 0.00
Latitude (^{0}N)	30.45	23.15	15.66	27.12	18.53
Longitude (^{0}W)	116.01	106.31	96.55	110.05	92.75

Data represent average from triplicate measurements ± standard deviations in each treatment TOC total organic carbon, VSS volatiles suspended solids, Fe iron, Mn manganese, BDL below detection limit, ND not determined.

2.3.3 Incubations to assess anammox activity in marine sediments

Anammox activity of studied sediments was assessed in glass serum bottles of 500 mL. Bottles were supplied with 450 mL of the previously described basal medium and inoculated with marine sediment to a final concentration of 1.5 g/L of volatile suspended solids (VSS). Nitrite and ammonium concentrations were stoichiometrically supplied at 7.13 and 5.41 mM, respectively. Bottles were sealed and the headspace was exchanged with argon gas for 15 min. It is noteworthy that the basal medium was also previously bubbled with argon to exclude oxygen from incubation bottles. Initial pH of all incubations was adjusted to 7.5 with 1 N HCl solution as needed and the temperature was maintained at 28 °C in a controlled temperature room. Importantly, in the experimental design three different controls were

included: control without electron donor (ammonium), control without electron acceptor (nitrite) and sterile control including ammonium and nitrite with autoclaved sediment. All experimental treatments were performed in triplicate. During the whole incubation period, samples were collected from each bottle to measure the concentration of NH_4^+, NO_2^- and NO_3^-. Sampling was carried inside an anaerobic chamber with an atmosphere of N_2/H_2 (95 %/5 %) to avoid oxygen interference in measurements. Once the concentration of ammonium and nitrite decreased below detection limit, a new pulse of ammonia and nitrite was supplemented to reestablish their initial concentration for several cycles.

2.3.4 Incubations to assess autotrophic denitrification activity in marine sediments

Enrichments derived from marine sediments performing anammox activity were also tested for their capacity to achieve autotrophic denitrification with sulfide and nitrite as electron donor and electron acceptor, respectively. Batch experiments were conducted in the same serum bottles used during enrichment of anammox activities. In an anaerobic chamber with an atmosphere of N_2/H_2 (95 %/5 %), bottles were decanted and 450 mL of fresh basal medium was supplied together with stoichiometric concentrations of sulfide and NO_2^- (2.68 and 7.13 mM, respectively) considering full oxidation of sulfide to sulfate according to the following equation:

$$3\ HS^- + 8\ NO_2^- + 5\ H^+ \longrightarrow 3\ SO_4^{2-} + 4\ N_2 + 4\ H_2O \qquad (2.2)$$

$$\Delta G^{0'} = -2944 \text{ kJ/mol}$$

Sulfide was added as $Na_2S \cdot 9H_2O$ from a concentrated stock solution. Bottles were sealed with rubber stoppers and aluminum rings and the headspace was purged with argon gas for

15 min to establish anaerobic conditions. Incubation conditions (pH and temperature) and experimental controls were similar to those described for anammox incubations.

2.3.5 Incubations to assess the coupling between anammox and autotrophic denitrification for simultaneous oxidation of ammonium and sulfide linked to nitrite reduction

Finally, enrichment cultures derived from marine sediments in which anammox and autotrophic denitrification activities were assessed, were also tested for their capacity to couple both respiratory processes: simultaneous oxidation of ammonium and sulfide linked to nitrite reduction. For this purpose, previously incubated bottles to determine anammox and auto- trophic denitrification activities were decanted and supplied with fresh basal medium. The applied concentration of sulfide, ammonium and nitrite were the same as in anammox and autotrophic denitrification experiments. Ammonium and sulfide were added with the same reducing capacity taking into account the stoichiometry of anammox and autotrophic denitrification (Eqs. 2.1 and 2.2) and nitrite was supplied to fully oxidize only one of the electron donors provided since it is expected that nitrite would be the limiting substrate in marine environments[24,25]. Bottles were sealed with rubber stoppers and metal rings and the head- space was exchanged with argon gas for 15 min to establish anaerobic conditions. Incubations were conducted under the same temperature and pH as described in the previous experiments. Experimental controls considered in this experiment were: sterile control (with sterilized sediment), control without NO_2^- and control without sulfide and NH_4^+.

2.3.6 Analytical methods

Ammonium was analyzed by the phenol-hypochloride method, nitrate with selective UV spectrometric method, nitrite was analyzed by spectrophotometry at 543 nm and sulfate was determined using standard turbidimetric method[26]. For all the above analysis, samples were centrifuged at 12,000 rpm and basal saline medium was used as a background level for all measurements. Sulfide was analyzed spectrophotometrically following the method described by Cord-Ruwisch[27], taking specially care that samples were measured immediately to avoid contact with oxygen. The spectrophotometer used was an UV–Vis Thermo Spectronic model Aquamate. VSS concentrations were analyzed according to standard methods[26]. The pH was measured with a pH meter (Thermo Scientific, Orion 4-star). Total organic carbon was measured using a Shimadzu TOCVCS/TNM-1 equipped with a solid sampling module (SSM-5000A).

2.4 Results

2.4.1 Anammox activity

Experiments to assess anammox activity were conducted at the same time for all marine sediments tested. Among all the littoral sites explored, anammox activity was evident in marine sediments collected from BC and SIN (Figure 2.2). During the first incubation cycles endogenous reduction of nitrite (in the absence of ammonium) was observed, but ceased after two cycles of incubation (Figure 1). In the case of sediment SIN, endogenous anaerobic ammonium oxidation (in the absence of nitrite) was also observed during the first three incubation cycles, probably due to the high concentrations of Fe and Mn present in this sediment (Table 2.1), which could have promoted anaerobic ammonium oxidation to N_2 linked to the reduction of these metals[28–30].

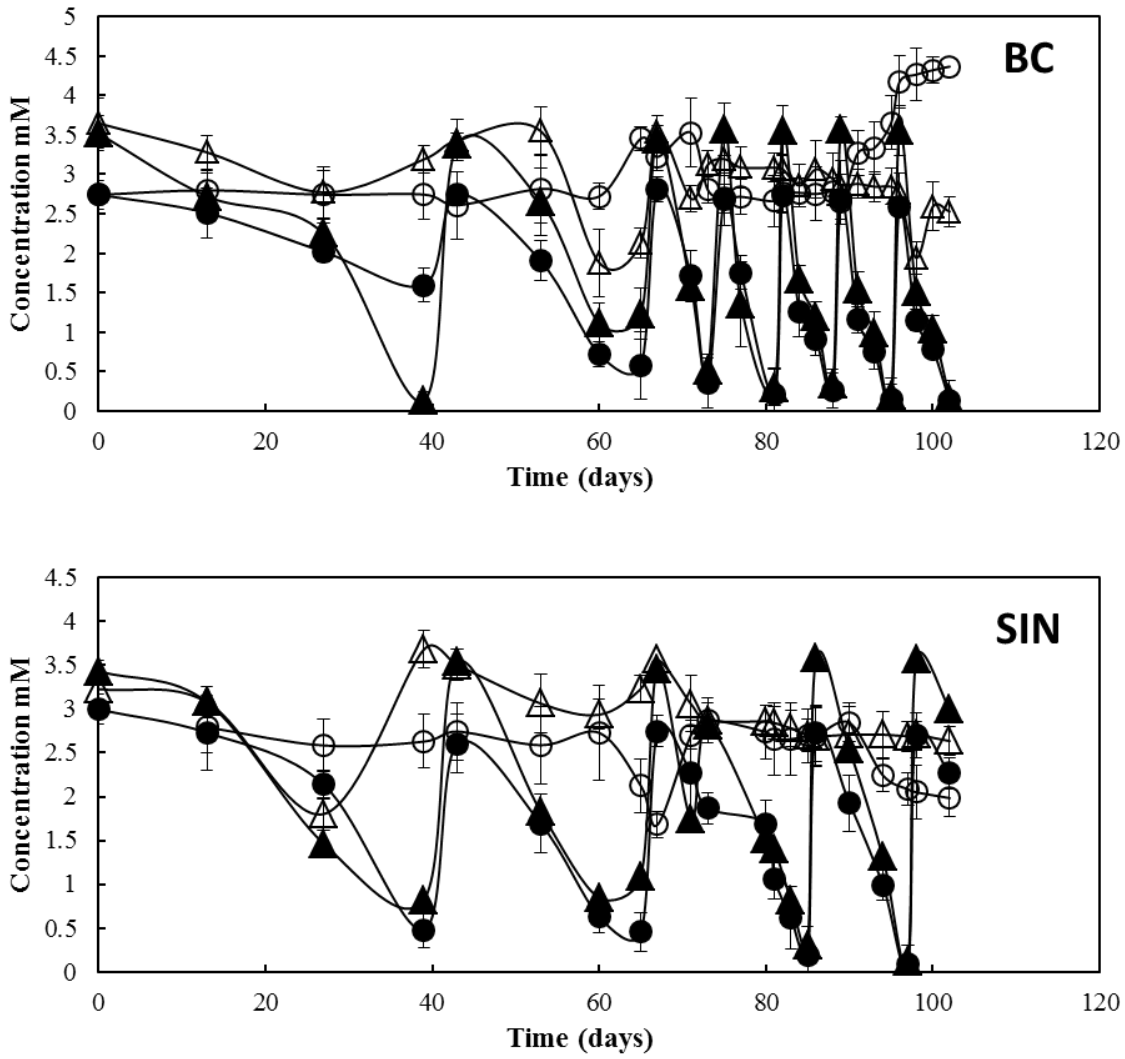

Figure 2.1. **Development of anammox enrichments cultures derived from marine sediments collected from Baja California (BC) and Sinaloa (SIN)**. Legends, (●), ammonium measured in treatment containing sediment, NH_4^+ and NO_2^-; (▲), nitrite measured in treatment containing sediment, NH_4^+ and NO_2^-; (○), ammonium measured in treatment containing sediment and NH_4^+, but without NO_2^-; (△), nitrite measure in treatment containing sediment and NO_2^-, but without NH_4^+.

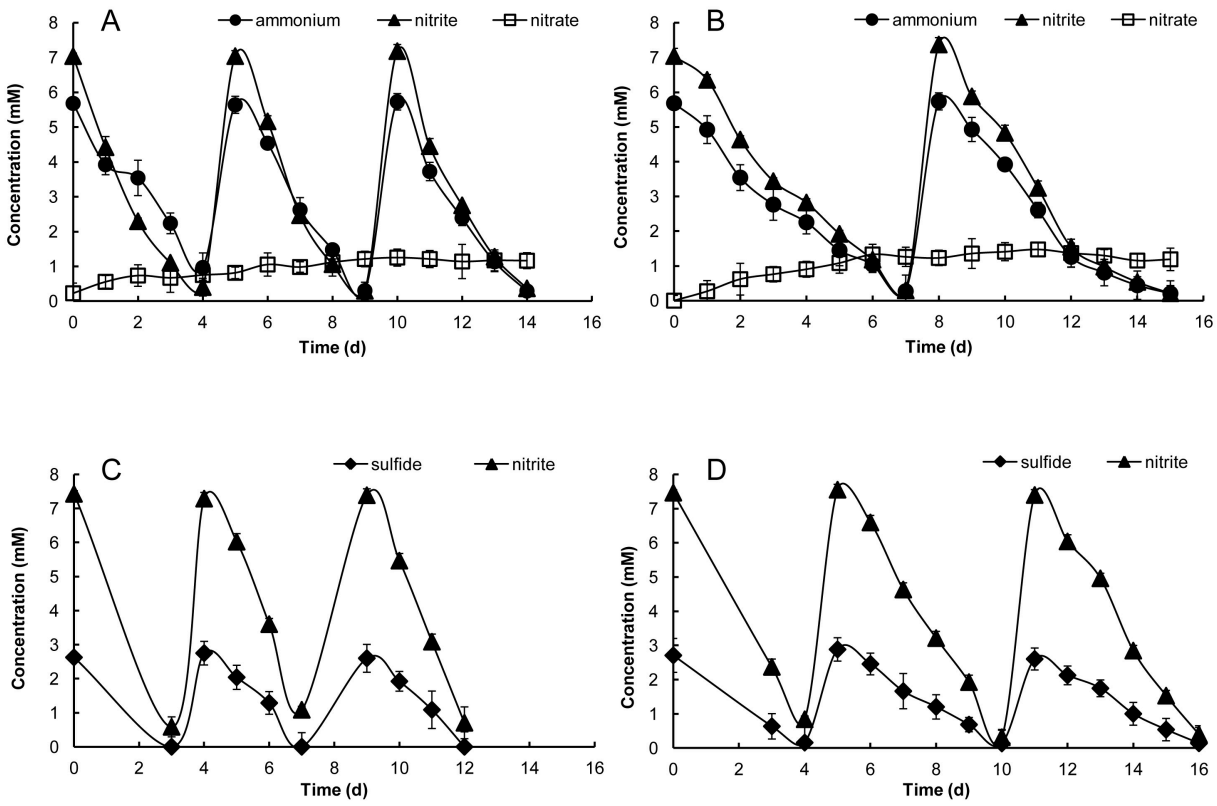

Figure 2.2 A, B Anammox activities by enriched sediments BC and SIN, respectively; and C, D autotrophic denitrification activities by enriched sediments BC and SIN, respectively. Data represent average from triplicate measurements and error bars, the standard deviation.

Moreover, considering the high concentration of sulfate present in the marine medium, sulfate-reducing anammox (SR-anammox) could also have occurred as has previously been documented[31,32] according to the following stoichiometry:

$$2\ NH_4^+ + SO_4^{2-} \longrightarrow N_2 + S^0 + 4\ H_2O$$

$$\Delta G^{0'} = -47.8 \quad kJ/mol \qquad 2.3$$

Table 2.2 Anammox and autotrophic denitrification activities measured in incubations with enriched sediments collected from BC and SIN.

Anammox		Autotrophic Denitrification	
BC	**SIN**	**BC**	**SIN**
meq/g VSS•h	meq/g VSS•h	meq/g VSS•h	meq/g VSS•h
1.48 ± 0.02	1.06 ± 0.03	1.98 ± 0.13	1.3 ± 0.04

Activities were calculated taking into account electrons transfer based on ammonium (anammox) and sulfide (denitrification) consumption and the units correspond to the milli-equivalents per g of volatile suspended solids per hour (meq/g VSS h). No anaerobic ammonium oxidation was observed without nitrite. Values for controls without nitrite were zero and values for controls without ammonium were 0.002, 0.004 in anammox incubations for sediments BC and SIN, respectively, and 0.037, 0.181 in autotrophic denitrification incubations also for sediments BC and SIN, respectively. Data represent average from triplicate measurements ± standard deviations in each treatment.

After several incubation cycles, endogenous activities were nullified and anammox activities could be measured (Fig. 2.2; Table 2.2). Enrichment derived from sediment BC showed higher anammox rate as com- pared to that obtained with sediment collected from SIN (Table 2.2). Interestingly, the two sediments performing anammox activity, presented the highest concentration of organic matter among the different sites explored (measured both as VSS and TOC, Table 2.1). Moreover, these two sediments contained the largest amount of Fe and Mn (Table 2.1). The fastest anammox activity could be established with enrichment derived from sediment originated from BC; which showed the first stoichiometric evidence of anammox activity around 40 days after sediment incubation started when all endogenous substrates were depleted. In the case of enrichment derived from sediment SIN, first stoichiometric evidence of anammox activity could be observed after 70 days of incubation

when all endogenous substrates had been consumed. The high content of organic matter present in both sediments explains the long incubation time required to accurately measure the anammox activity. Strong stoichiometric evidence of anammox activity was obtained from incubations for both tested sediments in the first cycle performed (Figure 2.1). Certainly, the ratio of NH_4^+ and NO_2^- consumption was very close to the value of anammox stoichiometry (1:1.32). Moreover, the ratio between produced nitrate and oxidized ammonium was also the stoichiometric value of the anammox reaction (0.26) in both cases. However, since no fresh medium was supplied during the subsequent additions of ammonium and nitrite, nitrate accumulated over time. Sterile controls did not show any consumption of ammonium and nitrite through the whole incubation period, confirming the biological nature of the process.

2.4.2 Autotrophic denitrification

Experiments to assess autotrophic denitrification activities were carried out with the same sediments performing anammox activity (sediments BC and SIN). Batch assays showed that nitrite and sulfide were readily consumed by the two sediments tested evidencing autotrophic denitrification activities (Figure 2.1). Sediment BC achieved higher autotrophic denitrifying activity compared with sediment SIN (Table 2.2). After three consecutive cycles, clear evidence was obtained indicating that sulfate was the main product derived from sulfide oxidation by both sediments. The quantified sulfate concentration was 2.26 and 2.14 mM (corrected for the background level in basal medium) for sediments collected from BC and SIN, respectively, at the end of the incubation period. These sulfate concentrations are consistent with the stoichiometry of the reaction (Eq. 2.2). Thus, autotrophic denitrification promoted almost complete oxidation of sulfide to sulfate in these cases. Comparing the

respiratory rates observed in these experiments, it can be concluded that anammox activity was 25 % lower than autotrophic denitrifying in the case of sediment BC and 18.5 % lower in the case of sediment SIN (Table 2.2). Sediment BC performed sulfide oxidation rate 1.5-fold faster than sediment SIN. Sterile controls did not show any consumption of sulfide and nitrite through the whole experimental period. Moreover, endogenous controls lacking either sulfide or nitrite showed negligible consumption of these substrates.

2.4.3 Coupling between anammox and autotrophic denitrification for simultaneous oxidation of ammonium and sulfide

After anammox and autotrophic denitrification activities were independently assessed for enriched sediments collected from BC and SIN (the two consortia showing both microbial processes), additional incubations were conducted to assess the capacity of both inocula to simultaneously oxidize sulfide and ammonium linked to nitrite reduction. Figure 2.3 shows two consecutive cycles clearly evidencing the coupling between anammox and autotrophic denitrification to achieve the concomitant oxidation of sulfide and ammonium by the two enriched sediments studied. For both incubations, autotrophic denitrification out-competed anammox for the available nitrite, which was the limiting substrate. Certainly, respiratory rates attributable to denitrification were 2.1- and 2.6-fold higher than those related to anammox for sediments collected from BC and SIN, respectively (Table 2.3). This scenario was reflected in complete removal of sulfide in both sediment incubations, while only 31 and 47 % of the supplied amount of ammonium was consumed by sediments from BC and SIN, respectively. Moreover, competition between anammox and autotrophic denitrification caused an incomplete oxidation of sulfide to sulfate. Indeed, sulfate concentration was 1.9 and 2.33 mM (corrected for the background level in basal medium) for sediments BC and

SIN, respectively, corresponding to 70 and 86 % of the stoichiometrically expected from Eq. (2) at the end of the incubation period.

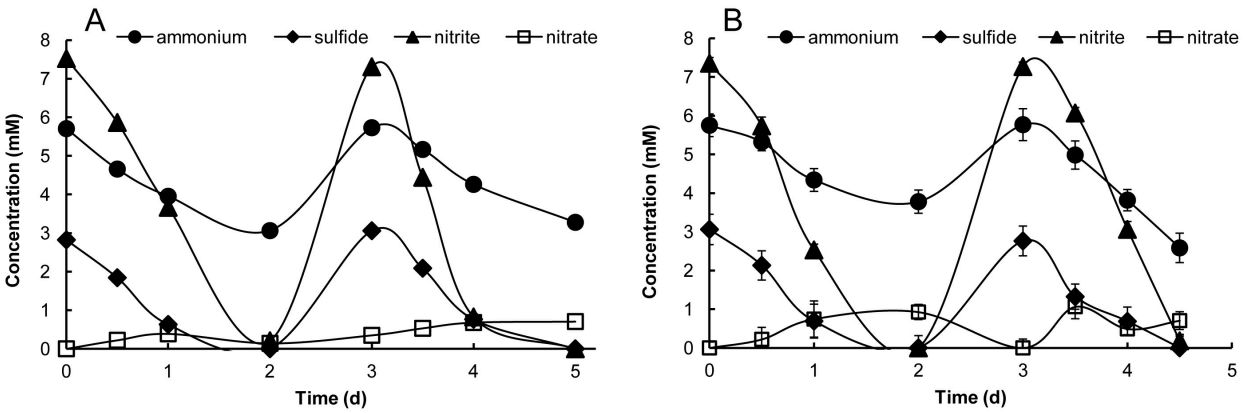

Figure 2.3 Simultaneous oxidation of ammonium and sulfide by the coupling between anammox and autotrophic denitrification in incubations performed with enriched sediments A BC and B SIN. Data represent average from triplicate measurements and error bars, the standard deviation.

Table 2.3 Anammox and autotrophic denitrification activities measured during simultaneous oxidation of sulfide an ammonium by enriched sediments collected from BC and SIN.

	Anammox	**Autotrophic denitrification**	**Total Respiration**
	meq/g VSS•h	meq/g VSS•h	meq/g VSS•h
BC	1.02 ± 0.08	2.17 ± 0.6	3.20 ± 0.3
SIN	1.2 ± 0.03	3.09 ± 0.9	4.30 ± 0.5

Activities were calculated taking into account electrons transfer based on ammonium (anammox) and sulfide (denitrification) consumption. Units expressed as milli-equivalents per g of volatile suspended solids per hour (meq/g VSS h). Data represent average from triplicate measurements ± standard deviations in each treatment.

Although S^0 was not quantified, qualitative evidence was obtained by the formation of white particles in these incubations, which agrees with previous studies where incomplete oxidation of sulfide has been observed[33–37]. Based on stoichiometric balances, partial oxidation of sulfide to S^0 would have been accounted for up to 7.3 % of reduced nitrite by the enriched sediments evaluated (Table 2.4).

Table 2.4 Contribution of anammox and autotrophic denitrification to nitrite reduction during simultaneous oxidation of ammonium and sulfide by enriched sediments BC and SIN.

Process	Contribution to nitrite reduction	
	BC	SIN
$HS^- \longrightarrow SO_4^{2-}$	5.3 mM (74.3%)	6.21 mM (87%)
$HS^- \longrightarrow S^0$	0.52 mM (7.3%)	0.25 mM (3.5%)
$NH_4^+ \longrightarrow N_2$	3.23 mM (45.3%)	3.40 mM (47.7%)
Total balance	126.9%	138.2%

Contribution of each process was calculated considering electron equivalents quantified from ammonium consumption (anammox) and sulfate production (denitrification) linked to nitrite reduction. For incomplete oxidation of sulfide to S^0, electron equivalents were calculated based on sulfide consumed minus sulfate produced. Percentage values are related to the initial nitrite concentration supplied (7.13 mM).

Therefore, the collected evidence indicates that anammox was more affected than autotrophic denitrification when both processes competed for nitrite in marine sediments incubations. Interestingly, mass balances (Table 2.4) indicated that reducing equivalents derived from ammonium and sulfide oxidation through the coupling between anammox and autotrophic denitrification by the two marine sediments studied, are far beyond those expected from the

quantified nitrite reduction. Indeed, 26.9 and 38.2 % more ammonium and sulfide (in terms of reducing equivalents) were oxidized than the stoichiometrically expected in incubations performed with sediments BC and SIN, respectively. Considering that negligible oxidation of ammonium and sulfide occurred in endogenous controls lacking nitrite (data not shown), it is postulated that nitrate originated from anammox activity would have been recycled back to nitrite by denitrifying activity fueling the coupling between anammox and sulfide-dependent denitrification (Figure 2.4). Moreover, the lower nitrate concentrations measured in sediment incubations performing simultaneous anammox and sulfide-dependent denitrification (Figure 2.3) as compared to concentrations observed in the absence of sulfide (with only anammox activity taking place, Figure 2.2) supports our hypothesis that sulfide-dependent denitrification was responsible for the replenishing of nitrite when the two respiratory processes were coupled. Nitrate production in anammox does not involve ammonium, only nitrite[38]. Thus, according to the stoichiometry described in Eq. 2.1, the ratio nitrate produced/nitrite consumed is 0.26/1.32. Therefore, the theoretical nitrate produced from anammox after the first recycling period would be 19.69 %of nitrite, then 3.87 %, 0.15 %, and so on for the following cycles. Although these stoichiometric calculations strongly support our hypothesis, they do not fully explain the extra oxidation of substrates quantified (Table 4). An additional mechanism that might have been involved in our incubations is SR-anammox according to Eq. 2.3, which might have contributed with additional oxidation of ammonium to N_2 (Figure 2.4).

2.5 Discussion

The aim of this study was to evaluate the capacity of marine sediments collected from the Mexican littoral to carry out anaerobic ammonium oxidation and sulfide-dependent

denitrification, and to study the coupling of these two respiratory processes to simultaneously remove ammonium and sulfide linked to nitrite reduction. Two out of five marine sediments tested performed anammox activity after all endogenous substrates were depleted. The prevalence of anammox in sediments BC and SIN might have been due to low O_2 concentrations and high availability of NH_4^+ and NO_x^- present in these ecosystems. Studies have reported anammox occurrence in anaerobic environments where NO_2^- occurs together with NH_4^+[38]. Collected sediments derived from sites frequently exposed to wastewater discharges with a high content of nitrogenous com- pounds (e.g. aquaculture effluents). Another factor, which might explain the occurrence of anammox activity in sediments BC and SIN is the relatively high content of organic matter present in these sites (Table 1). High concentration of organic matter can promote anaerobic conditions in these sites, which combined with high concentrations of nitrogenous compounds, might have created the proper niche for anammox bacteria. These observations agree with Babbin et al.[39], who correlated anammox activities with organic matter content in marine sediments. Although several studies have reported anammox activity in distinct marine ecosystems, the present study constitutes the first effort to elucidate the prevalence of anammox bacteria in different Northern Pacific sites. The first direct evidence documenting the presence of anammox bacteria in marine environments was provided by Thamdrup and Dalsgaard [40]. After that, anammox activity has been detected in a limited number of coastal, estuarine and marine sediments[41–44], mangrove sediments[45], in the suboxic zone of Golfo Dulce, Costa Rica[14], Black Sea[15], and in the Benguela oxygen minimum zone of the Namibian coast[46].

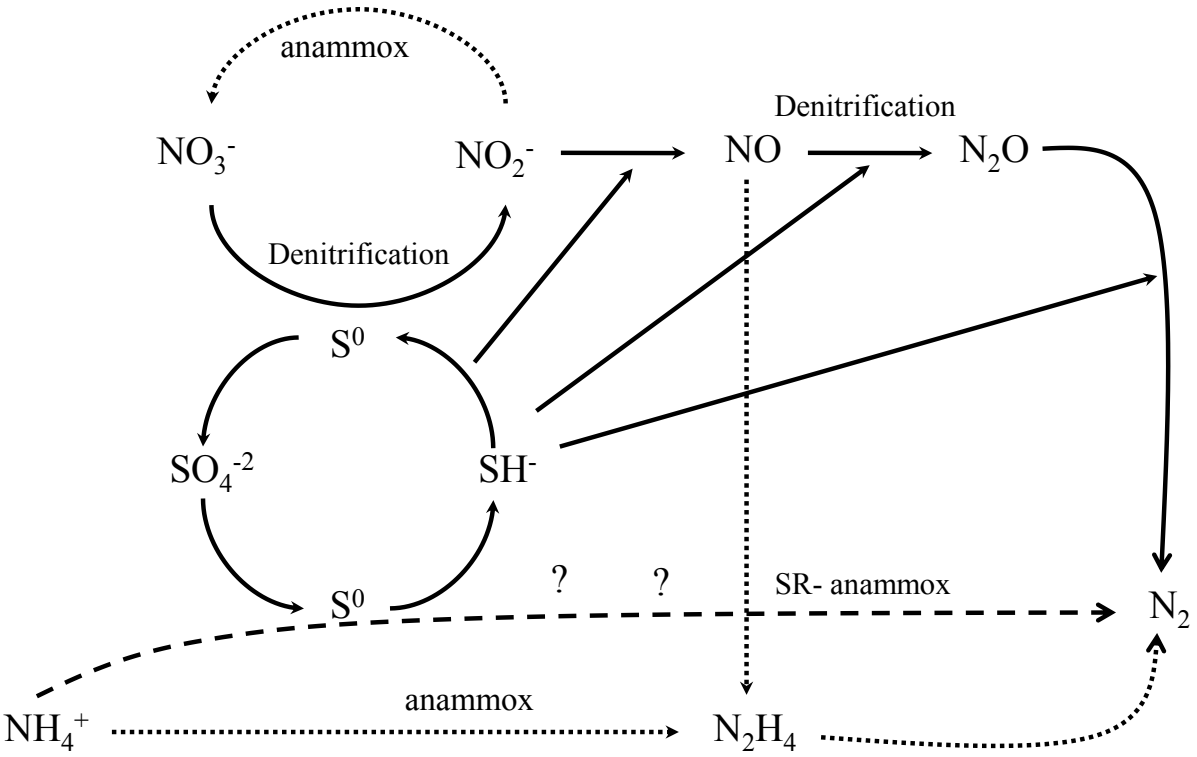

Figure 2.4 Schematic model describing the interconnection between nitrogenous and sulfurous compounds through simultaneous occurrence of anaerobic ammonium oxidation (anammox), sulfate-reducing anammox (SR-anammox) and sulfide-dependent denitrification. Question mark represents unknown intermediates of SR-anammox

Very recently, anammox activity was also reported on one site of the Mexican littoral of the Pacific Ocean [47]. In this study, the authors reported the symbiosis between anammox bacteria and nitrate sequestering sulfur-oxidizing *Thioploca* species and showed that *Thioploca*–anammox symbiosis intensifies benthic fixed nitrogen losses in anoxic sediments, by efficiently coupling the carbon, nitrogen and sulfur cycles. Our contribution further underlines the inter- connection between the nitrogen and sulfur cycles by explaining the contribution of anammox and sulfide- dependent denitrification on nitrogen and sulfur fluxes

in the studied marine sediments. A few recent studies have documented the link between anammox and sulfide-dependent denitrification [22,23,48]. In our study, mass balances revealed up to 38 % of additional oxidation of ammonium and sulfide (in terms of reducing equivalents) beyond the level expected from stoichiometry (Table 2.4). Although several studies have documented the coupling of anammox with organotrophic denitrification [18–21] as well as with litotrophic denitrification[22,23,48,49], the collected evidence in the present study suggests complex interactions between nitrogenous and sulfurous compounds during the coupling between anammox and sulfide-dependent denitrification (Figure 2.4). One of the mechanisms involved may be the recycling of nitrite, from nitrate generated through the anabolic part of anammox, contributing with additional oxidation of sulfide. Furthermore, it is postulated that SR-anammox might have contributed with additional ammonium oxidation to N_2 according to Eq. 2.3. Experiments performed in the present study were conducted with a relatively high nitrite concentration (7.13 mM) to reach quantifiable levels, which do not reflect the actual nitrite concentrations usually prevailing in marine environments; nevertheless, the collected evidence suggests that the complex interactions between nitrogenous and sulfurous compounds summarized in Figure 2.4 may significantly drive the nitrogen and sulfur fluxes in marine environments. Furthermore, the coupling between anammox and autotrophic denitrification is expected to play a significant role in wastewater treatment systems for achieving simultaneous removal of nitrogenous and sulfurous contaminants from saline discharges, such as those generated from aquaculture activities. Thus, identification of marine sediments with anammox activity is also relevant from the technological point of view since these sediments could serve as inocula to start up anammox reactors for the treatment of nitrogen-rich saline wastewaters. This is particularly important since adaptation of anammox consortia to high salinity concentrations is costly and time-

consuming[50]. The co-existence of anammox and autotrophic denitrifying bacteria is also important due to the oxidation of sulfide promoted by autotrophic denitrification, which could mitigate the inhibitory effects of this sulfurous substrate on anammox. The literature reports contradictory results related to the effects of sulfide on anammox processes[51–53]. For instance, Dapena-Mora et al. 2007[51] reported that 5 mM of sulfide led to complete loss of anammox activity, while the research of Van de Graaf et al. (1996)[49] showed an increased on anammox activity at the same sulfide concentration (5 mM). Thus, it is important to consider that inhibitory effects of sulfide are dependent on different aspects, such as substrate concentration, sulfide level and exposure time[53]. Sulfide toxicity is enhanced at low pH values due to the speciation moving towards the toxic species H_2S, and therefore high pH values (expected from the occurrence of denitrification) may contribute to mitigating sulfide toxicity. A pH value of 7.8 ± 0.2 was measured in our experiments at the end of the competition experiments, which might have mitigated the inhibitory effects of sulfide. Autotrophic denitrification occurred without lag phase and out-competed anammox in all sediment incubations performed, which suggests that sulfide- oxidizing denitrifying microorganisms were abundant and actively present in the two marine sediments studied. The prevalence of sulfide-dependent denitrification over anammox may be partly due to thermo- dynamics since oxidation of sulfide is more energetically favorable than ammonium oxidation linked to nitrite reduction (Eqs. 2.1 and 2.2). Moreover, anammox activity relies exclusively on the availability of nitrite while denitrification can proceed with both nitrite and nitrate (derived from anammox) as terminal electron acceptors as previously discussed. Our preliminary findings that marine sediment collected from SIN showed endogenous anaerobic ammonium oxidation (in the absence of nitrite) suggest that the high concentration of Fe and Mn present in this sediment (Table 1), as well as SR- anammox, would have been

promoted this unexpected oxidation of ammonium to N_2; these interesting experiments will be further elucidated in future studies.

2.6 Conclusions

Anaerobic ammonium oxidation coupled to sulfide- dependent denitrification for achieving simultaneous removal of ammonium and sulfide linked to nitrite reduction was demonstrated in two enriched sediments collected from different Northern Pacific sites. Competition between both respiratory processes caused incomplete oxidation of ammonium and sulfide when nitrite was the limiting substrate. Nevertheless, mass balances revealed up to 38 % more oxidation of these electron donors (based on electron balances) than that expected from stoichiometry, which is suggested to occur through complex interactions between nitrogenous and sulfurous compounds involving recycling of nitrite from nitrate generated from anammox, and SR- anammox, contributing with additional oxidation of sulfide and ammonium, respectively. These complex interactions may play a significant role on nitrogen and sulfur fluxes in marine environments.

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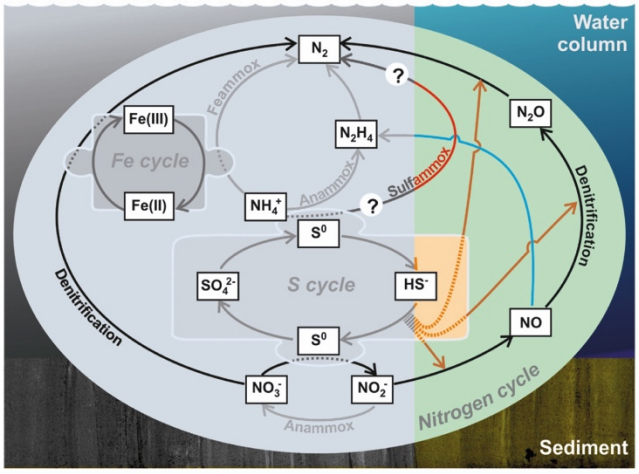

CHAPTER 3

Anaerobic ammonium oxidation linked to sulfate and ferric iron reduction fuels nitrogen loss in marine sediments

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3.1 Abstract

Availability of fixed nitrogen is a pivotal driver on primary productivity in the oceans, thus the identification of key processes triggering nitrogen losses from these ecosystems is of major importance as they affect ecosystems function and consequently global biogeochemical cycles. Denitrification and anaerobic ammonium oxidation coupled to nitrite reduction (anammox) are the only identified marine sinks for fixed nitrogen. Here we show that anaerobic ammonium oxidation coupled to the reduction of sulfate, the most abundant electron acceptor present in the oceans, prevails in marine sediments. Tracer analysis with ^{15}N-ammonium revealed that this microbial process, here introduced as **sulfammox**, accounts for up to 5 μg $^{30}N_2$ produced g^{-1} day^{-1} in sediments collected from the eastern tropical North Pacific coast. Raman and X-ray diffraction spectroscopies revealed that elemental sulfur and sphalerite (ZnFeS) were produced, besides free sulfide, during the course of **sulfammox**. Anaerobic ammonium oxidation linked to Fe(III) reduction (feammox) was also observed in the same marine sediments accounting for up to 2 μg $^{30}N_2$ produced g^{-1} day^{-1}. We suggest that these novel nitrogen sinks may significantly fuel nitrogen loss in marine environments. These findings call to revisit the interconnections among the oceanic biogeochemical cycles of N, S, and Fe.

3.2 Introduction

Nitrogen fluxes in marine environments are almost exclusively controlled by microbial processes. While diazotrophic microorganisms manage nitrogen fixation, creating the nitrogen pool for photoautotrophic carbon dioxide assimilation[1], denitrification has traditionally been assumed as the main nitrogen sink in the oceans releasing dinitrogen gas (N_2) back to the atmosphere[2]. More recently, anaerobic ammonium oxidation (the anammox reaction involving the oxidation of ammonium with nitrite as electron acceptor), a process proposed by Richards[3] 50 years earlier, has also been identified as a major nitrogen sink in marine environments accounting for up to 50% of nitrogen loss from these ecosystems[4–6]. The prevalence of the anammox process requires the proper niche in which ammonium and nitrite coexist, such as oceanic oxygen minimum zones (OMZs) in which anammox has been proposed to be the dominant N_2 producing process accounting for the loss of 80-150 Tg N yr^{-1} from the world´s OMZs[6,7]. Although the contribution of anammox to the total denitrification process in marine sediments has been estimated to be up to 80%, nitrite prevails in very low concentrations in most anoxic marine sediments. In contrast, sulfate is the most abundant electron acceptor in the oceans and can theoretically fuel anaerobic ammonium oxidation in marine sediments when nitrite is absent, according to the following thermodynamically favorable reaction:

$$8NH_4^+ + 3SO_4^{2-} \rightarrow 4N_2 + 3HS^- + 12H_2O + 5H^+ \qquad \Delta G^{\circ\prime} = -17.6 \text{ kJ mol}^{-1} \qquad 3.1$$

While the **sulfammox** (sulfate-dependent anammox) reaction has been documented in wastewater treatment systems[8,9], there are, to our knowledge, no reports of its occurrence or

activity in natural environments. Anaerobic ammonium oxidation coupled to Fe(III) reduction (known as feammox) has been documented in soils[10,11] and in intertidal wetlands[12]; however, no evidence of prevalence in marine sediments has been reported. An additional mechanism driving nitrogen loss in the oceans is autotrophic denitrification by Fe(II)-oxidizing[13] and sulfide-oxidizing bacteria[14], which replenish the Fe(III) and sulfate pool, respectively, to fuel the feammox and **sulfammox** reactions. Here, we reveal that the **sulfammox** and feammox processes prevail in two different marine sediments freshly collected from the eastern tropical North Pacific coast (Table 3.1 and Figure 3.1), and quantified the rates at which their microbial communities perform these processes.

Table 3.1 Characterization of sediment and water samples collected from Baja California Peninsula (BC) and from Sinaloa (SIN), north-western Mexico.

		BC	SIN
Temperature (°C)		28.9±0.0	29.6±0.0
SO_4^{2-} (mM)		26.04±1	40.6±0.8
HS^- (mM)		0.24±0.02	0.30±0.04
NH_4^+ (µM)	***Water***	0.10±0.01	4.07±0.14
NO_2^- (µM)		0.39±0.07	0.35±0.07
NO_3^- (µM)		4.36±0.6	0.85±0.21
pH		8.0	8.1
VSS (%)		1.4±0.09	9.04±0.61
TOC (mg g^{-1})	***Sediment***	5.2±0.03	11.8±0.05
Ca^{2+} (mg g^{-1})		0.53±0.0	0.12 ± 0.06
Total Iron (mg g^{-1})		0.30±0.0	0.42±0.0

Data represent average from triplicate measurements ± standard deviations in each parameter displayed. VSS, volatiles suspended solids; TOC, total organic carbon.

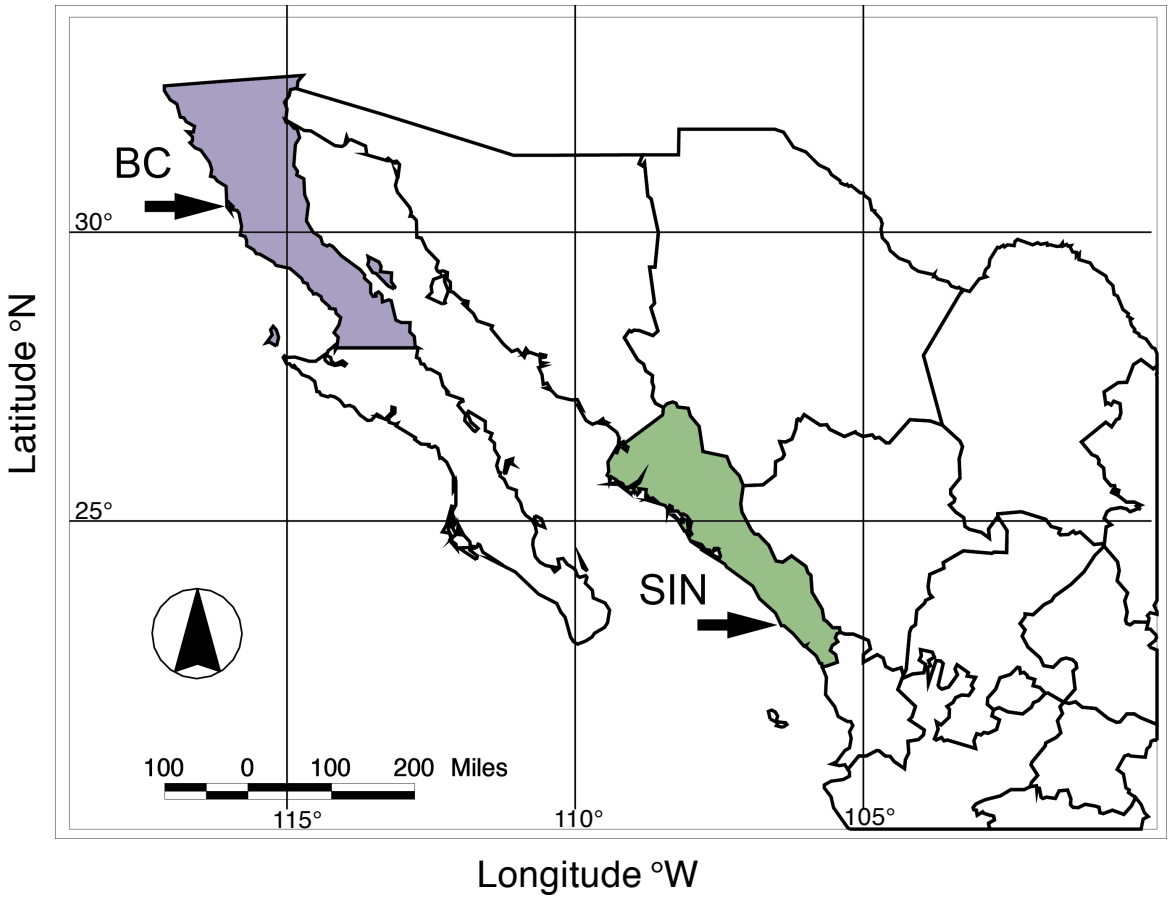

Figure 3.1 Location of sampling points of marine sediments from Baja California Peninsula (BC) and from Sinaloa (SIN).

3.3 Material and Methods

3.3.1 Sediments

Marine sediment cores were sampled from the coast of Baja California Peninsula (N 30.45° and W 116.01°) and Sinaloa (N 23.15° and W 106.31°), both located in the eastern tropical North Pacific coast (Figure 1). Coastal sediment cores with a depth of 15 cm were collected under a water column of 70 cm. Water samples were also collected from the area of sediment sampling points for characterization. All samples were sealed in hermetic flasks and were immediately placed on ice until arrival at the laboratory. Upon arrival, all samples were

stored at 4°C in a dark room until analysis and incubation. Sediment cores were opened and homogenized within an anoxic chamber (atmosphere composed of N_2/H_2 (95%/5%, v/v)) before characterization and incubation. Characterization of water and sediment samples is described in Table 3.1. Sediments are referred to as the sites from where they were collected: Baja California (BC) and Sinaloa (SIN).

3.3.2 Incubation experiments

Incubations to assess **sulfammox** and feammox activities were carried out in serological bottles with 120 ml of capacity. Sediment cores were thoroughly mixed inside an anoxic chamber and inoculation took place by adding 10 g of volatile suspended solids (VSS) l^{-1} to serological bottles containing 50 ml of artificial marine medium, which was prepared as previously described[15]. Initial ammonium concentration was set at 1 mM. All bottles were sealed with rubber stoppers and aluminum rings. Both the liquid phase and the headspace were flushed with argon gas for at least 20 min to obtain anaerobic conditions. Controls lacking ammonium and electron acceptors (sulfate and ferrihydrite) were also prepared by following an identical procedure. The Fe(III) oxyhydroxide, ferrihydrite, was synthesized according to Schwertmann and Cornell[16]. Briefly, 40 g of $Fe(NO_3)_3 \cdot 9H_2O$ were dissolved in 500 ml of distilled water, KOH (1 M) was added to adjust the pH to 7.8. The suspension was centrifuged and washed several times to remove salts. Ferrihydrite was re-suspended in marine mineral basal medium just after its synthesis to obtain an initial concentration of 3 mM. Sampling to measure all relevant parameters (ammonium, ferrous iron, nitrite, nitrate and sulfide) was always conducted inside the anoxic chamber. Initial pH of all incubations was 8.1 and remained constant throughout the incubation period. All incubations were placed in a dark room at 28 °C. All experimental treatments were performed in triplicate.

3.3.3 Nitrogen loss assessed with tracer analysis with $^{15}NH_4^+$

Incubations to quantify $^{30}N_2$ production were carried out in serological bottles with 60 ml of capacity. The bottles were inoculated with 2 g of wet sediment, supplied with 1 mM of $^{15}NH_4Cl$ (98 atom % ^{15}N, Sigma Aldrich, catalogue number 299251) and with the stoichiometric amount of ferrihydrite in 25 ml of marine basal medium. All experimental procedures were carried out inside the anoxic chamber. Both the liquid phase and the headspace were flushed with argon gas for at least 20 min to obtain anaerobic conditions. Additional incubations were performed in the presence of the sulfate reduction inhibitor, molybdate (50 mM). Each experimental treatment was carried out with five replicates. Calibration curve was established by preparing microcosms under the same experimental conditions and spiked with different amounts of $^{15}N_2$ (98 atom % ^{15}N, Sigma-Aldrich catalogue number 364584).

3.3.4 Measurements of nitrogenous compounds

Ammonium was analyzed by the phenate method. An intense blue compound, indophenol, is formed by the reaction of ammonia, hypochlorite, and phenol catalyzed by sodium nitroprusside, and the absorbance is measured at 640 nm. Nitrate was measured with selective UV spectrometric method at 220 nm. Because dissolved organic matter may also absorb at 220 nm and nitrate does not absorb at 275 nm, a second measurement was made at 275 nm to correct the nitrate value. Acidification with 1N HCl was designed to prevent interference from suspended particles. Nitrite was determined by colorimetric method, through formation of a reddish purple azo dye produced by coupling diazotized sulfanilamide with N-(1-Naphthyl)-ethylene diamine dihydrochloride. The applicable range of the method is 10 to 1000 µg NO_2^- -N l^{-1}, the color system obeys Beer´s law up to 180 µg N l^{-1} with a 1-cm light

path at 543 nm [17]. Before performing measurements, samples were centrifuged at 12,000 rpm and filtered through 0.22 µm nitrocellulose membranes. Basal marine medium was used as a background level for all measurements. The spectrophotometer used was an UV–Vis Thermo Spectronic model Aquamate.

3.3.5 Measurement of sulfide

Sulfide was analyzed spectrophotometrically following the method described by Cord-Ruwish[18], taking specially care that samples were immediately measured to avoid contact with oxygen.

3.3.6 Quantification of ferrous iron (Fe^{2+})

Fe^{2+} concentration was measured by the ferrozine technique according to Lovley and coworkers[19]. Samples of ~500 µl were taken from incubations with a syringe, a sub-sample of 200 µl was then put in contact with 200 µl of HCl 0.5 M for 30 min. Then a sub-sample was centrifuged at 12,000 rpm, afterwards 200 µl of supernatant were taken and added to 4.8 ml of ferrozine reagent (prepared with HEPES buffer 50 mM). Ferrous iron forms a purple complex along with ferrozine reagent, which has its maximum absorbance at 562 nm. All solutions employed in this determination were bubbled with argon gas for 30 min to ensure the absence of dissolved oxygen.

3.3.7 Characterization of inorganic fraction of sediments

A total of 29 elements were measured with inductively coupled plasma-optic emission spectroscopy (ICP-OES, Varian 730-ES). Samples of 2 g of sediment were in contact with a

mixture of HNO_3 and HCl at a ratio of 1:3 v/v to extract inorganic content. The mixture was washed several times with distilled water and filtered through 0.22 µm nitrocellulose membranes. We analyzed samples by triplicate.

3.3.8 Measurements of $^{30}N_2$

Quantification of $^{30}N_2$ production from $^{15}NH_4^+$ oxidation was conducted by mass spectrometry (MS) (Agilent 5975C Series GC/MSD) complemented by 7890A GC (gas chromatograph). Separation was achieved with a (30 m × 0.320 mm ID, 0.20 µm) HP-PLOT/Q+PT capillary column from Agilent Technologies. Helium was used as carrier gas at 0.2655 ml min^{-1} column flow rate. The temperatures of injector and MS source were maintained at 250 and 230 °C, respectively. The oven temperature is maintained at 70 °C (5 min). The injection volume was 50 µl and there was only one replicate of injection per bottle. The gas injected into the gas chromatograph was taken directly from the headspace of the incubations and immediately injected in to the GC port. The total time of the run had a duration of 6 min.

3.3.9 X-ray diffraction (XRD) analysis

X-ray diffraction analysis of sediments was carried out in an X-Ray diffractometer Bruker D8 Advance. XRD patterns were recorded from 20° to 90° 2θ with a step time of 2 s and step size of 0.01° 2θ.

3.3.10 Scanning electron microscopy (SEM)

SEM/EDS (energy dispersive spectroscopy) spectra were recorded in an environmental scanning electron microscope ESEM FEI-QUANTA 200. Samples (2 g) were dried at room

temperature for 12 h. Afterwards, a portion of each sample was placed on the top of a pin covered with carbon tape. Finally, samples were covered with a thin layer of gold to make their surface conductive.

3.3.11 Raman Spectroscopy

Raman spectra were obtained with an inVia Confocal Raman spectroscope (Renishaw, UK) using a 532 nm laser. The laser power on the sample was 20mW, and the exposure time for each sample was 10 s. The spectra were collected in parallel and in perpendicular polarization direction using a polarizer and a λ/2 plate. Samples were placed on a tape before measurements.

3.4 Results

3.4.1 Anaerobic ammonium oxidation driven by microbial reduction of sulfate and Fe(III) in marine sediments

Anaerobic ammonium oxidation readily occurred in both contrasting marine sediments studied, concomitant to the reduction of the sulfate and Fe(III) intrinsically present (Figure 3.2 and Figure 3.3). Sediment collected from the coast of Baja California Peninsula showed ammonium oxidation corresponding to 0.71 μg $N\text{-}NH_4^+$ oxidized g^{-1} day^{-1} stoichiometrically linked to **sulfammox** activity fueled by the sulfate present in its pore water, while reduction of the Fe(III) naturally present in this sediment accounted for 0.18 μg $N\text{-}NH_4^+$ oxidized g^{-1} day^{-1} (Figure 3.2 and Table 3.2). When additional sulfate was supplied with artificial marine medium, representing quasi natural conditions, **sulfammox** activity was responsible for 2 μg $N\text{-}NH_4^+$ oxidized g^{-1} day^{-1} (Figure 3.2 and Table 3.2).

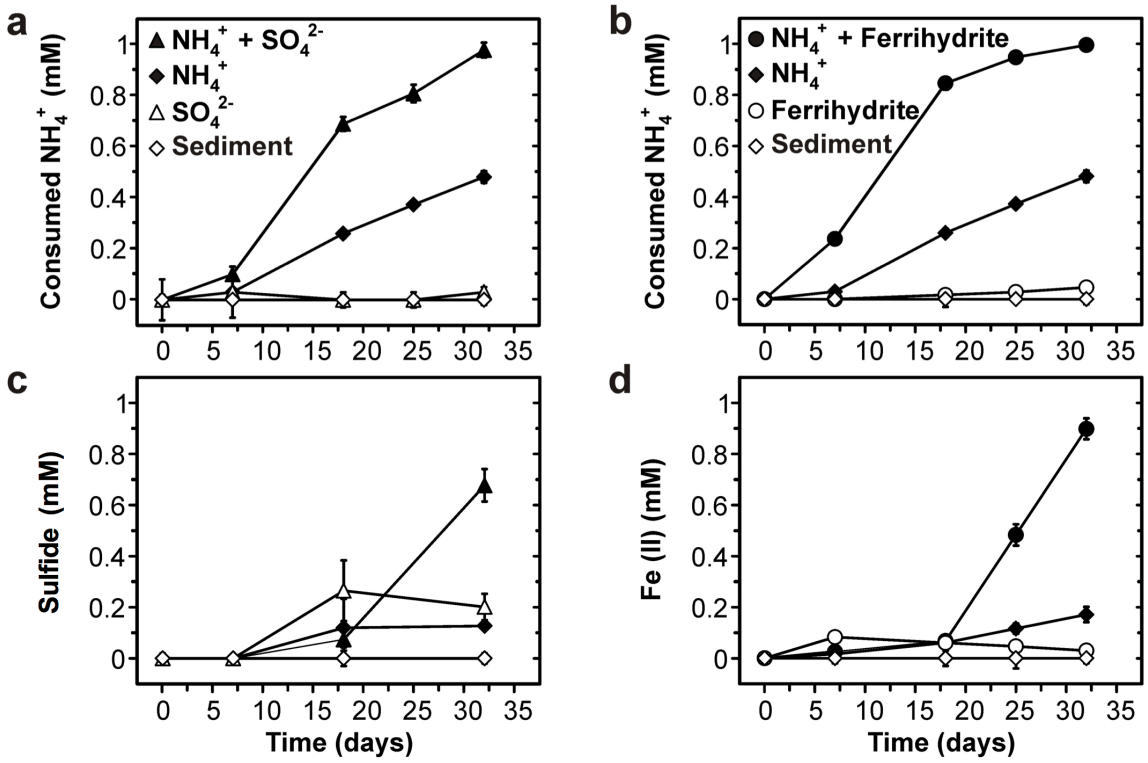

Figure 3.2 Anaerobic ammonium oxidation coupled to sulfate and Fe(III) reduction, measured by the production of sulfide and Fe(II), respectively, in marine sediments collected from Baja California Peninsula. a, Consumption of ammonium linked to the **sulfammox** process. **b,** Consumption of ammonium linked to the feammox process. **c**, Sulfide derived from the **sulfammox** process. **d**, Fe (II) derived from the feammox process. Data represent average from triplicate measurements and error bars, the standard deviation.

Elemental sulfur and sphalerite were identified as sulfurous products during the course of **sulfammox** (Figure 3.4), which may explain the scarce sulfide quantified during the first days of incubation (Figure 3.2). The detection of elemental sulfur as one of the products from the **sulfammox** process agrees with the following thermodynamically favorable reaction:

$$2HN_4^+ + SO_4^{2-} \rightarrow N_2 + S^0 + 4H_2O \qquad \Delta G^{\circ\prime} = -45.3 \text{ kJ mol}^{-1} \qquad 3.2$$

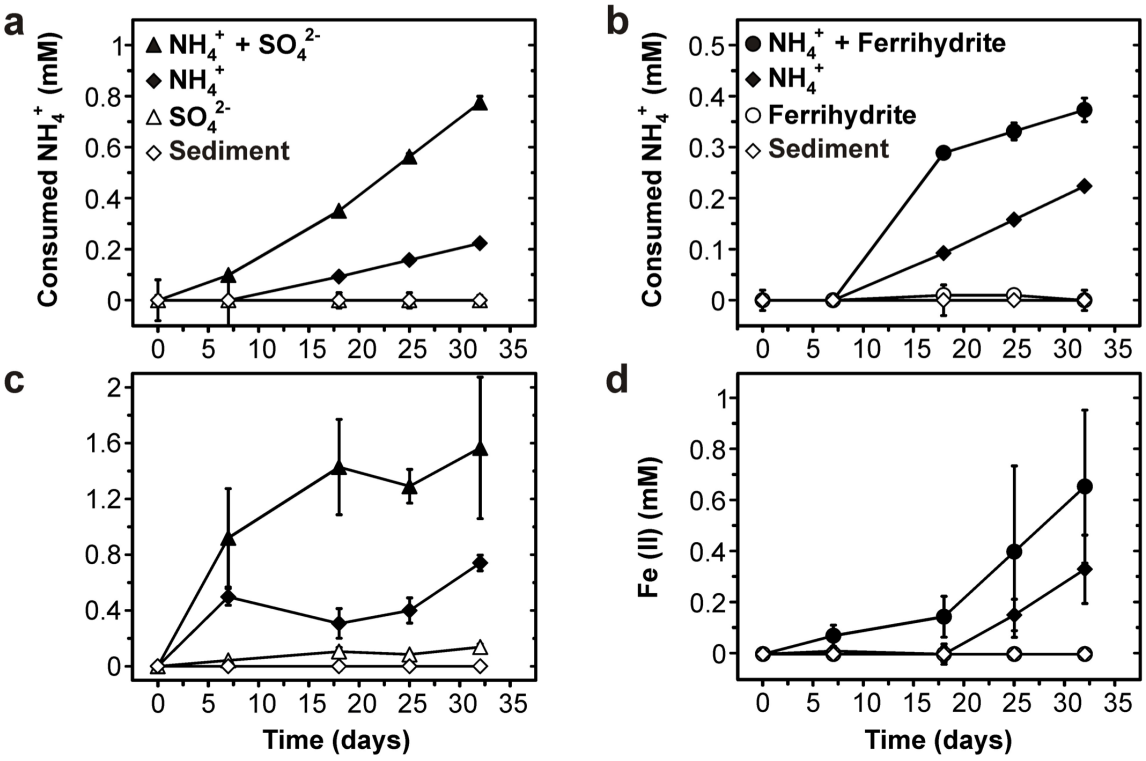

Figure 3.3 Anaerobic ammonium oxidation coupled to sulfate and Fe(III) reduction, measured by the production of sulfide and Fe(II), respectively, in marine sediments collected from Sinaloa. a Consumption of ammonium linked to the **sulfammox** process. **b,** Consumption of ammonium linked to the feammox process. **c**, Sulfide derived from the **sulfammox** process. **d**, Fe(II) derived from the feammox process. Data represent averages from triplicate measurements and error bars, the standard deviation.

Table 3.2 Contribution of sulfammox and feammox processes to NH_4^+ oxidation by sediments collected from Baja California Peninsula (BC) and Sinaloa (SIN).

Process	Contribution to NH_4^+oxidation			
	With electron acceptors added		Without electron acceptors added	
	BC	SIN	BC	SIN
	($\mu g\ N\text{-}NH_4^+\ g^{-1}\cdot d^{-1}$)			
$NH_4^+ + SO_4^{2} \rightarrow HS^- + N_2$	2.03	n.a.	0.71	1.2
NH_4^+ + Ferrihydrite $\rightarrow$ Fe(II) + N_2	0.93	0.19	0.18	0.32

Contribution of each process was calculated considering the measured amount of reduced sulfate (as sulfide) and Ferrihydrite (as Fe(II)), for **sulfammox** and feammox, respectively, and corrected for endogenous controls unamended with ammonium. The quantified amount of reduced sulfate and Ferrihydrite was then related to ammonium oxidation, according to the following stoichiometries:

$$8NH_4^+ + 3SO_4^{2-} \rightarrow 4N_2 + 3HS^- + 12H_2O + 5H^+$$

$$NH_4^+ + 3Fe(OH)_3 + 5H^+ \rightarrow 0.5N_2 + 3Fe^{2+} + 9H_2O \quad 3.3$$

n.a. = not applicable: Due to large endogenous sulfide production, which was much larger than the stoichiometric value, it was not possible to accurately assess this activity.

Spectroscopic screening of the original sediment did not detect any of these sulfurous compounds (Figure 3.5) probably because elemental sulfur was further metabolized and sphalerite prevailed at a very low concentration to be identified.

The other sediment collected south of the former (coast of the state of Sinaloa, Mexico), contained large amounts of organic matter (Table 3.1), which elicited high endogenous reduction of sulfate and Fe(III), interfering with the accurate assessment of the **sulfammox** and feammox activities. Nevertheless, ammonium oxidation occurred in parallel to the reduction of both sulfate and Fe(III) in this sediment, beyond the level observed in controls

incubated in the absence of added ammonium (Figure 3.3), strongly suggesting that the microbial biota of this site is also able to perform the **sulfammox** and feammox reactions.

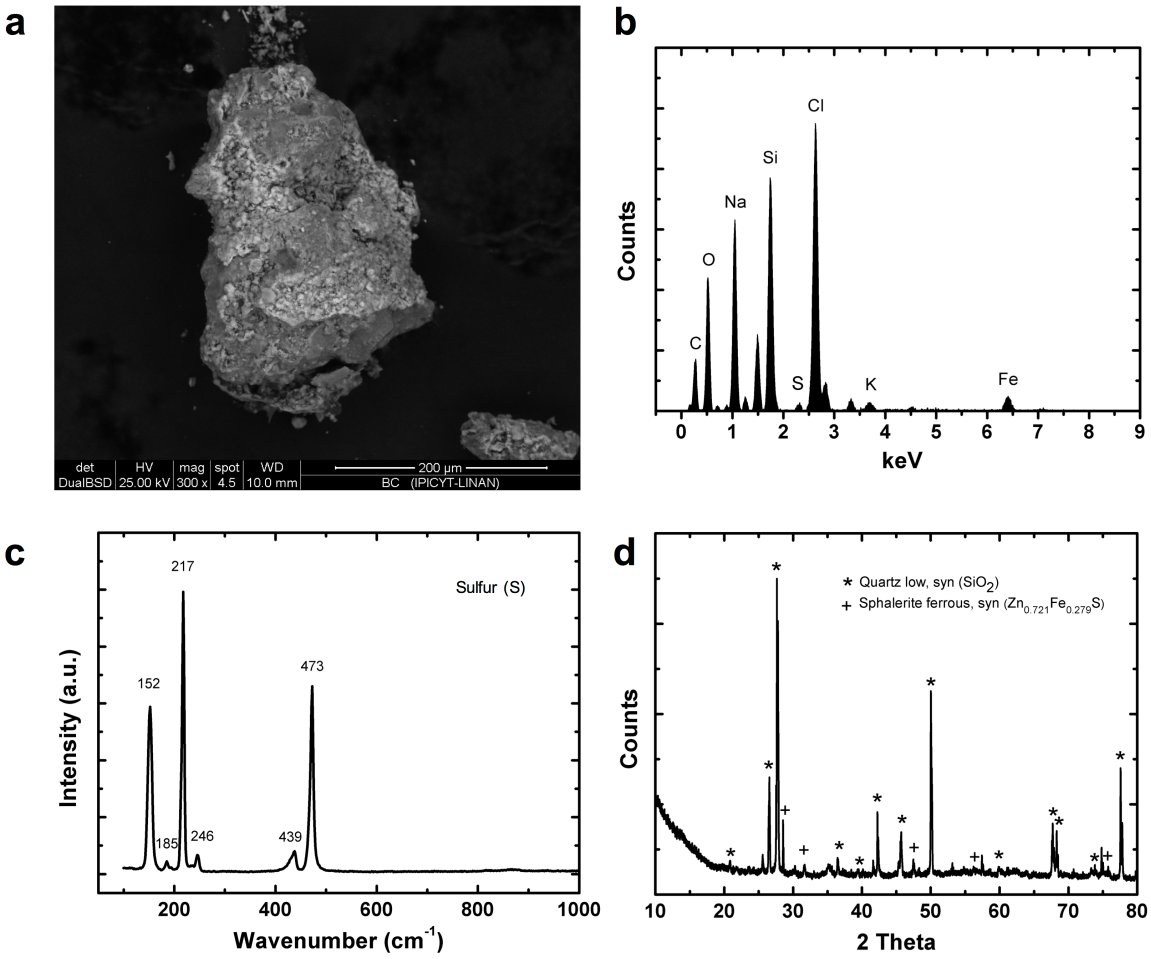

Figure 3.4 Evidence of sulfurous products derived from the sulfammox reaction performed by marine sediment collected from Baja California Peninsula. a and **b**, SEM-EDS spectrum indicating the precipitation of sulfurous compounds in sediment particles. **c**, Raman spectrum confirming elemental sulfur as one of the products derived from the **sulfammox** reaction and accumulated in sediment particles. **d**, XRD difractogram revealing the formation of sphalerite after the **sulfammox** reaction. A total of 5 samples were screened yielding similar results. The reference codes used in XRD analysis are 01-083-0539 for SiO_2, and 01-089-4938 for Sphalerite ferrous, syn.

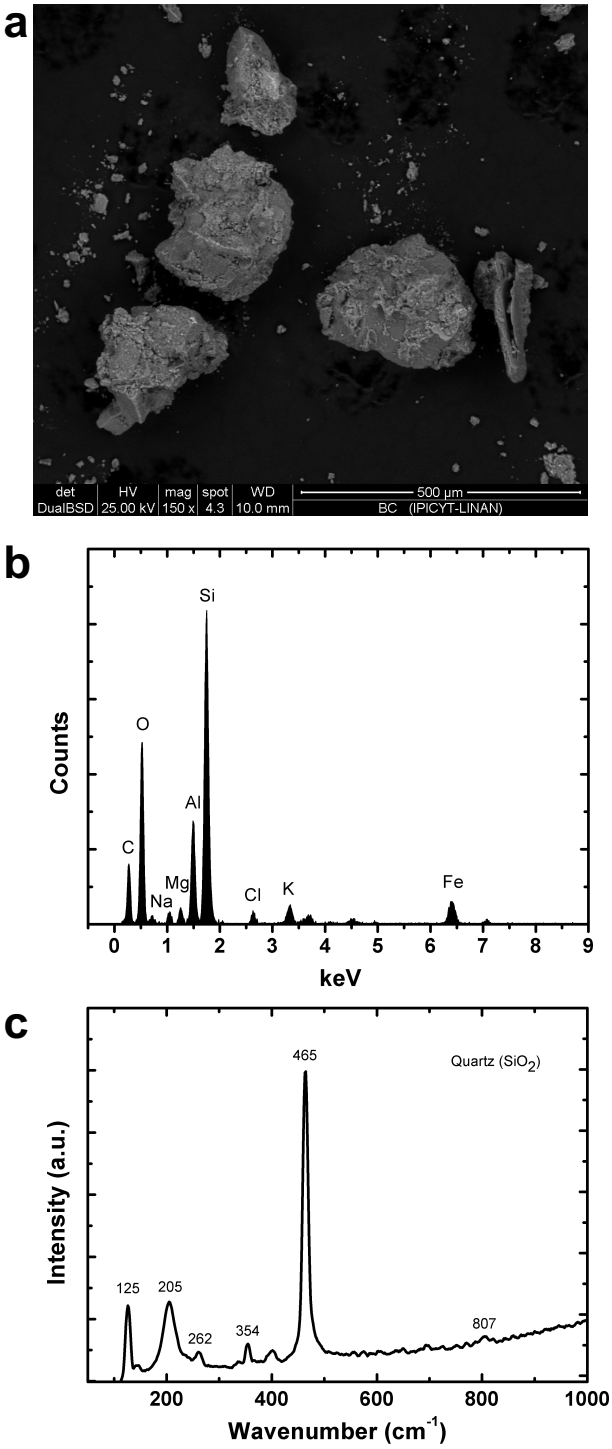

Figure 3.5 Representative characterization of marine sediment sample collected from Baja California Peninsula before incubation confirming the absence of sulfurous species. a, SEM image of sediment sample. **b**, EDS spectrum and **c**, Raman spectrum. A total of 5 samples were screened yielding similar results.

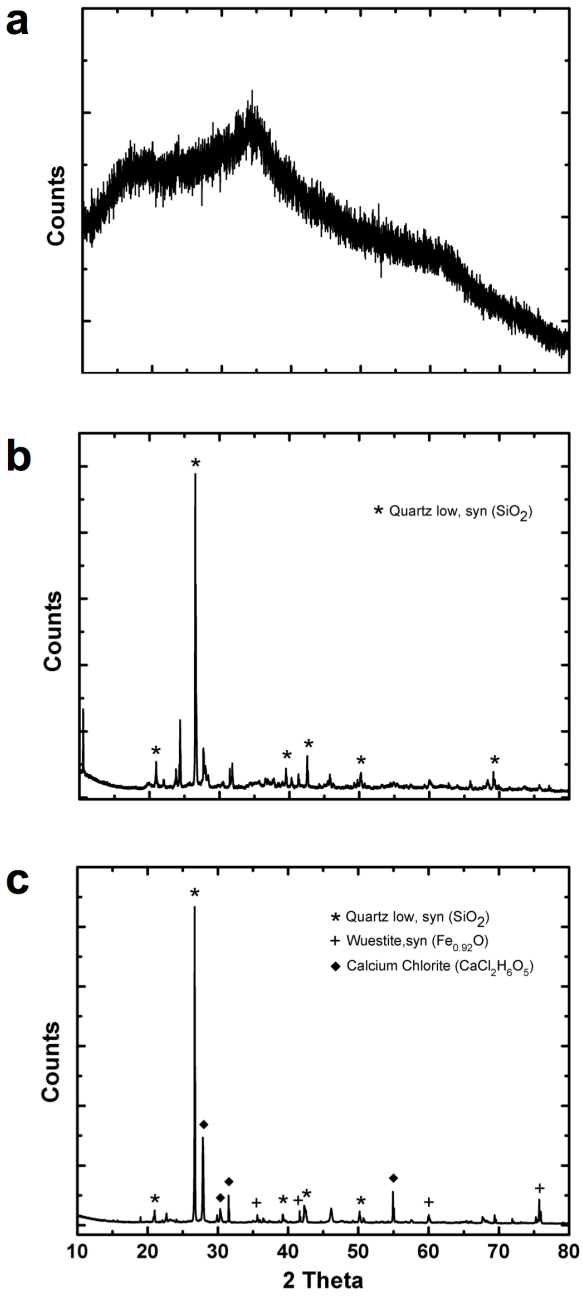

Figure 3.6 Characterization of ferrihydrite and marine sediment samples collected from Baja California Peninsula before and after the course of the feammox reaction. a, XRD spectrum of ferrihydrite synthesized via Schwertmann and Cornell method, before incubation. **b**, XRD spectrum of sediment sample before incubation. **c**, XRD spectrum at the end of the incubation period of sediment sample performing the feammox process. The reference codes used in XRD analysis are 01-083-2465 for SiO_2, 01-079-1969 for Wuestite, and 00-023-0868 for Calcium Chlorite.

Enrichment of both sediments with external ferric iron, in the form of ferrihydrite, confirmed that the biota present in both sites has the capacity to catalyze the feammox reaction (Figure 3.2 and Figure 3.3). Certainly, quantification of ferrous iron derived from ferrihydrite reduction accounted for 0.93 and 0.19 μg $N\text{-}NH_4^+$ oxidized g^{-1} day^{-1} by sediments collected from Baja California Peninsula and Sinaloa, respectively (Table 3.2), while negligible iron reduction occurred in the absence of added ammonium. Interestingly, during the course of the feammox process, wuestite was produced from ferrihydrite reduction, which was confirmed by X-ray diffraction spectra (Figure 3.6).

Sterile controls incubated with autoclaved sediments confirmed the biological nature of the **sulfammox** and feammox processes documented in both sediments studied as no activity was observed in these incubations. Furthermore, nitrite and nitrate did not accumulate in sediment incubations suggesting that N_2 was the ultimate product derived from both **sulfammox** and feammox.

3.4.2 Quantification of nitrogen loss fueled by sulfammox and feammox

We performed additional sediment incubations amended with $^{15}NH_4^+$ in order to assess nitrogen loss by the **sulfammox** and feammox processes. We selected the sediment collected from Baja California Peninsula for these incubations to minimize endogenous interference during the assessments. Tracer analysis revealed that **sulfammox** activity was responsible for 5 μg $^{30}N_2$ produced g^{-1} day^{-1} after 20 days of incubation (Figure 3.7). Only minor $^{30}N_2$ production occurred in incubations lacking external sulfate, presumably due to intrinsic electron acceptors (e.g. ferric iron and sulfate) present in the sediment. Additional incubations spiked with the sulfate reduction inhibitor, molybdate (50 mM), in the presence

of ammonium and sulfate, confirmed the dependency of anaerobic ammonium oxidation on this microbial process as no $^{30}N_2$ production was observed in this experimental treatment. Moreover, no $^{30}N_2$ production evolved in killed controls ratifying the biological nature of the **sulfammox** reaction observed. Furthermore, sediment incubations enriched with ferrihydrite showed feammox activity accounting for 2 μg $^{30}N_2$ produced g $^{-1}$ day^{-1} during the same incubation period, while minor $^{30}N_2$ production occurred in incubations lacking ferrihydrite (Figure 3.7) fueled by electron acceptors naturally present in the sediment (e.g. ferric iron and sulfate).

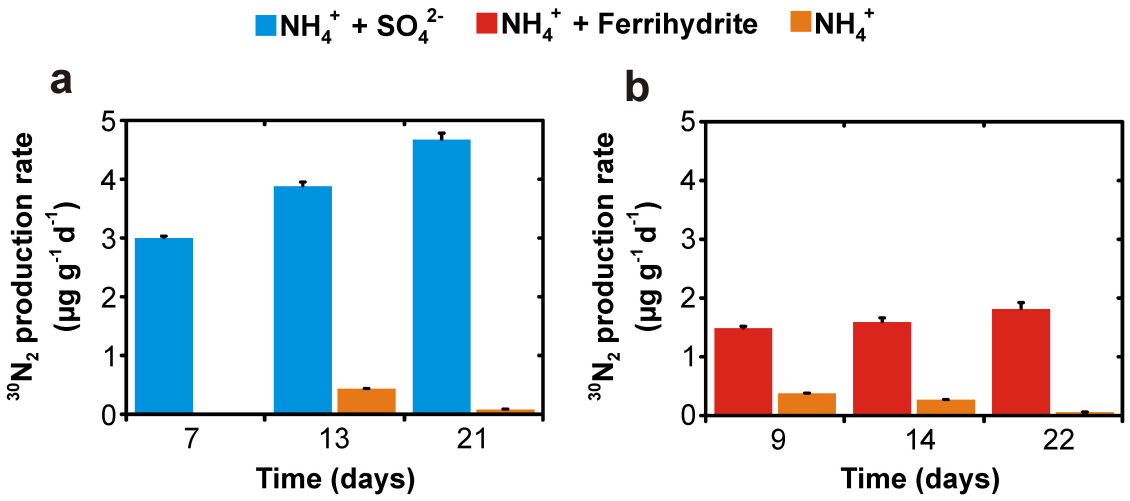

Figure 3.7 Mean $^{30}N_2$ production rates driven by sulfammox and feammox by marine sediments collected from Baja California Peninsula. a, Mean $^{30}N_2$ production rates following the addition of $^{15}NH_4^+$ + Sulfate or $^{15}NH_4^+$ alone to marine sediments (**sulfammox** process). **b**, Mean $^{30}N_2$ production rates following the addition of $^{15}NH_4^+$ + Ferrihydrite or $^{15}NH_4^+$ alone to marine sediments (feammox process). Error bars represent standard errors (n=5). Additional incubations were performed in the presence of the sulfate reduction inhibitor, molybdate (50 mM), amended with both sulfate and $^{15}NH_4^+$; without $^{15}NH_4^+$ addition in the presence of sulfate; and in sterilized controls including both sulfate and $^{15}NH_4^+$; in all these control incubations $^{30}N_2$ was not detected within a practical detection limit of 150 fg.

3.5 Discussion

Fixed nitrogen availability tends to limit primary productivity in the oceans and consequently controls photoautotrophic carbon dioxide assimilation, which ultimately has enormous impacts on global climate change. Thus, the elucidation of key processes driving nitrogen loss from marine environments is of major importance considering the colossal effects triggered by the scarcity of this crucial nutrient in the oceans functioning and in global biogeochemical cycles. Oceanic N_2-fixation rates, which are in the range of 100-200 Tg N yr^{-1} worldwide[7], rival vast losses of fixed N due to microbial processes, such as denitrification and anammox. Denitrification is thought to be responsible for the loss of 200 Tg fixed N yr^{-1} in marine sediments[20], while current estimates suggest that anammox may drive 10-40% of N_2 production in the oceans[20]. Within the oceans, sedimentary denitrification is thought to account for 50-75% of the total marine nitrogen loss, with the remainder occurring in the pelagic OMZ[20]. The two novel marine nitrogen sinks revealed in the present study (**sulfammox** and feammox) change the paradigm that denitrification and anammox are the only processes fueling nitrogen loss in the oceans and further exacerbates the nitrogen balance in these ecosystems.

Sulfate reduction produces large quantities of ammonia in marine sediments through remineralization of organic matter[2]. The predominance of sulfate as terminal electron acceptor in these environmental niches may constitute an important barrier, via the **sulfammox** reaction, to prevent ammonia diffusion up to the anoxic zones where denitrification and anammox prevail. Several decades ago, Richards[3] noticed that most of the ammonia that should be expected from anaerobic remineralization of organic matter was unaccounted for. While he proposed that the missing ammonia might have been anoxically oxidized to N_2 by unknown microbes using nitrate or nitrite as electron acceptor, our findings

suggest that **sulfammox** is probably the main nitrogen sink explaining this gap balance in marine sediments. This is in agreement with the observation that diffusion of nitrate into anoxic marine sediments does not fully account for the N_2 efflux from these sites[21] probably because the **sulfammox** reaction significantly contributes to N_2 production in marine sediments.

The feammox reaction discovered here also accounts for significant N losses and further challenges the nitrogen balance in marine environments. In fact, we measured N_2 production rates driven by feammox, which are very similar to those reported in terrestrial environments[10]. Our findings suggest that the interconnections among the oceanic biogeochemical cycles of N, S and Fe are much more complex than previously considered (Figure 3.8). Besides the four nitrogen sinks already identified (denitrification, anammox, **sulfammox** and feammox), several microbial processes also contribute to N_2 production in marine environments. While some microorganisms perform sulfide-dependent nitrate reduction to ammonium[22], some other reports suggest that coupling between sulfide-dependent denitrification and anammox may drive significant N losses in marine sediments[14]. Sulfate derived from sulfide-dependent denitrification can in turn oxidize ammonium through the **sulfammox** reaction. Fe(II)-dependent denitrification can also contribute to N losses from marine sediments[13] and the replenished Fe(III) can then fuel ammonium oxidation via the feammox reaction. Future quantification of the global marine N_2 production should consider the complex interactions among the biogeochemical cycles of N, S and Fe discussed here.

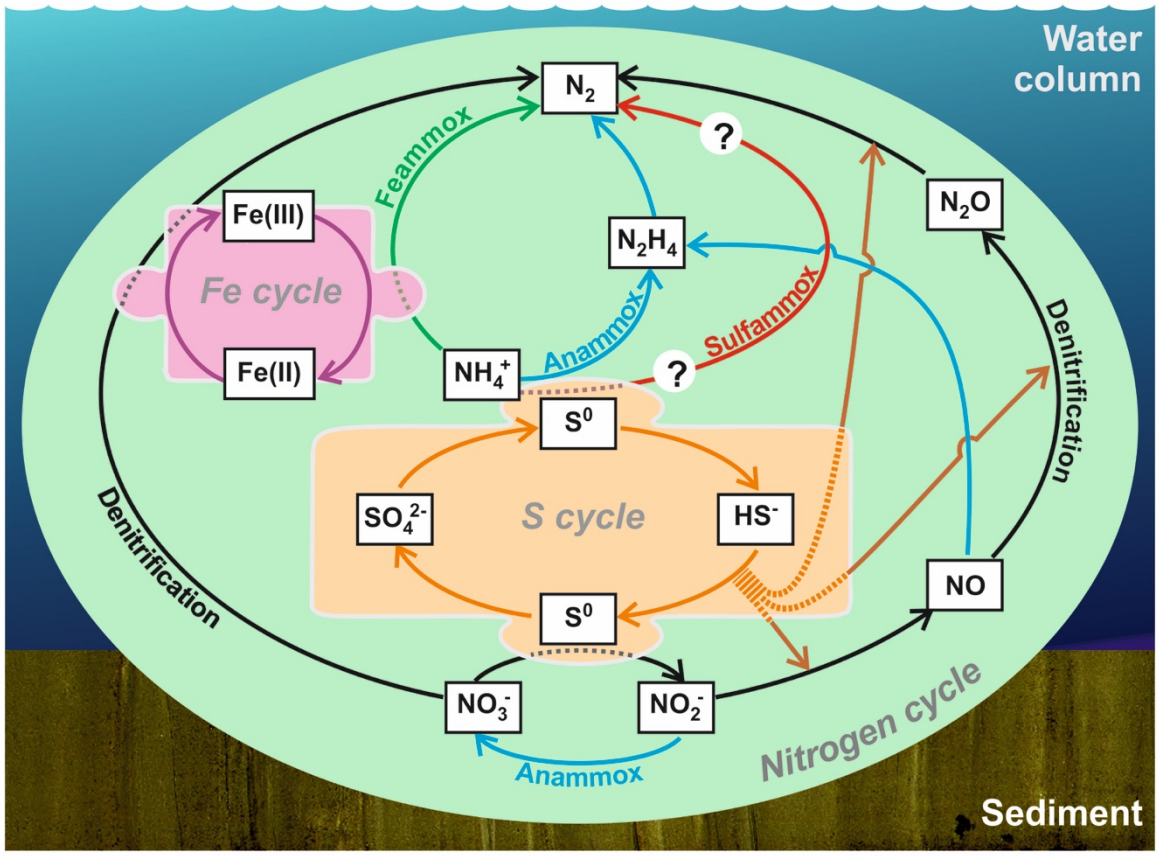

Figure 3.8 Schematic model describing the interconnections among the oceanic biogeochemical cycles of N, S and Fe. Question mark represents unknown nitrogenous intermediates of **sulfammox**.

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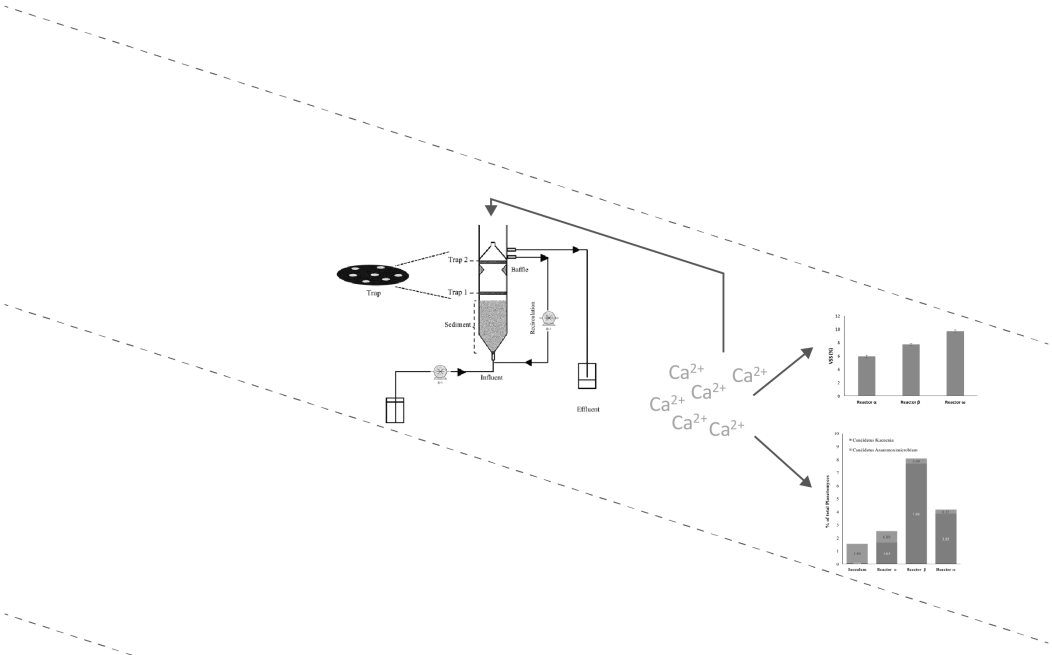

CHAPTER 4

Up-flow anaerobic sediment trapped (UAST) reactor as a new configuration for the enrichment of anammox bacteria from marine sediments

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Up-flow anaerobic sediment trapped (UAST) reactor as a new configuration for the enrichment of anammox bacteria from marine sediments

4.1 Abstract

A novel reactor configuration for the enrichment of anammox bacteria from marine sediments was developed. Marine sediments were successfully kept inside the bioreactors during the enrichment process by strategically installing traps at different depths to prevent the wash-out of sediments. Three up-flow anaerobic sediment trapped (UAST) reactors were set up (α, β and ω supplied with 50, 150 and 300 mg Ca^{2+}/L, respectively). Nitrogen removal rates (NRR) of up to 3.5 g N/L-d and removal efficiencies of >95% were reached. Calcium enhanced biomass production as evidenced by increased volatile suspended solids and extracellular polymeric substances. After the long-term operation, dominant families detected were *Rhodobacteracea*, *Flavobacteracea*, and *Alteromonadacea*, while the main anammox genera detected in the three reactors were *Candidatus Kuenenia* and *Candidatus Anammoximicrobium*. The UAST reactor is proposed as suitable technology for the enrichment of anammox bacteria applicable for the treatment of saline industrial wastewaters with high nitrogen content.

4.2. Introduction

Intensive discharge of nitrogen present in wastewaters to water bodies may cause serious environmental and public health problems. Specifically, wastewaters derived from textile dyeing, aquaculture, chemical and pharmaceutical sectors, as well as landfill leachates and seafood processing factories, contain large amounts of ammonium and salts[1].

Conventionally, sequential nitrification and heterotrophic denitrification are the typical biological treatment processes used for nitrogen removal from wastewaters, which require external organic matter, chemical reagents and aeration[2]. For these reasons, anaerobic ammonium oxidation (anammox) based technologies have been considered as promising alternatives for the traditional technologies because they are cost saving and energy-efficient[3]. The anammox process uses nitrite as an electron acceptor to achieve ammonium oxidation to nitrogen gas[4]. In addition, anammox is an autotrophic process without requiring external organic matter as a carbon source. Energy consumption can be decreased by 60% and sludge generation minimized in industrial applications, when anammox processes are implemented[5]. The stoichiometry of the anammox process was calculated according to Kleerebezem and Van Loosdrecht[6] as follows:

$$NH_4^+ + 1.19\ NO_2^- + 0.066\ HCO_3^- + 0.076\ H^+ \longrightarrow$$

$$1.02\ N_2 + 0.18\ NO_3^- + 0.066\ CH_2O_{0.5}N_{0.15} + 2\ H_2O \qquad (4.1)$$

$$\Delta G^{0'} = -358\ kJ/mol$$

Some of the main challenges in the development of the anammox process are the complex composition of wastewater, including inhibitory components[7,8], and the long time required for starting-up an anammox bioreactor due to the very slow growth rate of anammox bacteria with typical doubling time between 10 and 12 days[9]. Although the anammox process has been investigated for two decades, the long start-up period of anammox reactors remains as one of the main obstacles to the widespread implementation of the process. Different bioreactors configurations have been tested for the enrichment and retention of anammox biomass including lift gas reactor[10], sequential batch reactor (SBR)[9,11,12], rotating biological contactors[13,14], membrane bioreactors (MBR)[15,16] and UASB (up-flow anaerobic sludge

blanket) reactors[17–20]. The proper selection of reactor configuration is essential to decrease the start-up period of an anammox process. Biomass retention is also important to develop anammox processes intended for high nitrogen loading rates (NLR). Most studies have evaluated the feasibility of freshwater anammox consortia when adapting to high salinity wastewater[21–23] but a few studies have been focused on the enrichment of anammox bacteria from marine sediments for biotechnological applications[24–26]. It is interesting from the engineering and from the ecological point of view to investigate nitrogen removal from saline wastewater and to elucidate the role of anammox in marine environments. Hence the main purpose of this study was the enrichment of anammox bacteria from marine sediments in a novel reactor configuration, referred to as up-flow anaerobic sediment trapped (UAST) reactor. In addition, the effect of calcium on the performance of the anammox process was also studied as well as the taxonomic characterization of the anammox enrichments.

4.3 Materials and Methods

4.3.1 Inocula and basal medium

Marine sediments were collected from bays of the states of Baja California (BC) and Sinaloa (SIN) located in the Mexican littoral at the Pacific Ocean. Marine sediments were selected based on a previous study, showing anammox activity in batch experiments, in which specific characteristics and location are reported[27]. In the present study, a mixture of fresh sediments from BC and SIN were used, in a proportion of 80%/20% v/v, respectively, with an initial content of 3.2 % of volatile suspended solids (VSS). Basal medium used for the operation of the UAST reactors was prepared with the following composition (g l^{-1}): $KHCO_3$, 1.25; NaH_2PO_4, 0.029; $CaCl_2 \cdot 2H_2O$, 0.3; $MgSO_4 \cdot 7H_2O$, 0.2; EDTA, 0.00625; $FeSO_4$, 0.00625;

organic salt, 35; and 1.25 mL/L of trace elements solution. It should be noted that 35 g/L of organic salt contained the following composition (mmol kg^{-1}): Na, 462; K, 9.4; Mg, 523; Ca, 9.4; Cl, 521; and Sr, 0.19. The trace element solution is reported in Rios-Del Toro and Cervantes[27]. Ammonium and nitrite were added to the basal medium as needed in the form of NH_4Cl and $NaNO_2$, respectively.

4.3.2 Bioreactors set-up

Three UAST reactors were referred to as alpha (α), beta (β) and omega (ω). Bioreactor configuration is schematically represented in Figure 4.1 Characteristics of each UAST reactor are as follows: They are glass columns with a height of 60 cm, volume of 1 L and an internal diameter of 5.5 cm. The hydraulic retention time (HRT) was set at 12 h throughout all experiments. The reactors were designed with two traps strategically located at different heights (at 30 cm and at 45 cm). Traps prevented the wash out of marine sediments throughout the enrichment process to contribute to the well establishment of biomass in the bioreactors. The up-flow velocity was established at 1 m h^{-1} by a recirculation line. Each trap contained 7 holes with an internal diameter of 8 mm each, which promoted disruption of sediment blocks carried by biogas accumulated. By disrupting sediment blocks transported by biogas produced inside the bioreactors, allowed sediments to settle back to the bottom. The reactors were placed in the darkness in a controlled temperature room at 28 °C and the pH of the influent was maintained in the range of 7.5–7.7.

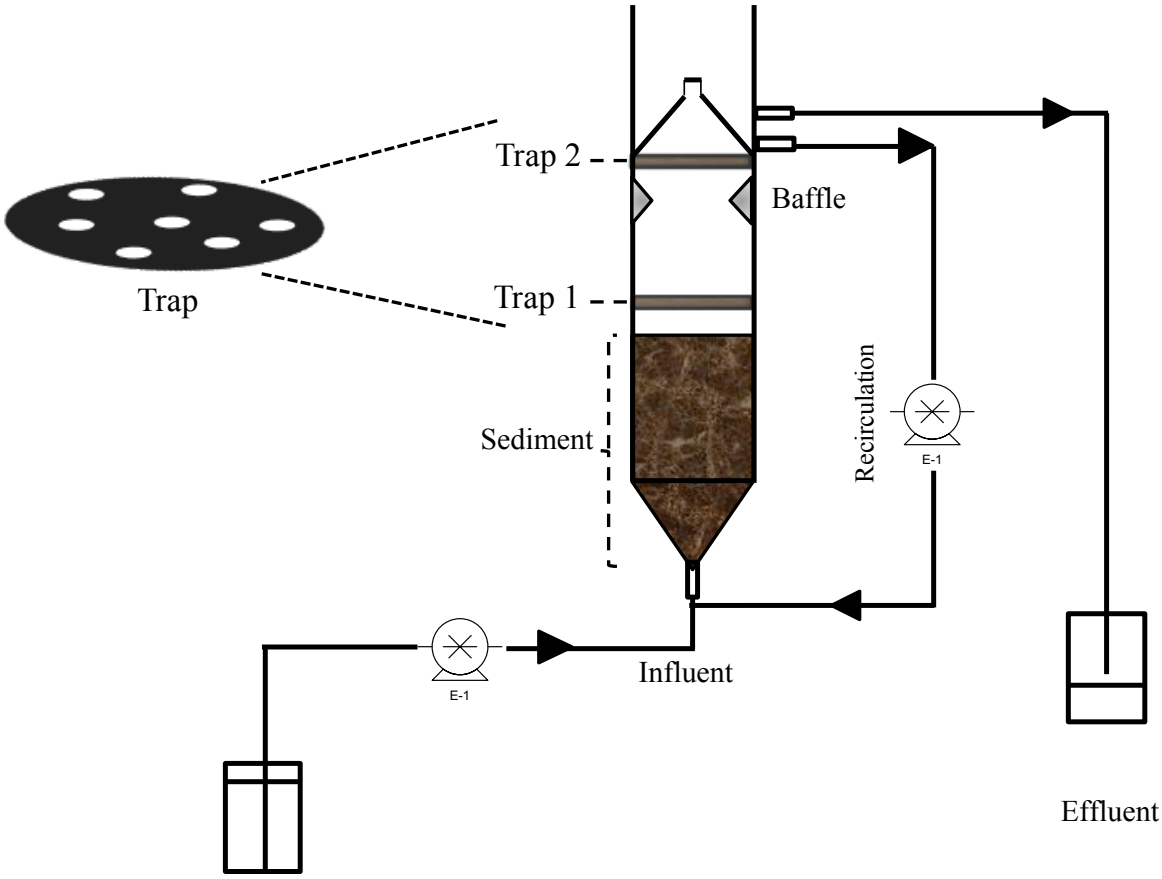

Figure 4.1 Schematic diagram of the up-flow anaerobic sediment trapped (UAST) reactor used for the enrichment of anammox bacteria from marine sediments.

The different concentrations of ammonium and nitrite used throughout the different periods of operation of bioreactors are presented in Table 4.1 Calcium was also added at concentrations of 50, 150 and 300 mg Ca^{2+}/L for α, β and ω reactors, respectively. Calcium was added to study the effect of this element both on the performance of the anammox enrichments and on the physical-chemical properties of the sediments.

In order to compare the performance of all reactors, Kruskal-Wallis test was carried out for nitrogen removal rate (NRR), as well as for NO_2^--N/ NH_4^+-N (Rs) and NO_3^--N/ NH_4^+-N (Rp) ratios, using software R version 3.3.1.

Table 4.1 Performance of UAST reactors at different influent substrate concentrations and at a constant HRT (12 h).

Table 4.1 Performance of UAST reactors at different influent substrate concentrations and at a constant HRT (12 h).

Stage	Period (days)	Influent Concentration (mg N/L)		NLR and NRR (g N/L-d)		NRE (%)	NLR and NRR (g N/L-d)		NRE (%)	NLR and NRR (g N/L-d)		NRE (%)
				Reactor α			Reactor β			Reactor ω		
		NO_2^--N	NH_4^+-N	NLR	NRR	NRE	NLR	NRR	NRE	NLR	NRR	NRE
1	0 - 65	98.4 ± 3.4	97.9 ± 3.7	0.39 ± 0.01	0.18 ± 0.07	46.1	0.39 ± 0.01	0.18 ± 0.07	46.7	0.39 ± 0.01	0.20 ± 0.01	50.9
2	66 – 194	197.1 ± 8.7	150.7 ± 6.9	0.70 ± 0.03	0.58 ± 0.06	91.5	0.69 ± 0.02	0.56 ± 0.07	91.4	0.70 ± 0.02	0.54 ± 0.10	81.9
3	195 – 227	304.6 ± 3.7	230.4 ± 6.5	1.07 ± 0.01	0.92 ± 0.06	99.4	1.07 ± 0.02	0.91 ± 0.06	99.4	0.95 ± 0.36	0.78 ± 0.37	95.3
4	228 – 248	510.8 ± 6.2	380.1 ± 9.7	1.77 ± 0.04	1.48 ± 0.10	99.5	1.79 ± 0.01	1.51 ± 0.08	99.8	1.78 ± 0.02	1.50 ± 0.08	96.2
5	249 - 259	762.78± 7.3	567.4 ± 10.5	2.68 ± 0.05	2.28 ± 0.10	99.5	2.66 ± 0.03	2.34 ± 0.10	99.8	2.64 ± 0.02	2.16 ± 0.01	99.1
6	260 - 346	1006± 9.0	766.3 ± 10.4	3.55 ± 0.02	3.08 ± 0.09	99.7	3.54 ± 0.02	3.10 ± 0.05	99.7	3.41 ± 0.70	2.89 ± 0.71	99.4

Nitrogen Loading rate (NLR) was calculated taking into account the ammonium and nitrite supplied. Nitrogen removal rate (NRR) was calculated as the total nitrogen removed. NRE = nitrogen removal efficiency. Reactor α with 50 mg Ca^{+2}/L, Reactor β with 150 mg Ca^{+2}/L, and Reactor ω with 300 mg Ca^{+2}/L.

4.3.3 Biomass characterization

Biomass from all reactors was characterized based on VSS, extracellular polymeric substances (EPS), calcium content, X-ray diffraction (XRD) analysis, scanning electron microscopy (SEM) and Illumina's 16S Metagenomic Sequencing. All samples were collected and analyzed immediately by triplicate.

4.3.4 Extracellular polymeric substances extraction

EPS extraction was carried out according to Liu and Fang[28]. To each sediment sample (6 g), 10 mL of deionized water and 0.06 mL of formaldehyde were added. Samples were left for 1 h at 4 °C and then 4 mL of 1 N NaOH were added and let stand for 5 h at 4 °C. Samples were centrifuged at 13,000 rpm for 20 min. Supernatants were filtered through a membrane of 0.22 μm and further purified with a dialysis membrane (3500 Da) for 24 h or until pH was 7.5 by constant washings with deionized water. Finally, samples were frozen and lyophilized at -50 °C. To the EPS purified fraction, carbohydrates and proteins were measured. Carbohydrate concentration was measured according to the proposed method by Dubois et al. (1956). Protein concentration was measured by Bradford's method based on the formation of a complex between the dye, brilliant blue G, and proteins in solution[30].

4.3.5 Calcium Content

Calcium content was obtained by inductively coupled plasma-optic emission spectroscopy (ICP-OES, Varian 730-ES). Samples of 2 g of biomass were in contact with a mixture of HNO_3 and HCl at a ratio of 1:3 v/v until boiling. The mixture was washed several times with distilled water.

4.3.6 X-ray diffraction (XRD) analysis

X-ray diffraction analysis of sediments was carried out in an X-Ray diffractometer Bruker D8 Advance. XRD patterns were recorded from 20° to 90° 2θ with a step time of 2 s and step size of 0.01° 2θ.

4.3.7 Scanning electron microscopy

SEM/EDS (energy dispersive spectroscopy) spectra were measured in an environmental scanning electron microscope ESEM FEI-QUANTA 200. Samples (2 g) were dried at room temperature for 12 h. Afterwards, a portion of each sample was placed on the top of a pin covered with carbon tape. Finally, samples were covered with a thin layer of gold to make their surface conductive.

4.3.8 DNA extraction and Illumina's 16S Metagenomic Sequencing

Composed sediment samples were obtained by mixing equal portions (10 g each) from the bottom, the medium and the top part of sediment blanket in each UAST reactor. Total DNA was extracted from homogenized samples using the Power Soil DNA extraction kit (MO BIO Laboratories, Carlsbad, CA, USA) following the protocol described by the manufacturer. DNA isolated from each sample was amplified using primers 341F and 785R, targeting the V3 and V4 regions of the 16S rRNA gene fused with Illumina adapter overhang nucleotide sequences32. Polymerase chain reactions (PCRs) were performed in 50 µl reactions using Phusion Taq polymerase (ThermoScientific, USA) under the following conditions: denaturation at 98 °C for 60 s, followed by 5 cycles of amplification at 98 °C for 60 s, 50 °C for 30 s and 72 °C for 30 s, followed by 25 cycles of amplification at 98 °C for 60 s, 55 °C

for 30 s and 72 °C for 30 s, followed by a final extension of 72 °C for 5 min. Two independent PCR reactions were performed for each sample. The products were indexed using Illumina's 16S Metagenomic Sequencing Library Preparation protocol and Nextera XT Index Kit v2 (Illumina, San Diego CA). Libraries were deep sequenced with the Illumina MiSeq sequencer. Analysis of 16S rRNA gene libraries was carried out using Mothur open source software package (v 1.34.4)33. Sequences containing homopolymer runs of 9 or more bases, those with more than one mismatch to the sequencing primer and Q-value average below 25 were dismissed. Illumina single end reads were analyzed for potential chimeric reads using the UCHIME algorithm. Group membership was determined prior to trimming of the barcode and primer sequence. High quality screened sequences were aligned against the SILVA 123 16S/18S rRNA gene template using the nearest alignment space termination (NAST) algorithm, and trimmed for the optimal alignment region. A pairwise distance matrix was calculated across the non-redundant sequence set, and reads were clustered into operational taxonomic units (OTUs) at 3% distance using the furthest neighbour method. Sequences and OTUs were categorized taxonomically using Mothur's Bayesian classifier and the SILVA 123 reference set. Obtained sequences have been submitted to NCBI GeneBank database.

4.3.9 Analytical Methods

Measurements of NO_3^--N, NO_2^--N, NH_4^+-N, pH, and VSS were performed according to the standard methods[31]. Details can be found in Rios-Del Toro and Cervantes (2016). Measurement of pH was done using a pH meter (Thermo Scientific, Orion 4-star).

4.4 Results and discussion

4.4.1 Anammox activity in UAST reactors

Enrichment of anammox bacteria from marine sediments was carried out in three UAST reactors (α, β and ω). All UAST reactors were operated under the same experimental conditions (HRT, up-flow velocity, pH, medium composition and temperature), except for the content of calcium supplied. Reactors α, β and ω were supplemented with 50, 150 and 300 mg Ca^{2+}/L, respectively, in order to assess the effects of this element to promote granulation of flocculent sediment during the enrichment of anammox bacteria. The UAST reactors were operated for nearly one year and their performance is shown in Fig. 4.2 Different stages can be distinguished depending on nitrite and ammonium concentrations in the influent (Table 4.1). Stage 1 was characterized mainly by the absence of ammonium consumption in the three reactors. At this stage only nitrite consumption was observed with endogenous substrates promoting denitrification. When nitrite concentration was doubled in stage 2, endogenous substrates were finally depleted and anammox activity started. Once ammonium consumption started in parallel with nitrite reduction, nitrate production also occurred in all UAST reactors. After about three months of operation, stoichiometric anammox activity could be established (Table 4.2) in all UAST reactors and a very good performance was observed in all of them achieving a total NRR of 0.56 g N/L-d when NLR was 0.70 g N/L-d in stage 2 (Table 4.1).

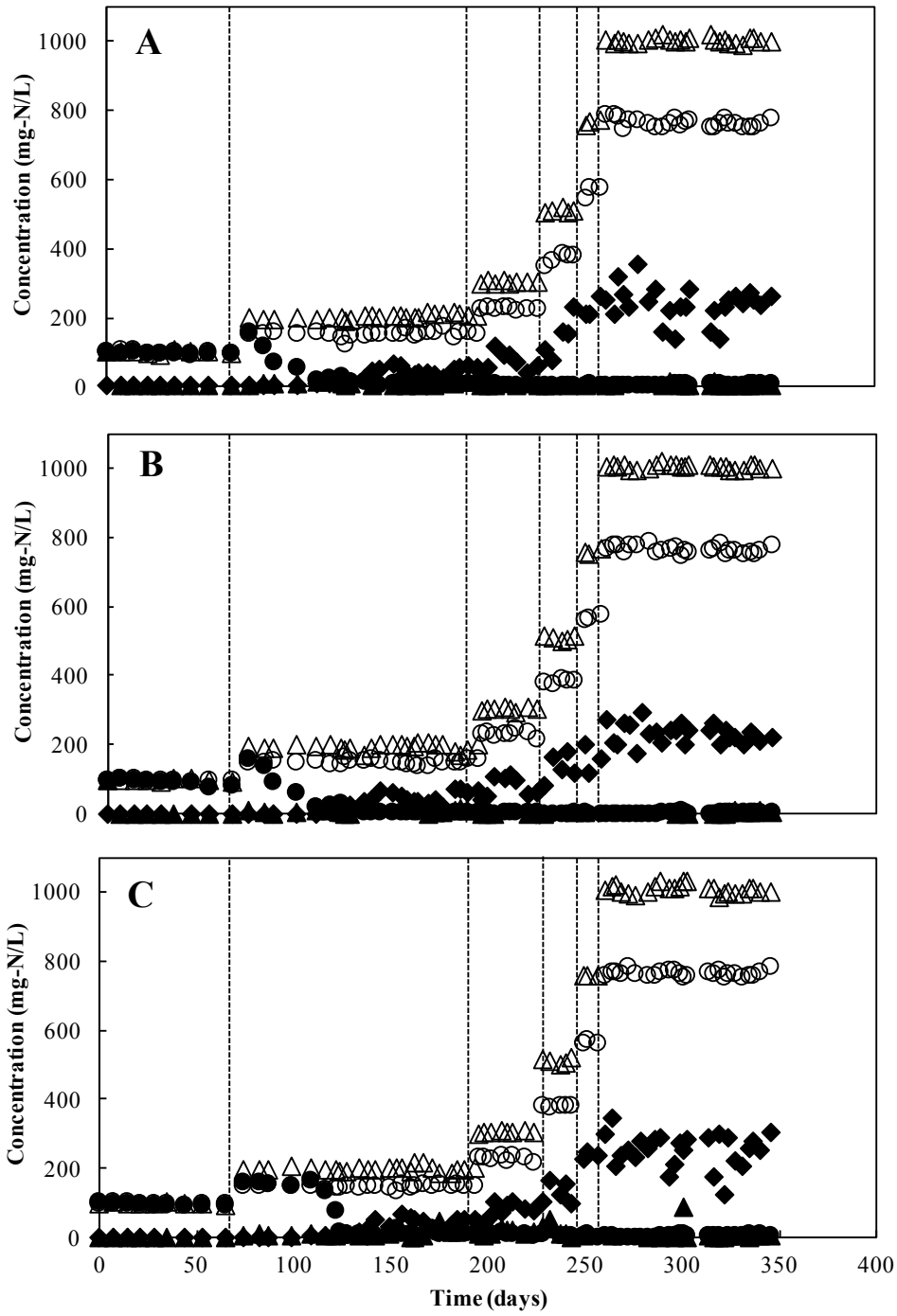

Figure 4.2 UAST reactors performance. A) reactor α with 50 mg Ca^{+2}/L, **B**) reactor β with 150 mg Ca^{+2}/L and **C**) reactor ω with 300 mg Ca^{+2}/L. Legends, (△), Nitrite influent; (▲), nitrite effluent; (○), ammonium influent; (●), ammonium effluent and (♦), nitrate effluent. Dotted lines indicate the different operational stages of the reactors.

In the following operational stages, ammonium and nitrite concentrations were gradually increased until reaching 1 g l^{-1} of nitrite at stage 6. Robust anammox activities could be established in all UAST reactors achieving nitrogen removal efficiency >95% throughout the remaining operational periods. Both Rs and Rp ratios appeared very close to the stoichiometric values throughout the experiments for all UAST reactors (Table 4.2).

Table 4.2 Stoichiometric ratios found during the operation of UAST reactors.

Stage	Reactor α		Reactor β		Reactor ω	
	Rs	Rp	Rs	Rp	Rs	Rp
1	-	-	-	-	-	-
2	1.35 ± 0.10	0.28 ± 0.10	1.31±0.16	0.26±0.15	1.34±0.15	0.28±0.18
3	1.35 ± 0.03	0.27 ± 0.10	1.32±0.02	0.34±0.13	1.32±0.03	0.33±0.12
4	1.37 ± 0.05	0.33 ± 0.10	1.34±0.06	0.36±0.10	1.33±0.03	0.36±0.09
5	1.35 ± 0.02	0.34 ± 0.06	1.35±0.02	0.28±0.10	1.35±0.02	0.43±0.02
6	1.31 ± 0.03	0.28 ± 0.08	1.32±0.02	0.28±0.02	1.32±0.02	0.33±0.06

Rs= substrate ratio, calculated as NO_2^- reduced/NH_4^+ oxidized. Rp= Product ratio, calculated as NO_3^-produced/NH_4^+oxidized. In stage I no anammox activity was observed. Reactor α with 50 mg Ca^{+2} l^{-1}, Reactor β with 150 mg Ca^{+2} l^{-1}, and Reactor ω with 300 mg Ca^{+2} l^{-1}.

Different reactor configurations have been explored for the enrichment of anammox bacteria, and the vast majority have been operated with fresh water anammox biomass, but anammox consortia are also demanded for the treatment of wastewaters containing high concentrations of ammonium and salinity, such as those generated from aquaculture activities. Several efforts to adapt fresh water anammox consortia to high salinity concentrations have been made[22,32], but this adaptation is time consuming and costly. Thus, anammox consortia derived from marine environments represent a suitable option to develop anammox biomass adapted to high salinity conditions. The present study introduces the novel bioreactor

configuration called UAST as an emerging system for the enrichment of anammox bacteria. The use of UAST configuration offers several advantages. First, excellent biomass retention can be achieved during the start-up period. The sandy and flocculent composition of marine sediments represents a challenge to maintain sediment particles inside bioreactors since accumulation of biogas can wash out fine particles and flocculent flocs. The strategic position of traps integrated inside the UAST reactors allowed sediment expansion and well mass transfer. The immobilizing mechanism (traps) of biomass inside the UAST reactor demands simple and cheap materials. No clogging occurred due to a proper combination of high up-flow velocity and relatively large open holes through the traps that allow disruption of flocs carried by biogas. The operation of the UAST reactor is easy and there is no need to change the operational conditions frequently as with others configurations. The efficient system designed to keep particles inside the UAST reactor could be a suitable technology to start up bioreactors inoculated with sediments or flocculent sludge for the treatment of different industrial wastewaters. The UAST reactor could be considered as a modified UASB reactor; however, the main novelty of the proposed design is that no matter the nature of the inoculum (granular or flocculent sludge, as well as marine sediments), the traps installed inside the UAST reactor will allow to keep biomass inside the reactor.

4.4.2 Biomass characterization

Biomass from the three UAST reactors was characterized as a function of VSS, EPS (Fig. 4.3), calcium content, X-ray diffraction, SEM/EDS (Fig. 4.4) and massive sequencing analysis (Figures 4.5 and Fig. 4.6). The highest biomass growth, based on VSS concentration (9.7%) was measured in reactor ω, which received the highest calcium supplied (Fig. 4.3).

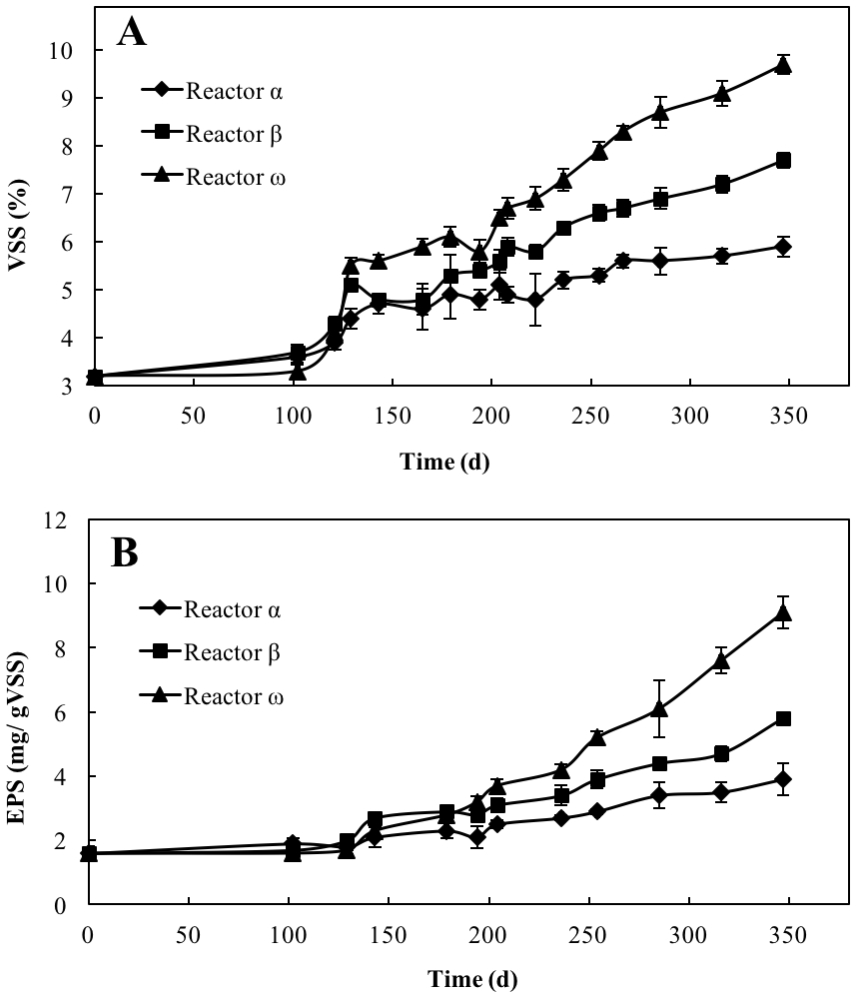

Figure 4.3 Content of volatile suspended solids (VSS) and extracellular polymeric substances (EPS) in the UAST reactors. Reactor α with 50 mg Ca^{+2}/L, Reactor β with 150 mg Ca^{+2}/L, and Reactor ω with 300 mg Ca^{+2}/L. The VSS and EPS content in the original inoculum were 3.2% and 1.6 mg/g VSS, respectively.

Reactor ω also showed the highest EPS content (9.1 mg/g VSS) at the end of the experiments. In fact, there was a direct correlation between calcium supplied and production of EPS in the UAST reactors with reactors α (with 50 mg Ca^{+2}/L) and β (with 150 mg Ca^{+2}/L) containing 3.9 and 5.8 mg/g VSS, respectively, at the end of the operation. Further analysis of extracted

EPS revealed a drastic change on the protein/carbohydrate ratio, with values of 6.55, 5.11 and 8.43 for reactors α, β and ω, respectively, after nearly one year of operation, which is very different from the value found in the original inoculum of 0.48.

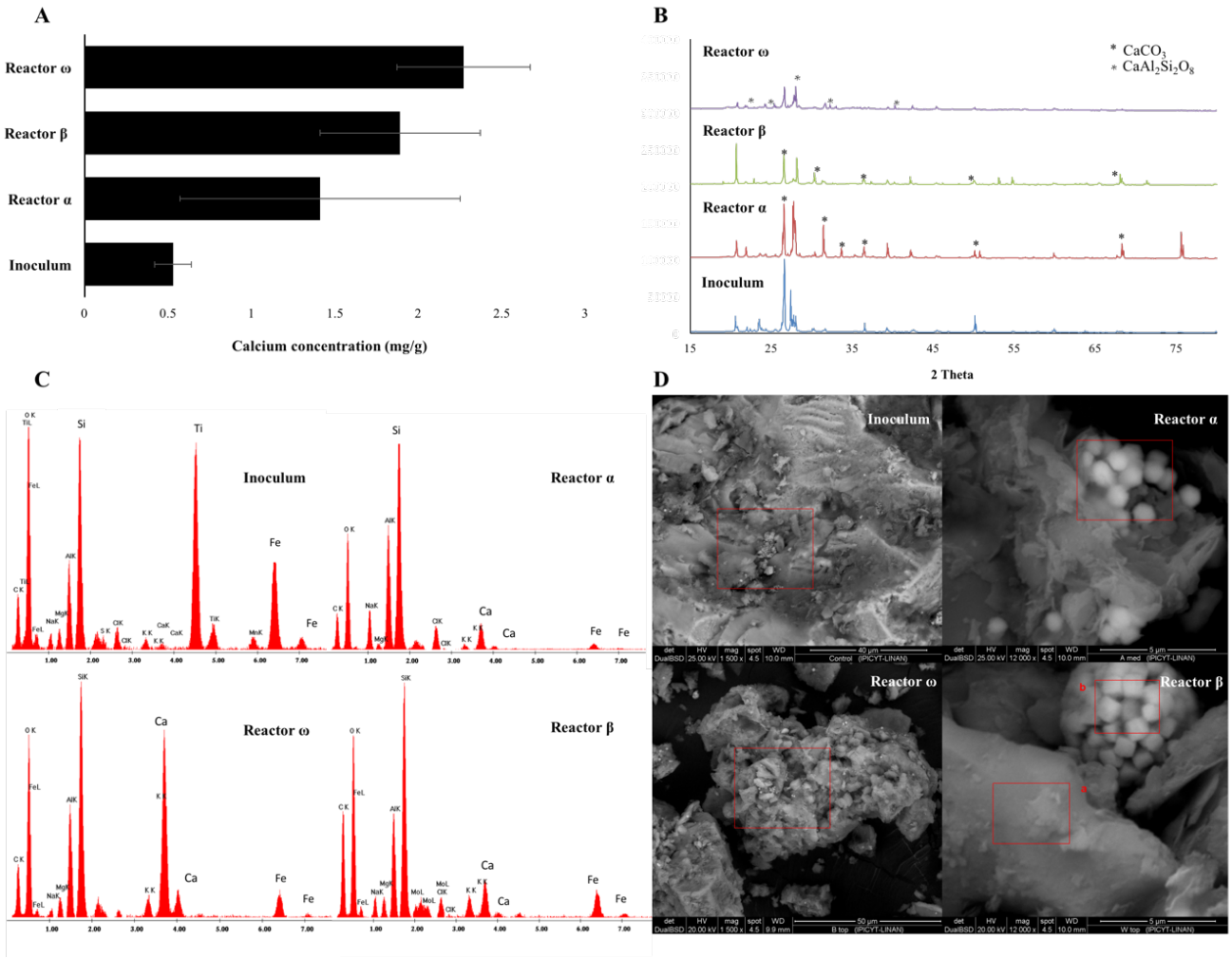

Figure 4.4 Biomass characterization of original inoculum and from the UAST reactors at the end of operational period. A) Calcium content, **B**) XRD spectra, **C**) EDS spectra of precipitates and **D**) SEM microscopies of precipitates.

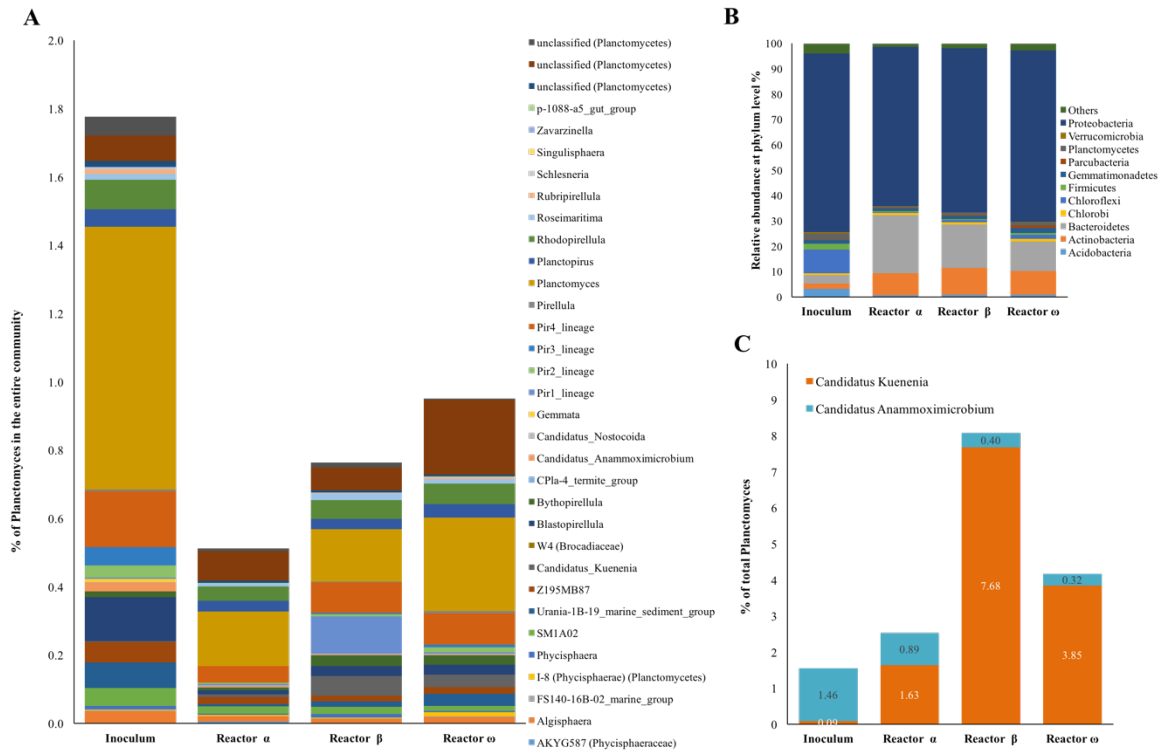

Figure 4.5 Microbial community composition of inoculum and from the UAST reactors at the end of operational period based on Illumina's 16S Metagenomic Sequencing. A) Relative abundance of all genera belonging to *Planctomycetes*, **B**) Relative abundance of phyla and **C**) Enriched anammox genera.

Total calcium concentrations in sediments collected from the three UAST reactors was also measured at the end of the experimental period. Results (Fig. 4.4) show an increase on calcium concentration in the sediments of bioreactors (1.41, 1.89 and 2.27 mg Ca/g for of α, β and ω reactors, respectively) compared with the original inoculum (0.53 mg Ca/g). Deep examination of sediments by XRD and EDS analysis revealed that calcium carbonate and calcium aluminum silicate were the main minerals sequestering calcium inside the UAST reactors (Fig. 4.4). On the other hand, calcium did not have any effect on reactors performance according to Kruskal-Wallis test (P=0.62, 0.19 and 0.35 for NRR, Rs and Rp values, respectively).

Taxonomic characterization of microbial communities developed in the UAST reactors was performed based on 16S rRNA gene sequencing of sediment samples. A total of 675,419 high quality reads of 16S rRNA gene were obtained for all libraries. For a better assessment, libraries were normalized to equal abundance of the sample with the least sequencing efficiency (131,430 reads per sample). The most abundant phyla (Fig. 4.5) were *Proteobacteria*, *Bacteroidetes*, *Actinobacteria* and *Gemmatimonadetes* in the three reactors, as well as in the original inoculum. The abundance of *Proteobacteria* was 62, 64 and 67 %, *Bacteroidetes* contributed with 23, 17 and 12 %, *Actinobacteria* with 9, 11 and 9 % and *Gemmatimonadetes* with 1, 1.3, and 1.9 % in reactors α, β and ω, respectively. *Bacteroidetes* is a phylum frequently reported in anammox reactors and our results agree with abundance levels reported by Egli et al. (2003) and by Li et al. (2009).

Relative abundance of all genera belonging to *Planctomycetes*, which have been related to the anammox process, are presented in Figure 4.5. Due to the selection imposed by operational conditions, the abundance of *Planctomycetes* decreased in the three reactors, compared to the inoculum. However, collected data suggest a correlation between the relative abundance of specific groups of *Planctomycetes* with respect to the amount of calcium added (0.51, 0.76 and 0.95 % for reactors α, β and ω, respectively). Some genera of *Planctomycetes*, such as *Rhodopirellula*, *Pir1 lineage*, *Planctopirus*, and an *unclassified* genus, were more abundant in the three reactors after the enrichment as compared to the original inoculum. Results also showed a decrease on the relative abundance of *Candidatus Anammoximicrobium* when calcium supplied increased (Fig. 4.5). The opposite occurred with *Candidatus Kuenenia*, which increased 18 times its relative abundance in reactor α and 85 times in reactor β as compared to its prevalence in the original inoculum.

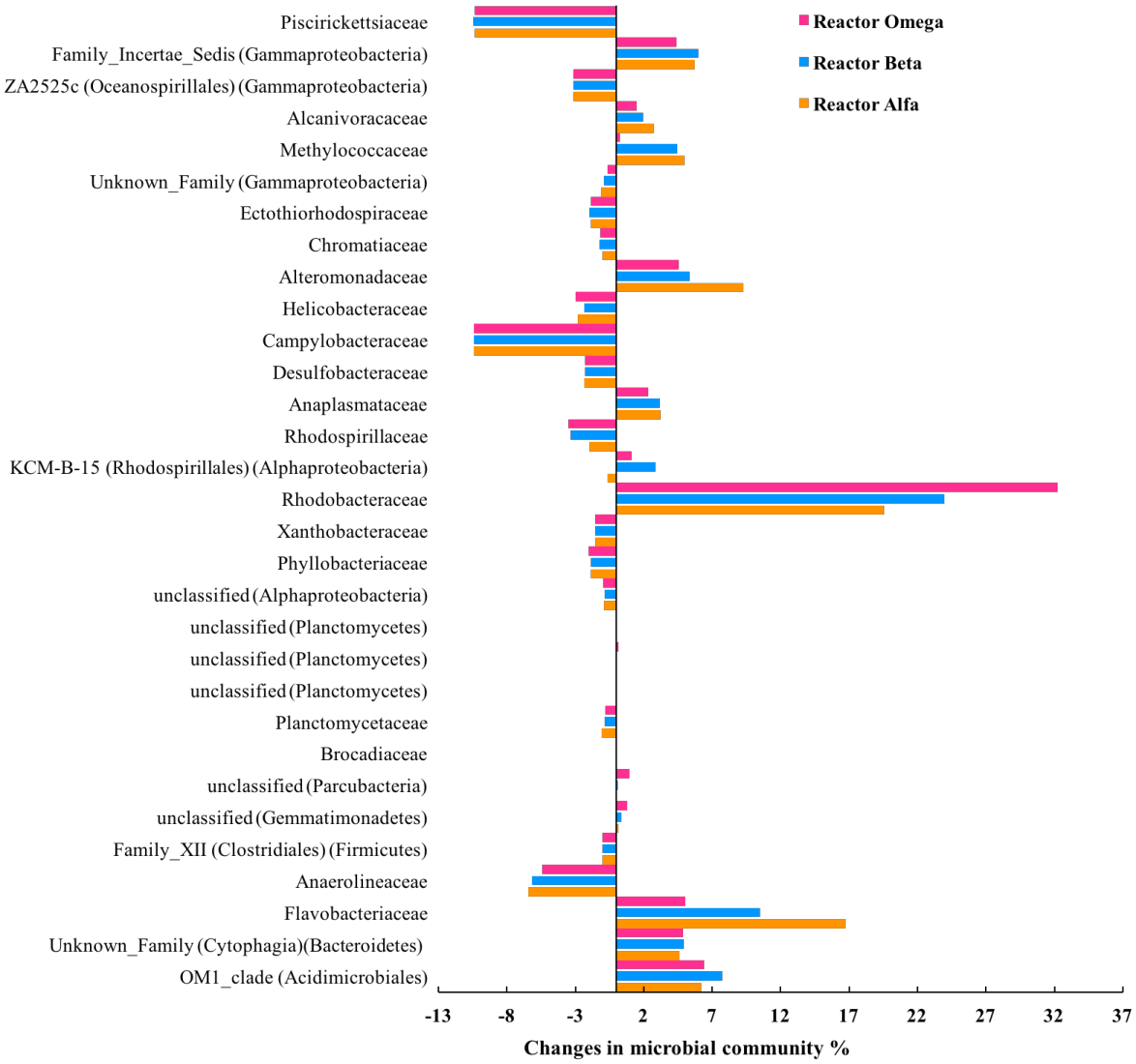

Figure 4.6 Changes in Bacterial families found in the UAST reactors at the end of operational period referred to the original inoculum. Negative values correspond to decrease on the abundance of families, while positive values correspond to enriched families.

Changes in the microbial community composition at family level with respect to the inoculum are showed in Figure 4.6. Among the principal families decreasing their abundance with respect to the inoculum were *Piscirickettsiaceae*, *Helicobacteraceae*, *Campylobacteraceae*, *Rhodospirillaceae* and *Anaerolineaceae*. Clear enrichment of

Rhodobacteraceae family was observed. Reactor ω obtained 46 % abundance of this family, followed by reactor β with 32 % and reactor α with 27 %. These results suggest that increased supply of calcium promoted a selective increase of *Rhodobacteraceae* family. *Rhodobacteraceae*, a big family which members had been reported as aquatic bacteria frequently thriving in marine environments[35]. Enrichment of this family in anammox reactors has been previously reported[36]. Within the main genera found belonging to *Rhodobacteraceae,* we obtained *Sediminomonas* (around 40%) and *Roseibacterium* (around 25%). *Sediminomonas* are strictly halophilic, require NaCl for growth, and are able to use a variety of carbon sources. Moreover, they are able to reduce nitrate to nitrite, thus they might have contributed to recycle the electron acceptor require for anammox (e.g. nitrite) in the UAST reactors, using endogenous substrates present in the sediments. *Roseibacterium* members have the genetic potential to utilize methylated amines as alternative nitrogen sources and requires Na^+ ion (or sometimes combined marine salts) for growth [35]. Conversely, families *Flavobacteriaceae*, *Alteromonadaceae*, *Incertae Sedis*, and *OM1 clade*, negatively responded to the addition of calcium. Certainly, the relative abundance of these families was higher in reactor α supplemented with the lowest calcium content (50 mg Ca^{2+} l^{-1}) as compared to reactor ω, which received the highest calcium supply (300 mg Ca^{2+} l^{-1}). Members of *Flavobacteriaceae* are chemoorganotrophic and widespread in nature [37]. *Alteromonadaceae* are mostly from marine origin and require sodium to grow. Members of this family do not form endospores or microcysts, are chemoorganotrophs, and can use oxygen or nitrate as electron acceptors[38]. These results suggest that anammox and other heterotrophic bacteria coexisted in the UAST reactors, supporting the idea that a diverse

microbial community is useful and probably necessary for the enrichment of anammox bacteria and for the stability of the anammox process[39].

4.5 Conclusions

Enrichment of slowly growing anammox bacteria from marine sediments was possible in the novel reactor configuration, UAST. The treatment concept was shown to be suitable for biomass retention and to establish anammox biomass to achieve high nitrogen removal rates. Addition of Ca^{+2} promoted greater production of biomass and enrichment of *Planctomycetes* in UAST reactors. The new reactor configuration could be a suitable option for the treatment of nitrogen-rich saline wastewaters, such as those generated from aquaculture.

4.6 References

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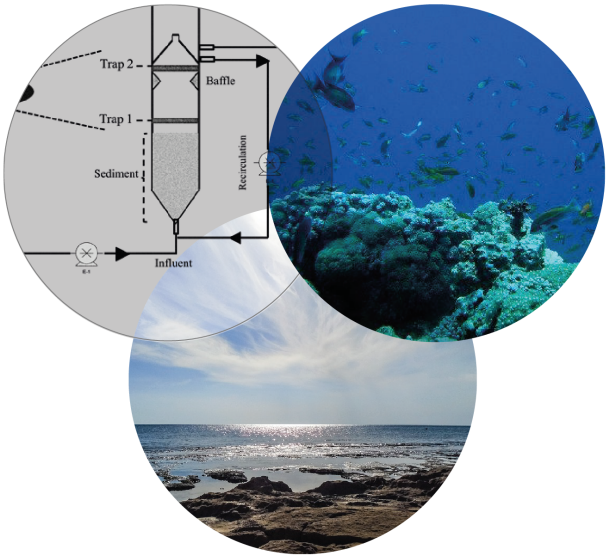

CHAPTER 5

General discussion

5.1 Introduction

When this Doctoral project began four years ago, the main purpose was looking for anammox activity from marine sediments from different sites of Mexico in order to identify suitable sources of biomass to apply the process on a large scale. Most knowledge about the anammox process is mainly reported in references generated from the European continent[1–4], since its discovery in The Netherlands in 1995. Nowadays, only a few reports exist related to the anaerobic ammonium oxidation in marine environments, although it plays a very important role in the biogeochemical cycles and despite the enormous potential to develop biotechnological processes for the treatment of wastewater. Furthermore, until 2013 most knowledge about the nitrogen cycle and its interconnection with the sulfur and iron cycles in marine environments was assumed to be almost complete[5–10]. Discoveries, such as the anaerobic ammonium oxidation coupled to iron reduction (feammox) in 2006[11] and the convincing evidence reported by Yang et al. [12], the biological interaction between the nitrogen and the sulfur cycle under anaerobic conditions[13] and their use in the treatment of high nitrogen-content wastewaters[8], changed the history.

We must always be open to new processes that could occur in nature, especially if they can proceed thermodynamically. This is the case in the present dissertation, which has led to discoveries, such as the coupling between anammox and autotrophic denitrification for the simultaneous removal of ammonium and sulfide by enriched marine sediments (Chapter 2). The anaerobic ammonium oxidation linked to sulfate reduction in marine sediments, proposed by many that could occur in nature[13–15], called suramox by others[16] and here discovered and introduced as the sulfammox process in Chapter 3, constitutes an important contribution of this work. Also, the occurrence of the feammox process in marine environments make the interconnections among the oceanic biogeochemical cycles of N, S

and Fe even more complex (Chapter 3). This general discussion is mainly focused on the interconnections among the oceanic biogeochemical cycles of N, S, C and Fe, taking into account the main contributions provided by this dissertation. Finally, the importance of these processes in the treatment of wastewaters, for the simultaneous removal of nitrogenous and sulfurous compounds is discussed.

5.2 Interconnections among the oceanic biogeochemical cycles of N, S, C and Fe

The interconnections among biogeochemical cycles is not a new issue. In fact, they existed since many years ago and are older than homo sapiens existence, they date back to the Earth´s history. Earth is ~4.5 billion years old, and during the first half of its evolutionary history, a set of metabolic processes that evolved exclusively in microbes would come to alter the chemical speciation of virtually all elements on the planetary surface. Consequently, our current environment reflects the historically integrated outcomes of microbial experimentation on a tectonically active planet endowed with a thin film of liquid water. The outcome of these events has allowed life to persist, even though the planet has been subjected to extraordinary environmental changes, from bolide impacts and global glaciations to massive volcanic outgassing. Although such perturbations led to major extinctions of plants and animals, to the best of our knowledge, the core biological machines responsible for planetary biogeochemical cycles have survived intact[17]. The biogeochemical cycles have evolved on a planetary scale to form a set of nested abiotically driven acid-base and biologically driven redox reactions that set lower limits on external energy required to sustain the cycles. These reactions fundamentally altered the surface redox state of the planet. Feedbacks between the evolution of microbial metabolic and geochemical processes create the average redox conditions of the atmosphere and the oceans. Hence, Earth's redox state is

an emergent property of microbial life on a planetary scale. The biological oxidation of Earth is driven by photosynthesis, which is the only known energy transduction process that is not directly dependent on preformed bond energy[17].

The fluxes of electrons and protons can be combined with H, C, N, O, S, and P to construct a global metabolic map for Earth.

Consequently, the coupling of carbon, nitrogen, iron and sulfur cycles could be another challenge to be settled concerning the global oceanic biogeochemical cycles. In chapter 2, we presented the coupling between anammox and autotrophic denitrification for the simultaneous removal of ammonium and sulfide by enriched marine sediments from two sites of the Mexican littoral. These anammox enrichments constituted the first effort to elucidate the prevalence of anammox bacteria in different sites of the Eastern Tropical North Pacific coast. During the course of anammox enrichments, ammonium consumption in controls lacking added nitrite or any other external electron acceptor was observed, which led to conduct additional experiments to elucidate microbial processes driving ammonium oxidation, besides the anammox process. In chapter 2, enrichments of anammox consortia from two marine sediments were achieved and proved that ammonium and sulfide could be removed simultaneously with nitrite as electron acceptor. Another interesting point is that the coupling between anammox and autotrophic denitrification caused an incomplete oxidation of sulfide, producing elemental sulfur as an intermediate. On the other hand, more ammonium and sulfide were oxidized than that expected from the stoichiometry (in terms of reducing equivalents). Therefore, it was postulated that nitrate originated from anammox activity would have been recycled back to nitrite by denitrifying activity (with sulfide) fueling the coupling between anammox and sulfide-dependent denitrification. Nevertheless, this recycling mechanism does not fully explain the extra oxidation of ammonium and

sulfide. It was then proposed an additional mechanism involved in these incubations, the sulfate-reducing anammox (SR-anammox) introduced here as sulfammox and discussed in detail in Chapter 3 (Figure 5.2).

Chapter 3 provides evidence that anaerobic ammonium oxidation linked to sulfate (sulfammox) and ferric iron (feammox) reduction prevails in marine sediments.

The first report of feammox process in natural environments was made by Yang et al.,[18] they described this activity in a tropical soil and later it was also found in paddy soils[19] and in an intertidal wetland[20].

Until now, only a few reports exist in the literature about the microorganisms involved in the feammox process. One of them by Huang and Jaffé in 2014,[21] who found that *Acidimicrobiaceae* bacterium, belonging to the *Acidimicrobiaceae* family, was responsible for the feammox process. Another study by the same research group corroborated the hypothesis of *Acidimicrobiaceae* bacterium was responsible for the feammox process[22]. Recently, another study was made by Bao and Li[23], who discovered a novel pathway for the feammox process, in a stable consortium, composed of *Anaerospora hongkongensis* and the facultative anaerobe, Comamonadaceae. In this process, *A. hongkongensis* reduces elemental sulfur, sulfite, and polysulfides to sulfide, which fuels ferrihydrite reduction. Sulfide, elemental sulfur, sulfite, and polysulfides serve as electron shuttles, completing the sulfur cycle between *A. hongkongensis* and ferrihydrite. In addition, *Comamonadaceae* showed ammonium oxidation potential under aerobic conditions, with nitrite as the main product. Finally, *Comamonadaceae* mediated simultaneous nitrification−denitrification coupled to iron redox cycling through nitrate/nitrite-dependent ferrous oxidation under anaerobic conditions. Hence, they suggested the feammox process can be driven by the sulfur redox cycling, coupling iron, nitrogen and sulfur cycle.

Anaerobic ammonium oxidation coupled to sulfate reduction (named here as sulfammox) is a process thermodynamically proposed since 2002[14], mentioned by some researchers[8] and recently resumed and called suramox,[16] but until now it had not been reported in nature. Sulfammox process is vastly reported in bioreactors for the simultaneous removal of nitrogenous and sulfurous compounds[8,13,24–28]; however, to our knowledge, there only exists one report of this process in marine environments by Schrum et al[15] in 2009. The results of Schrum and colleagues are based mainly in ΔG results and modest ammonium and sulfate consumptions profiles, which are not considered as compelling evidence of the occurrence of this process. Therefore, the results obtained in the present study (Chapter 3) are the first convincing evidence of sulfammox in marine environments.

In summary, Chapter 3 presents the discovery of feammox activity in marine environments linked to sulfammox activity, demonstrating that the biogeochemical cycles of sulfur, iron, and nitrogen can be coupled in marine ecosystems, and in this way, calling to revisit the interconnections among the oceanic biogeochemical cycles (Figure 3.8 and 5.2)

The existence of sulfammox process in these sediments is not surprising, negative ΔG values demonstrate that it is thermodynamically feasible (Eq. 3.1 in Chapter 3). Also, according to the redox potential values between the NH_4^+/N_2 and SO_4^{2-}/HS^- pairs, the electron transport can be carried out (Figure 5.1).

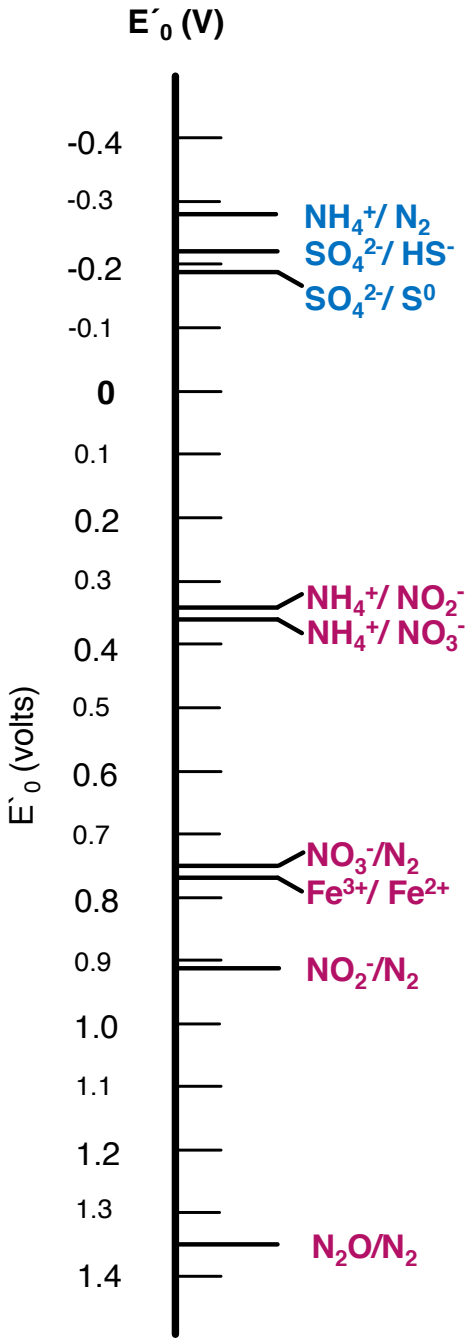

Figure 5.1 Energy scale for various oxidation/reduction couples. Energy scales are expressed in volts.

5.3 Global importance

The coupling among biogeochemical cycles of nitrogen, carbon, iron and sulfur has different implications, none less important than the other. One of them is the relevance in nature, and the other the development of biotechnological applications for the simultaneous removal of pollutants from wastewater.

Anammox activity in enrichments derived from marine sediments was established, and the discovery of feammox and sulfammox activities without enrichment in the same marine sediments was also observed. The sediments were obtained from the coasts of Baja California and Sinaloa (Table 2.1, Figure 3.1). Both sites belong to the oxygen minimum zones (OMZs)[29].

OMZs are major sinks for fixed nitrogen in nature[30], their expansion also causes changes in the cycling of trace gases such as methane (CH_4), nitrous oxide (N_2O) and carbon dioxide (CO_2). Although oceanic CH_4 emissions are minor (<2% of natural CH_4 emissions), the ocean accounts for at least one-third of all natural N_2O emissions, a large fraction of which are derived from OMZs via microbial respiration of nitrate (NO_3^-) and nitrite (NO_2^-)[31].

Recent studies also posit an essential role for sulfur cycling in OMZs, coupling the production and consumption of reduced sulfur compounds to dissimilatory NO_3^- reduction and the fixation of inorganic carbon[32]. The integration of carbon, nitrogen and sulfur cycles represents a recurring theme in the O_2-deficient water column, where electron donors and acceptors are actively recycled between lower and higher oxidation states[29].

Globally, 30-50% of the total N losses occur in OMZs[33], where heterotrophic denitrification has traditionally been recognized as the only significant process converting fixed nitrogen to gaseous N_2. This statement had to be reconsidered after the quantification of anammox activity in marine sediments[34] and now it will also need to be revised taking into account the

anaerobic ammonium oxidation linked to sulfate and ferric iron reduction (sulfammox and feammox processes) discovered in the present dissertation.

Recently, anammox activity was also reported in one site of the Mexican littoral of the Pacific Ocean by Prokopenko et al.[35] In this study, the authors reported the symbiosis between anammox bacteria and nitrate sequestering sulfur-oxidizing *Thioploca* species, showing that *Thioploca*–anammox symbiosis intensifies benthic fixed nitrogen losses in anoxic sediments, by efficiently coupling the carbon, nitrogen and sulfur cycles.

Undoubtedly, all indicates that there are still gaps in the nitrogen cycle, and further studies are needed to explain the interconnections among major biogeochemical cycles (Figure 5.2).

The coupling among different biogeochemical cycles allow the removal of pollutants from wastewater. Contamination by carbon, nitrogen and sulfur compounds in wastewaters and in natural water bodies is a critical problem[36]. Wastewaters, produced from different industries like livestock, antibiotics manufacturing factories, hoggery and meat processing companies, may contain a high concentration of organic compounds, sulfide and ammonia. Specifically, the food industry (i.e. yeast production) may have high content of ammonium and sulfate (>1,000 mg l^{-1} of each)[13]. Other relevant sector is the aquaculture, with important concentration of ammonium, which is produced in high amounts by cultured fish as a consequence of their high-protein diets.

Also, when ammonium is released into the environment, it can cause eutrophication of water bodies and deterioration of water quality, posing risk to fish stocks. A typical characteristic of aquaculture effluents is also their high concentration of salt (until 3.5%).

The application of the coupling between anammox and autotrophic denitrification presented in Chapter 2 is expected to play a significant role in the wastewater treatment systems for

achieving simultaneous removal of nitrogenous and sulfurous contaminants from saline discharges, such as those generated from aquaculture activities.

The use of marine sediments offers several advantages against the use of conventional activated sludge from freshwater environments. Some of them are:

- The start-up of reactor is faster since no adaptation to high salinity is required.
- Inhibition by salt is overcome.
- No requires the addition of compatible solutes, such as glycine betaine for alleviating salt toxicity.

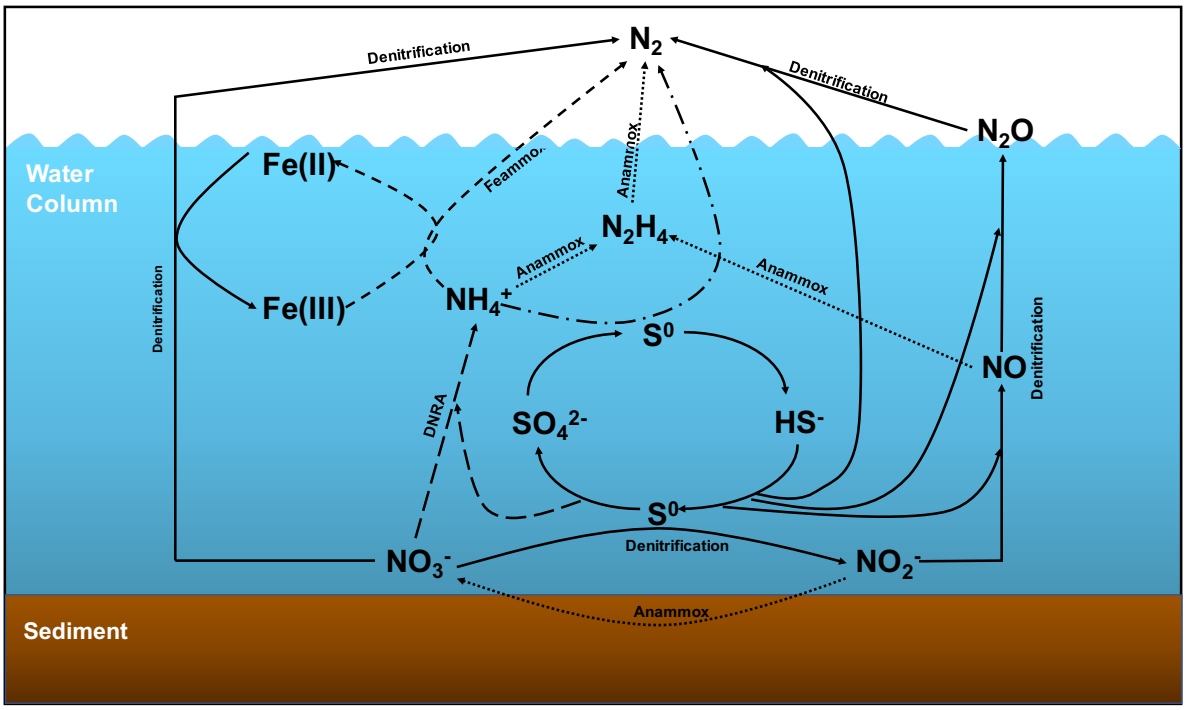

Figure 5.2 Schematic model describing the interconnections among the oceanic biogeochemical cycles of N, S and Fe. Integrating DNRA: dissimilatory nitrate reduction to ammonium.

The decrease in the adaptation time is an important aspect taking into account that some adaptions periods could take months[37–40], which can be translated into significant operating costs. Besides aquaculture, many industrial wastewaters rich in ammonium also contain high salt concentrations[41]. Examples are established effluents produced in the fish canning industry[42], land-fill leachate[43], leather industry[41], seafood processing, textile dyeing, chemical, pharmaceutical and petroleum industries, oil and gas production, tanneries, and livestock, among others[44]. Thus, the identification of marine sediments capable of carrying out anammox activity, besides the feammox and sulfammox processes, may also be relevant from the technological point of view since these sediments could serve as inocula to start up reactors for the simultaneous removal of nitrogen, carbon and sulfur.

5.4 Biotechnological applications of anammox bacteria to N-removal technologies

Anammox bacteria play a significant role in the transformation of fixed nitrogen. After the discovery of the process in waste water treatments plants, several anoxic environments have been screened for the detection of anammox bacteria. Consequently, a massive detection of different anammox species or anammox 16S rDNA sequences have been produced almost elsewhere the main conditions of anoxia. The more profuse were their findings, the more evident became their ecological role and quantitative importance in the N- transformations occurring in different kinds of natural and artificial environments.

The anammox process was discovered in a wastewater treatment plant, so it was no difficult to think immediately on its application. The use of anammox bacteria in water treatment saves costs by requiring less aeration since only half of NH_4^+ is needed to be oxidized to NO_2^-; no exogenous electron donor is required and sludge generation is minimum (0.06 mol

C/mol NH_4^+)[45]. Currently, anammox-based technologies are considered as one of the most promising systems for nitrogen removal in wastewater treatment. However, there are still some challenges in practical applications of anammox-based treatment processes. The biggest barrier for the application of the anammox process is the extremely low growth rates of anammox bacteria (doubling time ranging between 7 and 14 days), causing slow start-up of the process at full-scale. The influence of several environmental parameters, such as salinity, sulfide, temperature and dissolved oxygen, has also been taken into account to optimize the process[46].

Different effluents can be treated with the anammox process. This discussion will be focused on aquaculture as described before. Aquaculture, including the freshwater and marine aquaculture, is a rapidly growing primary production sector implemented to feed the increasing human population. Since 1970, aquaculture has grown at an average rate of 8.9% per year, and the percentage contribution of aquaculture to the total world fisheries has grown from 17.0 to 31.7 % from 1993 to 2003. Due to the worldwide decline of ocean fisheries and the continuous expansion of the human population, aquaculture will continue to grow greatly to satisfy the minimum protein requirement for human nutrition[47].

Chapter 4 describes the development of the UAST (Up-flow anaerobic sediment trapped) reactor concept, as a novel configuration for the treatment of this kind of effluents. The use of UAST configuration offers several advantages (Table 5.1).

Table 5.1 Advantages and disadvantages of UAST configuration.

Advantages	Disadvantages
• Low energy requirements	• Long start-up periods when a proper inoculum is not available
• Excellent biomass retention can be achieved during the start-up period	• Sensitive to inhibitors as nitrite, sulfide, heavy metals, antibiotics, among others
• The immobilizing mechanism (traps) of bio- mass inside the UAST reactor demands simple and cheap materials	• Needs to be optimize to decrease the hydraulic residence time
• No clogging occurred	
• The operation of reactor is easy and there is no need to change the operational conditions frequently as with others configurations	
• Small space needed	

However, there is no doubt that implementation of the UAST technology is a suitable option for the treatment of nitrogen-rich effluents with a high salt content.

The combination of autotrophic nitrification with heterotrophic denitrification has traditionally been the most widely used method for nitrogen removal in biological wastewater treatments. The nitrification-denitrification sequential process performs the aerobic conversion of ammonium to nitrite and further to nitrate, which is finally converted to N_2 in anoxic conditions using a variety of electron donors, including methanol, acetate, ethanol, etc. As nitrification and denitrification have different requirements, besides N-compounds

(oxygen for the former and organic carbon for the latter), and are conducted by different microorganisms these processes have to be separated in time or space.

Therefore, most of the novel technological developments are based on a combination of nitritation coupled to the anammox process, although their physiological requirements are rather different and the processes usually have to attempt to solve several shortcomings.

Several types of reactors have been employed for pilot scale and full-scale installations where anammox is the dominant process (under various names in Table 5.2)[48].

Recently, another configuration was reported, called IFAS for integrated fixed-film activated sludge reactor,[49] which operated under the same principle as the others, applying nitritation/anammox process.

Since the discovery of the anammox process, the mentioned reactor configurations were derived, and they have allowed the combination of biological processes, for the simultaneous removal of nitrogenous pollutants from wastewaters.

On the other hand, due to the anammox process was protected by the discoverers, every research group around the world that start working with anammox process has to start from cero with the enrichment of anammox biomass. Alternatively, if researchers have the opportunity to access to anammox biomass from a real-scale anammox reactor (what happens in rare occasions) the problem gets solution.

Table 5.2 Process options and names for nitrogen removal involving the anammox process (Modified from reference[48]).

Principle	Number of reactors	Source of nitrite	Alternative process names	First reference
two-reactor nitritation-anammox process	2	NH_4^+ nitritation	SHARON[1,2]-anammox	Van Dongen et al. (2001)[50]
			two stage OLAND[3]	Wyffels et al. (2004)[51]
			two stage deammonification	Trela et al. (2004)[52]
one-reactor nitritation-anammox process	1		aerobic deammonification	Hippen et al. (1997)[53]
			OLAND[3]	Kuai and Verstraete (1998)[54]
			CANON[4]	Third et al. (2001)[55]
			deammonification	Seyfried et al. (2001)[56]
			SNAP[5]	Lieu et al. (2005)[57]
			DEMON[6]	Wett (2006)[58]
one-reactor denitrification-anammox process		NO_3^- denitrification	anammox[7]	Mulder et al. (1995)[1]
			DEAMOX[8]	Kalyuzhnyi et al. (2006)[59]
			denammox[9]	Pathak and Kazama (2007)[60]

[1]Acronym of Sustainable High rate Ammonium Removal Over Nitrite; the name only refers to nitritation where nitrite oxidation is avoided by choice of residence time and operation at elevated temperature.

[2]Sometimes the nitrification-denitrification over nitrite is addressed by with this term.

[3]Acronym of Oxygen-Limited Autotrophic Nitrification-Denitrification.

[4]Acronym of Completely Autotrophic Nitrogen removal Over Nitrite.

[5]Acronym of Single-stage Nitrogen removal using the Anammox process and Partial nitritation; Name only refers to the process on a biofilm surface layer.

[6]Name only refers to the process in an SBR under pH-control.

[7]System where anammox was found originally. Whole process was originally designated as "anammox".

[8]Acronym of DEnitrifying AMmonium OXidation; This name only refers to denitrification with sulfide as electron donor.

[9]Acronym of DENitrification-anAMMOX process; this name only refers to denitrification with organic matter as electron donor.

5.4.1 Implications and challenges in the development of biotechnological application for wastewater treatment using anaerobic ammonium oxidizing bacteria

Anammox-based process has been recognized as efficient, cost-effective and low energy alternative to the conventional nitrification and denitrification processes. However, there are still some challenges in practical applications of anammox-based treatment processes. The biggest hurdle for the application of anammox process is the slow growth rates of anammox bacteria (doubling time, $T_{1/2}$, ranged between 7–14 days), causing slow start-up of the process at full-scale. The influence of other environmental parameters, such as salinity, sulfide, temperature and dissolved oxygen, has also been taken into account[46].

There are now 114 reported full-scale anammox installations around the world[61], and the number is increasing rapidly. Most of the anammox plants (88 out of 114) were constructed in Europe, followed by China and North America. Although the first anammox reactor was only 70 m^3, the volume capacity[62] of anammox plants is becoming large, and the number is increasing rapidly. Full-scale plants with more than 142,000 m^3 volume capacity are presently in operation, which can treat 134 tons per day of nitrogen load.

Different bioreactors configurations have been tested for the enrichment and retention of anammox biomass including lift gas reactor[63], sequential batch reactor (SBR)[45,64], rotating biological contactors[65], membrane bioreactors (MBR)[66,67] and UASB (up-flow anaerobic sludge blanket) reactors[68–71]. The proper selection of reactor configuration is essential to decrease the start-up period of an anammox process. Biomass retention is also important to develop anammox processes intended for high nitrogen loading rates. Most studies have evaluated the feasibility of freshwater anammox consortia when adapting to high salinity wastewater[41,42,72] but a few studies have been focused on the enrichment of anammox bacteria from marine sediments in wastewater treatment systems[38,39].

The use of marine sediments as inocula to start-up anammox bioreactors is promising since anammox bacteria seem to be widespread in marine environments (Table 5.3). A comparative summary of the different reactor configurations inoculated with marine sediments to achieve the anammox process is provided in Table 5.4.

From the beginning of the operation of the UAST reactors, several difficulties occurred that had to be overcome. The first problem was the presence of the dissolved oxygen in the system causing ammonium accumulation in the system. Then the lack of granules formation to immobilize the sediment led to the continuous loss of sediment in the effluent, and it was when the traps were implemented to develop the UAST system. Also, nitrate accumulation occurred due to the recirculation system. Finally, another important aspect to consider is the method of anammox biomass preservation, due to the difficulty to cultivate and maintain a large amount of active anammox biomass at once. There exist different methods of preservation. Vlaeminck et al., 2007[73], reported that anammox activity of OLAND biomass was lost after two-month storage at 20 °C. Recently, an effective preservation technique was introduced for anammox bacterial species ‘‘*Ca. Brocadia sinica*’’. Storage in nutrient medium containing 3mM of molybdate at room temperature with periodical (every 45 days) supply of ammonium and nitrite[74]. However, this technique has only tested in one anammox species, until now there is now consensus about which method of preservation is the most suitable to have more than 70% of reactivation.

Table 5.3 Relative importance of anammox on N_2 production documented in different marine environments. [ra% = (Anammox rate)/(total N_2 production rate)] (Updated from reference [75]).

ra%	Study area	Reference
0-22%	Chesapeake Bay (United States)	Rich et al. (2008)[76]
0-77%	Norwegian Trench	Trimmer et al. (2013)[77]
1-11%	United Kingdom estuaries	Nicholls and Trimmer (2009)[78]
10-15%	Gulf of Finland	Hietanen & Kuparinen 2008[79]
10-20%	North Sea	Neubacher et al. (2011)[80]
17-77%	Cávado Estuary (Portugal)	Teixeira et al. (2012)[81]
2-37%	Medway Estuary (United Kingdom)	Rooks et al. (2012)[82]
23-47%	Gullmar Fjord (Sweden)	Brandsma et al. (2011)[83]
33% (average)	North Atlantic Shelf-slope transect	Trimmer and Nicholls (2009)[84]
37%	Sagami Bay (Japan)	Glud et al. (2009)[85]
4-79%	Long Island Sound (United States) and the Skagerrak	Engström et al. (2005)[86]
42% (average)	Cascadia Basin (northeast Pacific)	EngstrØm et al. (2009)[87]
45-48%	Eastern Tropical North Pacific coast	Rios-Del Toro and Cervantes (2016)[88]
5-23%	Arctic fjords	Gihring et al. 2010[89]
Anammox unimportant	Southwest Pacific island estuaries	Dong et al. 2011[90]
Anammox unimportant	Goa Estuary (India)	Fernandes et al. 2010[91]
Anammox unimportant	Gulf of Finland	Jantti et al. 2011[92]

Table 5.4 Different reactor configurations and their performance inoculated with marine sediments to achieve the anammox process with saline wastewaters.

Reactor type	days	NLR	NRR	Reference
	days	(g N/L-d)		
Fixed bed reactor	550	0.08	0.06	Nakajima et al. (2008)[38]
Column reactor	320	0.16	0.13	Kawagoshi et al. (2010)[37]
Up-flow Column Reactor	594	3.33	2.17	Kindaichi et al. (2011)[39]
UAST	346	3.5	3.0	Rios-Del Toro et al. (2017)[93]

UAST= Up-flow anaerobic sediment trapped reactor, NLR= nitrogen loading rate and NRR= nitrogen removal rate.

The fact that the sulfammox process can occur in marine sediments opens the possibility to start-up bioreactors inoculated with consortia from these environments to achieve the simultaneous removal of ammonium and sulfate from saline wastewaters, such as those generated from aquaculture and seafood processing factories, as well as textile, pharmaceutical, among other industrial effluents. Furthermore, elemental sulfur has been identified as intermediate from sulfammox reaction (Chapter 3), thus it is conceivable that it could be recovered as a valued by-product from this process. Table 5.5 shows different reactor configurations operated with the sulfammox process. However, all these reports on the sulfammox process were studied for achieving sulfate and ammonium removal from different wastewaters by inocula not derived from marine sediments, which needed some adaptation to high salinity conditions.

Table 5.5 Sulfammox process carried out in bioreactors for the removal of ammonium and sulfate from wastewaters.

Name	Type of reactor	Inocula	Reference
-	GAC fluidized-bed reactor.	sludge from an industrial anaerobic contact reactor treating wastewater from a yeast factory	Fdz-Polanco et al. (2001)[24]
SRAS	UASB reactor	nitrifying sludge	Yang et al. (2009)[27]
SRAO	MBBR and UASB	anammox biofilm/ anaerobic sludge	Rikmann et al. (2012)[8]
SRAO	UASB	anaerobic sludge containing Anammox bacteria	Rikmann et al. (2014)[25]
SRAO	MBBR and UASB	anammox biofilm/ anaerobic sludge	Rikmann et al. (2016)[28]

GAC = granular activated carbon, SRAS = simultaneous removal of ammonium and sulfate, UASB = up-flow anaerobic sludge blanket reactor, SRAO = sulfate-reducing anaerobic ammonium oxidation

5.5 Perspectives and future opportunities

The coupling between anammox and autotrophic denitrification can be scaled to UAST reactors to be used in the treatment of wastewater. Also, it is needed the implementation of UAST technology, together with sulfammox process, in experiments to test its capacity to remove nitrogenous and sulfurous compounds from wastewaters.

The sulfammox process was introduced here showing evidence of tracer analysis, that the process occurred in marine sediments from two different sites. At the same sites, also feammox process ensued. The specific metabolic pathway of feammox and sulfammox processes has not been reported yet, it is necessary to perform specific experiments of molecular biology to elucidate it. While metagenomics approaches (as performed in chapter

4) provide information on gene content, these technologies do not describe the activities of the microbial community and how these activities vary with respect to space, time, environmental factors or biotic interactions. Metatranscriptomics, or the large-scale sequencing of mRNAs retrieved from microbial communities, can shed light on microbial activities and their regulation. Metatranscriptomics overcomes the targeted nature of quantitative PCR (qPCR) and microarrays to measure gene expression in the cultures. However, metatranscriptomics is yet to be applied widely to study the activity of anammox, feammox and sulfammox communities due to several technical challenges, including the low relative abundance of mRNAs (1–5%), the difficulty of isolating prokaryotic mRNA due to the lack of poly(A) tails, and the short half-life of mRNA.

Experiments to define the microbial community structure of feammox and sulfammox communities using qPCR and 16S rRNA gene sequencing are missing.

In order to implement the UAST reactor at full-scale for the treatment of aquaculture effluents, and others with similar characteristics, the treatment concept needs to be optimized so that shorter hydraulic residence times could be applied during its implementation.

5.6 Concluding remarks

We live on an ocean-dominated planet, and the collective metabolic expression of cellular life in the ocean has a profound influence on the evolution of the biosphere. Cellular life in the ocean is in turn dominated by microbial communities that form interaction networks, which are both resilient and responsive to environmental perturbation. Determining how these interaction networks form, function and change over time reveals otherwise hidden links between microbial community structure and higher order ecological and biogeochemical processes[29].

Until now, the sulfammox and feammox processes had not been reported with convincing results in marine environments. The evidence provided by this dissertation demonstrated the occurrence of both processes in marine sediments. Also, collected evidence suggested that the coupling between sulfide-dependent denitrification and anammox may drive significant N losses in marine sediments. Sulfate derived from sulfide-dependent denitrification can in turn oxidize ammonium through the sulfammox reaction. Fe(II)-dependent denitrification can also contribute to N losses from marine sediments and the replenished Fe(III) can then fuel ammonium oxidation via the feammox reaction.

Also, it was demonstrated that the implementation of the UAST technology allowed the enrichment of anammox bacteria from marine sediments and this reactor configuration could be suitable for the treatment of nitrogen-rich wastewaters, such as those generated from the aquaculture sector.

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