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A tale of two stories from the underground: soil microbial diversity and N cycling

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ENC  **21** EUROPEAN NITROGEN CYCLE
21st Annual Meeting

Information and Abstract Book

5-7th September 2016



University of East Anglia

Welcome to ENC21

We are excited to be welcoming you to UEA for ENC21, for what looks to be a very exciting programme. We are delighted that the Biochemical Society will be sponsoring prizes for the best poster and the best offered talk. Here is some further information to help you get the most out of the conference.

Transport:

Norwich is accessible by road, train, bus or coach. Please refer to some information detailed on the [UEA website](#) providing transport links and information.

If you're driving, please park in the main car park upon arrival; there is clear signage when you enter the campus. A car parking validator will be available at the registration desk from lunchtime onwards, enabling free parking throughout your stay. You may leave your car in the car park for the whole duration of the conference. If you are doing so, please print off and bring a permit (see attached) with you, filling in the required fields, to display in your windscreen throughout your visit. We will validate your tokens during your stay.

Accommodation:

If you've booked for a residential place, please retrieve your keys from The Enterprise Centre upon arrival, which is very close to the main car park as well as bus stops, if you're coming by public transport.

For those of you arriving later than Monday, please retrieve your keys from the Security Lodge.

Twitter:

We will have a live feed of all tweets associated with the conference, please use #ENC21UEA for any Nitrogen Cycle related tweets during your stay. You can keep track on the feed [here](#).

Other important information:

- Please note there are no available plug sockets in The Enterprise Centre to gain Passivhaus status; please ensure your electronic devices are well charged prior to attending. Plug sockets are available in our residences.
- Wireless connection is powered by The Cloud; you will be provided with information on how to join at registration. The Cloud is available across campus. If your institution already uses Eduroam, you will be able to access this on electronic devices once on campus but may need to adjust your permissions whilst here. If you have any issues in doing so, please call UEA IT Helpdesk on 01603 592345 (open Monday to Friday, 9am to 5pm)

If you have any enquiries, please contact online.registration@uea.ac.uk or g.rowley@uea.ac.uk
We look forward to meeting you all.

Andy Gates, David Richardson, Gary Rowley

Schedule

Monday 5th September

- 1400 - 1800 Registration at The Enterprise Centre (including Accommodation keys)
- 1800 - 1815 Welcome to UEA and N cycle
- 1815 - 1915 Plenary Speaker:

Professor Laurent Philippot,; *A tale of two stories from the underground: soil microbial diversity and N cycling*
- 1915 - 2100 Drinks, buffet and poster session

Tuesday 6th September

Session 1: Biochemistry and regulation I

- 0830 - 0900 Coffee
- 0900 - 0930 Stuart Ferguson
TBC
- 0930 - 0950 Victor M. Luque-Almagro
Integrating transcriptomic and proteomic data applied to cyanide biodegradation
- 0950 - 1010 Daniel Mania
Elucidating the aggressive N₂O reduction capability of nodule-forming Bradyrhizobium strains
- 1010 - 1030 Isabel Johnston
Contribution of the NsrR regulon to nitric oxide detoxification: linking the nitric oxide and tellurite responses of Salmonella Typhimurium
- 1030 - 1100 Maria Delgado
A coordinated pathway for nitrate assimilation and nitric oxide metabolism in plant-associated endosymbiotic bacteria
- 1100 - 1130 Coffee

Session 2: Biochemistry and Regulation II

- 1130 - 1150 Manuel J Soriano-Laguna
Copper maturation of nitrous oxide reductase of Paracoccus denitrificans
- 1150 - 1210 Cintia Carreira
Marinobacter hydrocarbonoclasticus nitrous oxide reductase - The active form in vivo

1210 – 1230 Pawel Lycus
“Forewarned- forearmed; to be prepared is half the victory”– respiratory survival strategies in P. denitrificans

1230 – 1250 Linda Bergaust
Quorum sensing systems LasI-LasR and RhII-RhIR regulate N₂O reduction in Pseudomonas aeruginosa

1300 - 1400 Lunch and posters

Session 3: Biochemistry and Regulation III

1400 - 1430 Hannah Gaimster
Genome-wide discovery of novel sRNAs in Paracoccus denitrificans : A potential strategy for greenhouse gas mitigation

1430 - 1450 Sophie Bennett
Copper detoxification/trafficking and Nitrous Oxide Reductase copper centre biogenesis in Paracoccus denitrificans

1450 - 1510 Natalie Lim
Metatranscriptomics show high nosZ transcription but severely delayed N₂O reduction in acid soil

1510 - 1530 Christina Ferousi
Exploring the mechanism of biological hydrazine synthesis

1530 - 1600 Coffee

1600 - 1620 Maria Torres
Disparate response to microoxia, FixK2 and NnrR of Bradyrhizobium diazoefficiens norCBQD and nosRZDYFLX genes

1620 - 1640 Nick Watmough
Using Vertebrate Globins to Understand the Mechanistic Basis of Heme Catalysed Nitrite Reduction

1640 - 1710 Lola Roldan
Transcriptional and translational adaptation to aerobic nitrate anabolism in Paracoccus denitrificans

Evening: BBQ at Wood Hall. Coaches leave UEA Congregation Hall at 1830.

Wednesday 7th September

Session 4: Physiology and Ecophysiology

Coffee available from 0815.

- 0840 - 0910 Jeff Cole
Regulation, sensory domains and roles of two Desulfovibrio desulfuricans Crp family transcription factors, HcpR1 and HcpR2, in response to nitrosative stress.
- 0910 - 0930 Monica Conthe
Microbes living on N₂O respiration: a chemostat study
- 0930 - 0950 Mingsheng Jia
Evaluating the potential for nitrate reduction through DNRA by anammox bacteria for municipal wastewater treatment
- 0950 - 1010 Daniel Milligan
Phylogeny vs denitrification phenotype; regulatory plasticity across a selection of Pseudomonas stutzeri strains
- 1010 - 1030 Javier Torregrosa-Crespo
First physiological study on NO_x emissions in haloarchaea: the case of Haloferax mediterranei.
- 1030 - 1100 Asa Frostegard
Ecophysiology of denitrifying bacteria; regulatory networks and phenotypic plasticity
- 1100 - 1130 Coffee

Session 5: Ecology of the N-cycle I

- 1130 - 1200 Sara Hallin
The many facets of denitrification as revealed by phylogenomics - a framework for ecological inference
- 1200 - 1220 Marcus Horn
Arctic peat circles host a remarkable denitrifier community capable of N₂O consumption at acidic pH
- 1220 - 1240 Linda Hink
The consequences of ammonia oxidiser niche specialisation for mitigation of nitrous oxide emissions
- 1240 - 1310 David Berry
Exploring N utilisation by the gut microbiota using single cell isotope probing
- 1310-1400 Lunch and posters

Session 6: Ecology of the N-cycle II

- 1400 - 1430 Jim Prosser
Eco-Evo studies of ammonia oxidisers: questions, surprises and partial answers
- 1430 - 1450 Ugo Marzocchi
Impact of settled algae-aggregates on benthic nitrogen cycling
- 1450 - 1510 Victoria Griessmeier
Investigation of the different routes of nitrogen reduction in denitrification beds as a function of carbon source and microbiome
- 1510 - 1530 Martina Putz
Effects of nitrogen fertilization on soil N₂O reducing bacteria and their importance for mitigating N₂O emissions
- 1530 - 1550 Dianming Wu
Large NO and HONO emissions from biological soil crusts regulated by drying-wetting cycles and temperature
- 1550 - 1615 Coffee

Session 7: Interconnectivity of the N-cycle with other biogeochemical cycles

- 1615-1635 Julea Butt
Cysteine Ligated Hemes and Biogeochemical Cycles
- 1635-1655 Jon Todd
Insights into sulphonium and nitrogenous osmolyte production in model marine microbes
- 1655-1715 Clive Butler
Bacterial selenate respiration
- 1715-1745 Colin Murrell
Exploring methylamine metabolism using stable isotope probing

1900 Bar opens at the Sainsbury Centre for Visual Arts

1930 Seated for 3 course dinner with closing remarks.

2200 UEA Scholars Bar for those that are keen.

Abstracts for Oral Presentations

Integrating transcriptomic and proteomic data applied to cyanide biodegradation

Luque-Almagro VM¹, Sáez LP¹, Escribano MP¹, Manso IM¹, Cabello P², Moreno-Vivián C¹, Roldán MD¹

¹Departamento de Bioquímica y Biología Molecular and ²Departamento de Botánica, Ecología y Fisiología Vegetal. Universidad de Córdoba. 14071-Córdoba-Spain

Cyanide is one of the most toxic compounds, which has been widely used in the chemical industry. Mining and metal processing activities are the main producers of cyanide-containing wastewaters, provoking serious environmental problems. *Pseudomonas pseudoalcaligenes* CECT5344 is an alkaliphilic cyanide-utilizing bacterial strain that is considered as candidate for bioremediation of cyanide-contaminated habitats. The whole genome sequence of the strain CECT5344 has been completed by constructing a fosmid library and closing the gaps by primer walking, and further PacBio single molecule sequencing. The *Pseudomonas pseudoalcaligenes* CECT5344 genome was resequenced and its methylome analyzed by applying the single molecule real-time (SMRT) sequencing technique. This genome has been the first of a *P. pseudoalcaligenes* strain sequenced as well as the first genome available from a cyanide-assimilating organism. The assessment of the whole genome sequence of the strain CECT5344 has been essential to constructing DNA microarrays in response to sodium cyanide and the jewelry cyanide-containing wastewater. This analysis revealed the induction by sodium cyanide and the cyanide-rich wastewater of four nitrilase-encoding genes, including the *nitC* gene that is essential for cyanide assimilation, the cyanase *cynS* gene involved in cyanate assimilation, the *cioAB* genes required for the cyanide-insensitive respiration, and the *ahpC* gene coding for an alkyl-hydroperoxide reductase that could be related with iron homeostasis and oxidative stress. In cells grown with the jewelry wastewater were specifically induced, among others, several genes coding for metal extrusion systems. A quantitative proteomic analysis by using liquid chromatography-mass spectrometry (LC-MS/MS) has been applied, thus corroborating the vast majority of the available transcriptomic data. New generation 'cyan-omic' techniques are very powerful in displaying a holistic view on the *P. pseudoalcaligenes* CECT5344 cyanide metabolism.

This work was funded by MINECO/FEDER (BIO2015-64311-R) and Junta de Andalucía (Grant CVI-7560), Spain. The authors thank FCC-Ámbito, SAVECO, AVENIR and MAGTEL for their collaborations.

Elucidating the aggressive N₂O reduction capability of nodule-forming *Bradyrhizobium* strains

Daniel Mania¹⁾, Kedir Jillo¹⁾, Tulu Degefu¹⁾, Endalkachew Wolde-meskel²⁾ and Åsa Frostegård¹⁾

¹⁾ Norwegian University of Life Sciences, Ås, Norway, ²⁾ International Livestock Research Institute, Ethiopia

Bradyrhizobium strains that form symbiotic root-nodules with soybean, peanut and cow pea and are increasingly used as inocula to improve crop yields and reduce fertiliser use. Some *Bradyrhizobium* strains can also denitrify. Rotting nodules are hot-spots for denitrification and therefore potential sources of N₂O produced by various soil bacteria. We screened forty *Bradyrhizobium* strains, isolated from nodules of legumes growing in Ethiopian soils, for their ability to reduce NO₃⁻, NO₂⁻ and/or N₂O under denitrifying conditions. The strains were phylogenetically diverse, as seen from MLSA. Most of the complete denitrifiers in our collection belong to the *B. japonicum* group. Genome sequencing of selected strains confirmed genes encoding periplasmic nitrate reductase NapA, but not membrane bound nitrate reductase NarG, similar to other strains of this genus. N₂-producing strains showed a strong preference for N₂O reduction over NO₃⁻ reduction. To elucidate whether this was due to transcriptional control, we did a detailed analysis of mRNA copies per cell over time for one strain, in response to O₂ depletion. The results suggest a common regulation of transcription for *nirK*, *norB* and *nosZ* by O₂ and NO₂⁻ and/or NO. The transcription pattern of *napA* was inverse to that of the other denitrification genes. N₂O did not repress transcription of *napA*. Our results suggest that the preference of N₂O over NO₃⁻ is exerted on the protein level and controlled neither by transcription nor by protein synthesis. We hypothesise that NapA cannot compete for electrons with nitrous oxide reductase N₂OR, and that N₂O reduction rates are significantly higher than NO₂⁻ and NO reduction rates in these *Bradyrhizobium* strains. With their aggressive N₂O reduction, these naturally occurring *Bradyrhizobium* strains show promising traits for the development of potent inocula, that also act as powerful sinks for greenhouse gas emissions from agricultural soil.

Contribution of the NsrR regulon to nitric oxide detoxification: linking the nitric oxide and tellurite responses of *Salmonella* Typhimurium

Isabel Johnston, Anke Arkenberg, Hannah Gaimster, Andrew Gates, David Richardson, Gary Rowley

School of Biological Sciences, University of East Anglia, Norwich Research Park, Norwich, Norfolk NR4 7TJ, U.K.

The intracellular pathogen *Salmonella* encounters nitric oxide (NO), a highly cytotoxic compound during its lifecycle. Exposure occurs endogenously where NO is formed as a respiratory by product of anaerobic nitrate reduction and, exogenously when challenged by the host defense inside macrophages. Effective NO detoxification systems are therefore essential for survival and virulence. This study identified three tellurite resistance (Te^R) genes, *tehB*, *STM1808* and *yeaR*, which were up-regulated by exogenous NO when *Salmonella enterica* Typhimurium SL1344 was cultured under anaerobic nitrate reduction. The putative role of these proteins for tellurite resistance was confirmed. Functional overlap in anaerobic NO protection was observed between the three genes and an accumulation of NO in a $\Delta tehB \Delta STM1808 \Delta yeaR$ triple mutant was observed during anaerobic nitrate respiration. Additionally, there is a link between protection against tellurite and NO in *Salmonella*, where strains lacking proteins known to be important for NO detoxification also have growth defects in the presence of tellurite. Survival of the triple mutant is significantly attenuated in activated macrophages, more so than $\Delta hmpA$, which is known to be important for virulence; this shows the importance of these three genes in combination and warrants further study. Interestingly, the hypersensitive triple Te^R mutant is not more sensitive to either selenite, which produces higher levels of reactive oxygen species (ROS) than tellurite, or H₂O₂ implying the phenotype is not due to ROS as previously considered. The mechanism by which these proteins provide resistance against both tellurite and NO therefore needs to be elucidated.

A coordinated pathway for nitrate assimilation and nitric oxide metabolism in plant-associated endosymbiotic bacteria

María J. Delgado¹, Ana Salas¹, Juan Cabrera¹, Maria J. Torres¹, Alba Hidalgo¹, Germán Tortosa¹, Socorro Mesa¹, Eulogio J. Bedmar¹, Lourdes Girard³, David J. Richardson², Andrew J. Gates²

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More than 60% of global nitrous oxide (N₂O) emissions are emitted from agricultural soils mainly by microbial nitrification and denitrification that are directly affected by soil nitrogen fertilization (1). In this context, biological nitrogen fixation by legume-rhizobia symbiosis is an economical and environmentally friendly agricultural practice to increase soil fertility and crop yield without adding N fertilizers. However, rhizobia species such as *Bradyrhizobium diazoefficiens*, the endosymbiont of soybeans, are also able to grow via denitrification that requires the *napEDABC*, *nirK*, *norCBQD* and *nosRZDYFLX* genes, encoding for nitrate, nitrite, nitric oxide and nitrous oxide reductases, respectively. It is well established that denitrification by *B. diazoefficiens* is the main process involved in NO and N₂O production in soybean nodules (2, 3). Nevertheless, a putative haemoglobin, Bjgb, that has been implicated in NO detoxification in free-living cells might also reduce NO to N₂O inside the nodules. Bjgb is encoded in a gene cluster that also codes for a number of proteins with important roles in nitrate assimilation including the large catalytic subunit of the assimilatory nitrate reductase (NasC), a major-facilitator superfamily-type nitrate/nitrite transporter, and a FAD-dependent NAD(P)H oxidoreductase (4). Recent results concerning the physiological role and regulatory link between nitrate assimilation and nitric oxide metabolism in *B. diazoefficiens* will be discussed.

Rhizobium etli CFN42 forms symbiotic interactions with *Phaseolus vulgaris* (common bean), another important legume crop in agriculture. Unlike *B. diazoefficiens*, *R. etli* lacks genes for respiratory nitrate reductases and nitrous oxide reductases and instead possesses only the *nirK* and *nor* genes. Here, we demonstrate that for *R. etli*, free-living cells, as well as common bean nodules are able to produce NO and N₂O from nitrate probably through the action of the *R. etli* assimilatory nitrate reductase and the NirK and cNor denitrification enzymes.

References

- (1) Torres et al. (2016) Adv Microb Physiol, 68, 353-433.
- (2) Sanchez et al. (2010) Mol Plant-Microbe Interact 23, 702-711.
- (3) Tortosa et al. (2015) Symbiosis 67, 125-133.
- (4) Cabrera et al. (2016) Biochem J. 473, 297-309.

Acknowledgements. This work was supported by ERDF co-financed grants AGL2013-45087-R from MINECO and PE2012-AGR1968 from Junta de Andalucía, and the International Exchanges 2014/R1 Royal Society Programme (Ref: IE140222). Continuous support from Junta de Andalucía to group BIO275 is also acknowledged.

Copper maturation of nitrous oxide reductase of *Paracoccus denitrificans*

Manuel J Soriano-Laguna, Matthew J Sullivan, Gary Rowley, David J Richardson and Andrew J Gates

School of Biological Sciences, University of East Anglia, Norwich Research Park, UK

Nitrous oxide (N₂O) is a very potent greenhouse gas and an intermediate produced during the respiratory reduction of nitrate by denitrifying soil bacteria. When extracellular copper concentrations are low, denitrification is interrupted and N₂O is the main product that is released. One of the main reasons for this is that nitrous oxide reductase (N₂OR), the only known enzyme capable of destroying N₂O, is highly dependent on copper. N₂OR is a functional dimer and each monomer contains 6 copper atoms divided into two different copper centers: the catalytic center CuZ and the electron transfer center CuA. The maturation of these two centers require the action of copper chaperones, these are proteins that binds copper and assist during the maturation of the active N₂OR. The *nos* gene cluster contains *nosDFYL* which encode proteins thought to be responsible for the maturation of the CuZ center. Meanwhile, a new gene cluster composed by a hypothetical gene, *pcuC* and *senC2* has recently been reported and is thought to be responsible for the maturation of the CuA center, which shares structural similarity to that present in the electron-transfer domain of cytochrome c oxidase. Here we report that disruption of any of these genes leads to the accumulation of N₂O by *P. denitrificans* cells, and the characterization of the biochemical properties of these proteins required to deliver copper to N₂O and O₂ dependent terminal reductases

Marinobacter hydrocarbonoclasticus* nitrous oxide reductase - The active form *in vivo

Cíntia Carreira¹, Rute F. Nunes¹, Olga Mestre¹, Isabel Moura¹, Sofia R. Pauleta¹

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Nitrous oxide is a very potent greenhouse gas that can be detoxified by nitrous oxide reductase (N₂OR), in a metabolic pathway named denitrification, during which nitrate is converted, via nitrite, to nitric oxide and nitrous oxide, and lately to the inert dinitrogen gas. Part of the atmospheric N₂O comes from the incomplete denitrification due to drop in pH in the environment^[1], as well as, anthropomorphic activities.

N₂OR has two copper centers, CuA responsible for electron transfer to the catalytic center, CuZ^[2]. This is a tetranuclear copper center that has been isolated as CuZ*(4Cu1S) or CuZ(4Cu2S). There has been a debate as which is the biological relevant form of the catalytic center, and in this work we demonstrate that when whole cells of *Marinobacter hydrocarbonoclasticus*, a model for marine microorganisms, are able to reduce nitrous oxide, N₂OR is isolated with CuZ as CuZ(4Cu2S). Nevertheless, the isolated enzyme has low specific activity and an activation mechanism is envisaged through a sulfur-shift.

The work presented here, include a study of the effect of environmental acidification on the denitrification pathway (with gene expression profiling and determination of metabolic intermediates), as well as, isolation of N₂OR from cells grown at different pHs. The results indicate an accumulation of NO₂⁻ and a very low rate of N₂O reduction by the whole cells at low pH.

Moreover, a proteomic approach identified differences in the respirasome of *Marinobacter hydrocarbonoclasticus*, which provide clues to explain why at low pH, N₂OR is isolated with its CuZ center as CuZ*(4Cu1S), and cells are not able to reduce N₂O. An activation mechanism for N₂OR is also proposed for CuZ(4Cu2S) to explain the high ability of the cells to reduce N₂O *in vivo*.

We thank FCT for financial support to CC (SFRH/BD/87898/2012) and to IM (PTDC/QUI-BIQ/116481/2010, PTDC/BBB-BQB/0129/2014).

[1] Saggar *et al* (2013) *Sci Total Environ* 465:173-95.

[2] Brown *et al* (2000) *Nature* 3(7):191-195.

“Forewarned- forearmed; to be prepared is half the victory” – respiratory survival strategies in *P. denitrificans*

Pawel Lycus¹, Manuel Soriano-Laguna², Andrew J Gates², David J Richardson², Linda Bergaust², Åsa Frostegård¹, and Lars R Bakken¹

1 Norwegian University of Life Sciences- Aas- Norway - 2 School of Biological Sciences- University of East Anglia- Norwich Research Park- Norwich NR4 7TJ- U.K.

Denitrification is a facultative trait that allows sustained respiratory growth in environments where oxygen tensions fluctuate. The enzymes driving nitrogen oxide reduction are expressed when oxygen becomes growth-limiting but their synthesis and assembly is energetically costly and there is no guarantee that anoxia will be prolonged. Therefore a sensible strategy for energy-limited microbes could be to allow a select sub-population to express denitrification proteins- e.g. nitrite reductase (NirS) in response to oxygen depletion. This would enhance overall population survival by securing anaerobic respiration for the fraction that carries NirS while minimizing energy costs for the fraction that does not. Upon reappearance of oxygen denitrification proteins could be preserved in sub-populations through generations of aerobic growth to serve in the event of another anoxic spell. Here we report such bet-hedging phenomena in the denitrifying bacterium *Paracoccus denitrificans*. Inspection of electron flow kinetics during oxygen depletion- combined with dynamic modelling approaches has previously revealed that initial transcription of the *nirS* gene is stochastic with a low probability ($r=0.5\% \text{ h}^{-1}$) consistent with only a fraction of cells switching to denitrification in response to oxygen depletion. Moreover- phenotypic data strongly suggest that- once developed- the denitrification proteome does not undergo degradation during aerobic growth; instead it is asymmetrically distributed among daughter cells ensuring the presence of a denitrification machinery in the population. Here we provide direct evidence for these two modes of cell differentiation by tracking NirS in *P. denitrificans* through generations of aerobic and anaerobic growth using a chromosomal NirS-mCherry reporter fusion.

Quorum sensing systems LasI-LasR and RhII-RhIR regulate N₂O reduction in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is a gamma-proteobacterium widely distributed in the environment. A number of strains are notorious causative agents of nosocomial infections in immunocompromised patients. One representative is PAO1, whose quorum sensing (QS) systems and regulons are well studied. It is also a full-fledged denitrifier, carrying all the enzymes necessary for the reduction of nitrate to N₂. *P. aeruginosa* uses a hierarchy of at least two different N-acyl homoserine lactone (AHL) QS systems, LasI-LasR and RhII-RhIR, where Las with a few exceptions is superordinate. Both circuits have been shown to transcriptionally regulate the denitrification machinery, but their influence on the organism's propensity to emit NO and N₂O during anaerobic growth has not been described. In a series of experiments addressing the denitrification regulatory phenotype (DRP) of a selection of *Ps. aeruginosa* strains, we observed that in PAO1, there was a strong positive correlation between cell density and N₂O accumulation during anaerobic growth. In contrast, a *lasI/rhII* deletion mutant kept N₂O at nM concentrations throughout the anoxic phase. Addition of N-butyryl-L-homoserine lactone (C4-HSL, sensed by RhIR) or N-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C12 HSL, sensed by LasR) to QS deficient cultures resulted in increased N₂O accumulation; in the 3-oxo-C12 HSL treated cultures to an extent comparable to wild type. This demonstrates that N₂O reductase is subject to repression through the QS systems, predominately the Las circuit, either directly through transcriptional regulation of NosZ, or via other factors in the QS regulon. This is interesting from an environmental perspective because, if it is a more general trait, it may have implications for the release of gaseous N-oxides e.g. from hotspots in soil or biofilms in wastewater. Detailed analyses of gas kinetics and transcription of denitrification genes in the PAO1 parent strain and the *lasI/rhII* deletion mutant are ongoing.

Genome-wide discovery of novel sRNAs in *Paracoccus denitrificans*: A potential strategy for greenhouse gas mitigation

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Nitrous oxide (N₂O) is a stable, ozone depleting greenhouse gas. Emissions of N₂O into the atmosphere continue to rise, primarily due to the use of nitrogen-containing fertilizers by soil denitrifying microbes. It is clear more effective mitigation strategies are required to reduce emissions. One way to help develop future mitigation strategies is to address the currently poor understanding of transcriptional regulation of the enzymes used to produce and consume N₂O. With this ultimate aim in mind we performed RNA-seq on a model soil denitrifier, *Paracoccus denitrificans*, cultured under high N₂O and low N₂O emitting conditions to identify small RNAs (sRNAs) with potential regulatory functions transcribed under these conditions. sRNAs are short (□40–500 nucleotides) non-coding RNAs that regulate a wide range of activities in many bacteria. 167 sRNAs were identified throughout the *P. denitrificans* genome which are either present in intergenic regions or located antisense to ORFs. Furthermore, many of these sRNAs are differentially expressed under high N₂O and low N₂O emitting conditions respectively, suggesting they may play a role in production or reduction of N₂O. Expression of 16 of these sRNAs have been confirmed by RT-PCR. 90% of the sRNAs are predicted to form significant secondary structures. Predicted targets include transporters and number of transcriptional regulators. A number of sRNAs were conserved in other members of the α-proteobacteria. In order to ascertain their biological role, a number of sRNAs were cloned into an over expression vector. Promising initial results suggests that over expression of some sRNAs results in altered N₂O emissions, suggesting that sRNAs play a role in the production and/or consumption of N₂O. Better understanding of the sRNA factors which contribute to transcription of the machinery required to reduce N₂O will, in turn, help to inform strategies for mitigation of N₂O emissions.

Copper detoxification/trafficking and Nitrous Oxide Reductase copper centre biogenesis in *Paracoccus denitrificans*

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Agricultural practices have presented large amounts of anthropogenic nitrogen to soil microbes over the past century, which has led to a significant rise in the emission of nitrous oxide (N₂O), a potent ozone depleting and greenhouse gas. The α -proteobacterium *Paracoccus denitrificans* (Pd1222) can use these nitrogen species as alternative electron acceptors under anaerobic conditions to survive, reducing nitrate stepwise to nitrogen gas in the process known as denitrification. The final step, yielding N₂ from N₂O, is catalysed by NosZ, a homo dimeric multi-copper enzyme. Copper deficiency leads to a loss of NosZ catalytic activity and thus N₂O emissions. Understanding how the copper metal centres of NosZ are assembled and the effect of copper on organisms that require this metal is an essential pre-requisite for efforts towards mitigating N₂O emissions.

Here, we report bioinformatic analysis of the *P. denitrificans* genome, revealing the absence of any common copper detoxification or trafficking system. It is noteworthy that both β - and γ -proteobacteria that synthesise NosZ do contain recognisable copper detoxification/trafficking systems. A global analysis of the transcriptome under sub-toxic copper levels is currently underway to identify any novel copper handling systems.

In addition, investigation of the roles of *nosL* and *nosX* present in the *nos* gene cluster reveal they are required for N₂O reduction in *P. denitrificans*. A strain lacking functional versions of these genes presents the same phenotype as a *nosZ* deletion with regard to culture growth and N₂O production. N₂O reduction in *nosLX* strains cannot be restored by additional copper and genetic complementation with *nosL*. NosL heterologously expressed in *E. coli* has the ability to bind copper, suggesting a potential role in metallo-centre biogenesis. A role for NosX and the individual contributions of NosL and NosX in biogenesis of NosZ will be the focus of future work.

Metatranscriptomics show high *nosZ* transcription but severely delayed N₂O reduction in acid soil

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The negative correlation between pH and N₂O emissions is well documented, but the reasons for this phenomenon are less well understood. In earlier studies of *Paracoccus denitrificans* and extracted soil communities, we demonstrated marginal N₂O reductase activity at pH ≤6.1 despite significant transcription of *nosZ*. This post-transcriptional effect of low pH was taken to explain the oft-observed correlation between pH and N₂O reduction in soils. It remains to be shown however, if this accounts for a wider range of denitrifying bacteria, since PCR primers only capture a fraction of the community. Moreover, attempts to investigate transcription in soil with lower pH has hitherto often been unsuccessful due to problems with mRNA extraction. We used an improved extraction protocol to analyse the metagenome/metatranscriptome (HiSeq) from two soils (pH 3.8 and 6.8) during anoxic incubation in the presence of nitrate. As expected, the pH 6.8 soil showed immediate onset of N₂ production, while the acid soil produced N₂ only after 40 hours. The *nosZ* genes were 1.4 times more abundant in the neutral than in the acidic soil. *nosZ* gene transcript levels quadrupled from 0.5 to 3 h in the neutral soil. In the acidic soil, *nosZ* transcript levels remained the same during the same period, and was comparable to the neutral soil throughout the experiment. This, and lack of N₂ production, strongly supported our hypothesis of a post-transcriptional effect of low pH on the N₂OR. *nirK* genes were >10 times more abundant than *nirS* in both soils, contradicting our qPCR results, thus indicating a narrow range for existing *nirK* primers. Proteobacteria was a major denitrifying bacterial class present in the soil, and highly active in both soils. Ongoing work with denitrification gene sequence assembly, linking genes/transcripts to organism groups, will be presented.

Exploring the mechanism of biological hydrazine synthesis

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Anaerobic ammonium-oxidizing (anammox) bacteria are ubiquitously present in nature and contribute substantially to the global release of dinitrogen gas (N_2) to the atmosphere. One of the biological novelties of the anammox process is the production of the reactive intermediate hydrazine (N_2H_4) which gets subsequently oxidized to N_2 yielding the necessary electrons for energy conservation. The protein complex catalyzing the formation of hydrazine from nitric oxide (NO) and ammonium (NH_4^+) hydrazine synthase (HZS)- has been purified to homogeneity as a dimer of heterotrimers ($\alpha\beta\gamma$)₂ and crystallized at 2.7 Å resolution. Our working model for hydrazine synthesis comprises two half reactions facilitated by two heme c active sites. First- NO undergoes a three-electron reduction to hydroxylamine (NH_2OH) at the active site of the subunit (heme I). Hydroxylamine is then transferred to the α -subunit through an intraprotein tunneling system that is presumably regulated by a short amino acid stretch of the β -subunit. The condensation of ammonia (NH_3) and NH_2OH to the end product N_2H_4 occurs at the second active site (heme al). Interestingly- the analyses of the HZS structure revealed several particularities with regards to the architecture of the active site hemes. The subunit is structurally similar to cytochrome c peroxidase and methylamine utilization protein G but displays a third covalent attachment between a Cysteine residue and the porphyrin ring of the I heme moiety. Heme al also differs considerably from the canonical heme c site- as the histidine residue of the CXXCH heme binding motif does not coordinate the heme iron- but a Zinc ion instead. The fifth ligand of heme al appears to be a Tyrosine- resembling the active site of catalases. To gain further insights into the unusual- yet important mechanism of biological hydrazine synthesis- electrochemistry- electron paramagnetic resonance (EPR) and magnetic circular dichroism (MCD) are currently being employed.

Disparate response to microoxia, FixK₂ and NnrR of *Bradyrhizobium diazoefficiens* *norCBQD* and *nosRZDYFLX* genes

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Bradyrhizobium diazoefficiens (formerly *Bradyrhizobium japonicum*) is a gram-negative soil bacterium associated symbiotically with soybean plants. This bacterium is able to growth via denitrification which depends on the *napEDABC*, *nirK*, *norCBQD* and *nosRZDYFLX* genes, encoding for nitrate, nitrite, nitric oxide and nitrous oxide reductases, respectively. In this bacterium, the regulatory network and environmental signals controlling *nor* genes involved in N₂O synthesis have been unravelled. However, much remains to be discovered regarding the regulation of the key enzyme to mitigate N₂O emissions, the respiratory N₂O reductase. In this work, we have demonstrated that the *B. diazoefficiens* *nosRZDYFLX* genes are co-transcribed from a major promoter located upstream of the *nosR* gene. Two transcriptional start sites were determined within the *nos* 5' untranslated region (UTR) by using the Rapid Amplification of cDNA 5' Ends (RACE) method. Expression analyses from a *nosR-lacZ* fusion in *B. diazoefficiens* wildtype and *fixK₂* and *nnrR* mutant backgrounds showed that low oxygen (microoxia) was sufficient to induce *nos* genes compared to oxic conditions, and that is FixK₂ the main regulator involved in such control. In contrast, *nor* expression was unresponsive to microoxia but it requires also nitric oxide (NO), and both FixK₂ and NnrR transcription factors, being NnrR the candidate to directly interact with the *norCBQD* promoter. These results suggest a disparate response of *B. diazoefficiens* *nor* and *nos* denitrifying genes with regard to their dependency on microoxia, and the regulatory proteins FixK₂ and NnrR. In this control, microoxic expression of *nor* genes requires NO and directly depends on NnrR, while microoxia and FixK₂ are the main factors involved in *nos* genes expression.

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Using Vertebrate Globins to Understand the Mechanistic Basis of Heme Catalysed Nitrite Reduction

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Some elements of the nitrogen cycle, notably the nitrate-nitrite-nitric oxide pathway, play an important role in human physiology and provide an alternative source of the key signaling molecule NO under anaerobic conditions. Dietary nitrate reduced to nitrite by commensal anaerobic bacteria in the oral cavity and gut is absorbed into the circulatory system. Under hypoxic conditions, nitrite is further reduced to NO by a number of redox enzymes the most significant of which is myoglobin; a structurally well-defined 17 kDa monomeric heme protein usually as a considered to be a short-term O₂ reservoir that sustains respiration.

The ability to engineer and produce large amounts of pure recombinant sperm-whale myoglobin (SWMb) makes it an attractive subject for mechanistic studies of nitrite reduction and in particular understanding the nature of the collaboration between the heme cofactor and the protein environment. Substitution of the conserved histidine (His-64) in the heme distal pocket each of five different amino acids leads to decreased rates of nitrite reduction. Conversely replacement of the proximal histidine ligand (His93) with lysine, which normally serves as the proximal ligand to the active site heme in the penta-heme nitrite reductase NrfA, enhances the rate of nitrite reduction.

To understand the basis of these observations we used we used a combination of stopped-flow methods, magnetic circular dichroism and EPR spectroscopies to study the binding of nitrite to oxidized wild-type SWMb and the six variants. Our data is consistent with a model in which the reactive species is free nitrous acid (HNO₂), His-64 directs binding of the substrate in the nitrito conformation and a requirement for a bound water molecule in the distal pocket to facilitate nitrite reduction.

Transcriptional and translational adaptation to aerobic nitrate anabolism in *Paracoccus denitrificans*

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A proteomic approach of second generation (LC-MS/MS) has been applied to *Paracoccus denitrificans* PD1222 cells grown under different oxygenic conditions with nitrate as sole nitrogen source. Ammonium was used as alternative nitrogen source in the aerobic growth or ammonium plus nitrate in the anaerobic cultures. A large number of proteins with a regulatory or structural role have been identified and a comparative bioinformatics analysis has been carried out. One of the process studied in more detail has been the assimilation of nitrate in the strain PD1222. Data from the proteomic analysis related with the nitrate assimilation metabolism in *P. denitrificans* have been compared with a previous DNA microarray analysis generated to analyze the response to nitrate *versus* ammonium under aerobic conditions. The assimilation of nitrate in *P. denitrificans* was positively regulated by nitrate through the *nasTS* regulatory genes. The nitrate and nitrite transporters and the nitrate and nitrite reductases are encoded by the *nasABGHC* genes. The *nasTSABGHC* genes were detected independently of the nitrogen source tested, except with ammonium that was a repressor, although NADH-dependent nitrate reductase activity was detected only when nitrate was present. Mutagenic and phenotypic analysis of the *P. denitrificans* *nas* genes have been performed and a function to each gene has been assigned. The ammonium repression of *Nas* was exerted through the *Ntr* system in *P. denitrificans* PD1222 as corroborated by mutational studies.

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Regulation of *Desulfovibrio desulfuricans* response to nitrosative stress by HcpR1 and HcpR2.

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We recently reported that, in *Escherichia coli*, the hybrid cluster protein, Hcp, is a high affinity nitric oxide reductase. Synthesis of Hcp requires a functional Fnr protein, so Hcp accumulates only during anaerobic growth. Its synthesis is further induced under conditions of nitrosative stress when the NsrR repressor protein is inactivated.

Many sulphate reducing bacteria encode proteins of the Crp-FNR family. One of them is HcpR, which regulates *hcp* expression in free-living bacteria such as *D. vulgaris*. Unlike *D. desulfuricans*, *D. vulgaris* is unable to reduce nitrate. The genome of *D. desulfuricans* encodes two copies of HcpR that differ significantly in primary sequence. Structures of the *D. desulfuricans* *hcpR1*, *hcpR2* and *hcp* operons will be reported. The *hcpR1* gene is located distantly from the *hcp* gene, but both genes are induced by nitrosative stress during growth in the presence of nitrate, nitrite or nitric oxide (NO). In contrast, the *hcpR2* gene is located close to *hcp*, but HcpR2 synthesis is not induced by nitrosative stress. HcpR1 is likely to be a *b*-type cytochrome. HcpR2 is an iron-sulfur protein, and therefore able to bind nitric oxide. We propose that HcpR1 and HcpR2 use different sensory mechanisms to regulate nitrate reduction and the response to nitrosative stress. We also note a correlation between the presence of two copies of *hcpR*, the ability to reduce nitrate, and ability to survive in the human body. We speculate that a second copy of *hcpR*, possibly together with the *hcp* gene, was transferred laterally into *D. desulfuricans*. Subsequently the two copies of *hcpR* have evolved to fulfil independent regulatory roles in nitrate reduction and the response to nitrosative stress. Diversification of signal perception and DNA recognition by these two proteins would enable *D. desulfuricans* to adapt to its particular environmental niche.

Microbes living on N₂O respiration: a chemostat study

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Nitrous oxide (N₂O), a byproduct and intermediate of a variety of microbial nitrogen transformations in the environment, is a potent greenhouse gas. However it is also a powerful electron acceptor, making N₂O reduction to more environmentally innocuous compounds such as N₂, thermodynamically, a very favorable reaction.

N₂O reduction to N₂ catalyzed by N₂O reductases, present in a wide range of microorganisms, is to date the only known biological N₂O “sink”. Favoring the growth and presence of N₂O reducing microorganisms could be a promising mitigation strategy for ecosystems with a high nitrogen turnover (e.g. agricultural soils and wastewater treatment plants). Thus, there is an increasing interest to learn more about the ecophysiology of N₂O reducers.

Here we describe work with an N₂O reducing enrichment in a chemostat, inoculated with activated sludge, fed with N₂O as a sole electron acceptor and acetate as an electron donor and carbon source (pH 7 and 20°C).

Enrichment and growth of the culture- dominated by γ and β Proteobacteria with NosZ type I- for a great number of generations proved that N₂O respiration can fully sustain the bioenergetic needs of a cell, with a bioenergetic efficiency of growth comparable to that of full denitrification. Interestingly, if the culture was grown under N₂O limiting conditions biomass yields were significantly higher than during acetate limiting conditions. We speculate that this is caused by cytotoxicity of the high levels of N₂O present during the acetate limiting conditions.

Evaluating the potential for nitrate reduction through DNRA by anammox bacteria for municipal wastewater treatment

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To shift currently energy-intensive wastewater treatment plants (WWTPs) towards energy neutrality, the implementation of the anammox technology in the mainstream of WWTPs for enhanced nitrogen removal is recommended. However, the lower temperature and ammonium concentration in the mainstream make it difficult to suppress the process disturbing nitrite oxidizing bacteria (NOB), which compete with anammox bacteria for nitrite. In addition, high organic matter content (COD) in the mainstream promotes the growth of the heterotrophs and will lead to the washout of the slow-growing autotrophic anammox bacteria if nitrogen is relatively low (high COD/N ratio). One important discovery besides the conventional anammox conversion (combination of ammonium and nitrite to nitrogen gas) is that anammox bacteria have the capacity to perform dissimilatory nitrate reduction to ammonium (DNRA) with nitrite as intermediate, coupled to the oxidation of volatile fatty acids (VFAs). This pathway allows anammox bacteria to utilize the VFAs in the mainstream and reduce the excess nitrate produced by NOB and/or anammox bacteria itself. The objective of this research is to gain understanding of the DNRA metabolic pathway of anammox bacteria. In this study, batch tests with anammox and heterotrophic bacteria showed the capacity of *Candidatus* 'Brocadia fulgida' to perform the DNRA coupled to the anammox reaction (DNRA-anammox) without prior adaption to VFA. In addition, thermodynamic calculations revealed that low influent COD/N ratios may favour the DNRA-anammox transformation over heterotrophic conversions since more Gibbs free energy is gained per mole of VFA (1984 kJ acetate-mol⁻¹ during DNRA-anammox vs. 797 kJ acetate-mol⁻¹ during heterotrophic denitrification). Furthermore, we suggest the implementation of an innovative nitrogen removal system in which the nitrate produced by NOB and/or anammox bacteria is converted during the DNRA-anammox pathway, resulting in sustainable nitrogen removal from municipal wastewater in which NOB do not compete with anammox but supply them with substrate.

Phylogeny vs denitrification phenotype; regulatory plasticity across a selection of *Pseudomonas stutzeri* strains

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Many aerobic bacteria carry out denitrification as an alternate respiratory mechanism when oxygen is transiently not available. They conserve energy by the partial or complete reduction of nitrate or nitrite to N₂. Some denitrifiers accumulate high concentrations of one or several intermediates, before their further reduction. The pattern of intermediate accumulation is characteristic of an organism, and a trait encompassed by the denitrification regulatory phenotype (DRP). It could have implications for the final products ultimately released into the atmosphere, and it also raises questions regarding the ecology and evolution of these organisms.

The γ -proteobacterium *Pseudomonas stutzeri* is ubiquitous in the environment, and it is a model organism for our understanding of the regulation and biochemistry of denitrification. More recently, research has expanded to look at a broader diversity of strains and their metabolic pathways. We have studied growth of a selection of *P. stutzeri* strains during the transition from aerobic respiration to denitrification. The results show that the DRP varies greatly at the strain level, particularly with respect to the accumulation of nitrite. There are two groups with some phylogenetic support, of which one accumulates significant amounts of nitrite, while the other strains maintain very low nitrite concentrations. The nitrite accumulators can in turn be divided into those that simultaneously reduce nitrate and nitrite and those that show delayed reduction of nitrite, only after significant accumulation of the intermediate. The growth kinetics of this final group indicate a gradual recruitment of bacterial cells to expressing nitrite reductase (NirS), leading to a subdivision of clonal cultures into NirS⁺ and NirS⁻ sub-populations. These findings illustrate a surprising regulatory plasticity among strains that carry highly similar denitrification machineries.

First physiological study on NO_x emissions in haloarchaea: the case of *Haloferax mediterranei*.

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Haloarchaea are extremophiles inhabiting saline and hypersaline media such as salt lagoons and salty ponds. Knowledge about denitrification in these microorganisms is scarce. The few studies executed so far have dealt with biochemical aspects of the pathway.¹ However, to date there are no physiological experiments on the emission of nitrogenous gases (NO_x gases) nitric oxide (NO) and nitrous oxide (N₂O) by the haloarchaea.

This work is the first comprehensive study on NO_x emissions in real time from an extremophilic archaeon, the haloarchaeon *Haloferax mediterranei*. Using an automated incubation system, we have studied the kinetics of all gases related to the denitrification pathway (CO₂, O₂, NO, N₂O, N₂)² under a range of conditions. The results confirm that *Hfx. mediterranei* is a complete denitrifier, capable of using nitrate and nitrite as final electron acceptors and reduce them to N₂. Under standard conditions (1% initial O₂ in headspace and 1-2 mM nitrate or nitrite), transition from aerobic to anaerobic respiration appeared smooth, with no dramatic drop in electron flow. All the available nitrate or nitrite was recovered as N₂ with transient accumulation of nM concentrations of NO and N₂O. However, anoxic cultures were sensitive to high concentrations (> 5 mM) of nitrite, which resulted in growth arrest due to accumulation of toxic levels of NO.

Considering the large areas that haloarchaea inhabit on Earth and the increase of nitrates and nitrites concentrations in these media, these results propose the haloarchaea as new group related to NO_x emissions and climate change.

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Ecophysiology of denitrifying bacteria; regulatory networks and phenotypic plasticity

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Details of the enzymatic mechanisms involved in denitrification are to date only known for a few model organisms, but yet reveal a surprisingly large diversity in the organization of the regulatory networks of this process. This in turn leads to large variations in denitrification phenotypes even between closely related bacteria, seen as differences in the timing of the onset of denitrification in response to O₂ depletion, and in the accumulation of intermediate and end products. During this talk I will give examples from our recent research showing how transcriptional regulation as well as post-transcriptional phenomena and metabolic control mechanisms generate a variety of denitrification phenotypes, further affected by abiotic factors such as pH and temperature. I will also discuss the relevance of such ecophysiological studies of denitrifying bacteria, taken into account the challenges encountered when trying to extrapolate results from pure cultures to complex microbial communities to understand how different regulatory phenomena affect the propensity of a soil to emit NO and N₂O.

Arctic peat circles host a remarkable denitrifier community capable of N₂O consumption at acidic pH

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Permafrost-affected acidic peat circles (PC) in the Eastern European Tundra harbor up to 2 mM of nitrate and are 'hot spots' for denitrification derived N₂O emissions. Denitrification potentials for PC were high, and N₂O/(N₂+N₂O) ratios approximated 100% within the first 24 hours of incubation under anoxic conditions at pH 4. N₂O consumption started at pH 4 after 24 h. N₂O/(N₂+N₂O) ratios approximated 0 when PC soil that was pre-incubated for 8 days under anoxic condition was supplemented with nitrate. Supplemental [¹³C]-acetate stimulated ¹³[C]O₂-production and denitrification potentials in the presence of nitrate. ¹³[C]O₂ represented 80% of total CO₂, suggesting that acetate was preferred over endogenous electron donors. Delineated recovery of nitrate N in N₂ ranged from 60-100%, which was in agreement with marginal concentrations of fermentation products, NH₄⁺, Fe²⁺ and initial SO₄²⁻ during the incubation time, suggesting that denitrification was the major electron sink. Incubations at pH 6 yielded similar results, although denitrification rates were higher than at pH 4. Amplicon sequencing of nitrate and nitrous oxide reductase genes concomitant to isolation studies indicated the presence of *Actinobacteria*, *Alpha-*, *Beta-*, and *Gammaproteobacteria* as well as environmental sequences. *Rhodanobacter denitrificans* is a model acid-tolerant, gram negative denitrifier of the *Gammaproteobacteria*, which is capable of complete denitrification at pH 3. *R. denitrificans* was thus chosen to study genes potentially involved in denitrification. *R. denitrificans* was incubated under oxic and micro-oxic/denitrifying conditions with electron donors. *In vitro* subtractive transcriptomics suggested stimulated expression of putative oxygen and nitrate sensors distantly related to those already known, unusual denitrification genes, and genes of unknown function under denitrifying conditions. The collective data indicate that PC denitrifiers are limited by easily available carbon limited, capable of complete denitrification under acidic conditions, and that unusual genes are utilized by organisms capable of N₂O reduction at low pH.

The consequences of ammonia oxidiser niche specialisation for mitigation of nitrous oxide emissions

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While the activities of both ammonia oxidising bacteria (AOB) and archaea (AOA) contribute to nitrous oxide (N₂O) emissions from aerobic soil, N₂O yields of AOA are lower, suggesting lower soil N₂O emission when ammonia oxidation is dominated by AOA rather than AOB. There is evidence that AOB growth is favoured at high ammonia supply, while AOA are favoured at low concentrations of ammonia, derived from mineralised organic nitrogen. However, the influence of ammonia concentration on AOA and AOB has not been tested rigorously. In this study, we tested the hypothesis that ammonia supplied continuously at a low concentration is preferentially oxidised by AOA, thereby generating a lower yield of N₂O per nitrate produced than would be expected by an AOB dominated process. A nitrogen fertiliser, with fast and slow release fractions, was added to soil microcosms providing an initial fast release, followed by a prolonged slow release of ammonium. The addition of acetylene (which inhibits both AOB and AOA) or 1-octyne (which inhibits only AOB) was used to distinguish AOA and AOB growth, activity and associated N₂O emissions. DNA-stable isotope probing with ¹³CO₂ was performed to identify active growing ammonia oxidisers. Following complete oxidation of ammonia from the fast release fraction, the rate of oxidation of ammonia from the slow release fraction was significantly higher than that of ammonia derived only from endogenous mineralisation. AOB dominated depletion of the initial high ammonium supply, whereas AOA dominated growth and activity during the slow release phase. This second phase was associated with N₂O yields that were similar to those when ammonia was derived from mineralisation only, and half of those when AOB dominated ammonia oxidation. The results therefore inform fertilisation strategies and suggest potential N₂O mitigation strategies based on different physiologies of AOA and AOB.

Eco-Evo studies of ammonia oxidisers: questions, surprises and partial answers

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The phenomenon of nitrification in acidic soils is of economic and environmental significance. A high proportion of soils are acidic, many of these are arable soils and high rates of nitrification in these soils reduce nitrogen fertiliser use efficiency and generate substantial nitrous oxide. Paradoxically ammonia oxidising bacteria, cultivated using traditional methods, cannot grow in liquid batch culture below pH 7, even when isolated from acid soils. Ecophysiological studies provided partial explanations, through protection in biofilms and ureolytic growth, but analysis of natural ammonia oxidiser communities, and potential selection for acidophiles, was impossible before application of molecular techniques. 16S-rRNA gene- and functional (*amoA*-) gene-based studies provided evidence for high diversity of ammonia oxidisers and evidence for pH-associated selection of bacterial ammonia oxidisers and, following their discovery, of archaeal ammonia oxidisers. More importantly, there was evidence for greater activity of ammonia oxidising archaea than bacteria at low pH and an obligate acidophilic archaeal ammonia oxidiser was subsequently isolated. This organism belongs to a phylotype that is globally distributed in acid soils and genomic and comparative genomic studies are suggesting mechanisms for acidophilic growth in archaea, and its absence in ammonia oxidising bacteria. These studies therefore addressed one of two major questions arising from our recently acquired ability to study microbial community ecology: so what? That is, does community composition matter for ecosystem function. They are also now being used to address the second question: why? That is, why is microbial diversity so high? *amoA* sequence data are being used to determine the role of pH in the evolution of archaeal ammonia oxidisers and to estimate rates of diversification. The results of these studies and the implications for other microbial groups and ecosystem processes will be considered, along with lessons to be learnt from application of different approaches.

EFFECT OF SETTLED DIATOM-AGGREGATES ON BENTHIC NITROGEN CYCLING

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The marine sediment hosts an unresolved mosaic of microenvironments. Algae-aggregates settling from the water column have been recently shown to contribute in altering the benthic O₂ topography by locally supplying organic material and limiting O₂ diffusion. Our data show how this microscale O₂ re-zonation profoundly influences key processes in benthic nitrogen cycling. In sediment flow-through incubations, the settlement of diatom-aggregates (diam.: 2-5 mm; density: 1 aggregate x 2 cm⁻² sediment) enhanced benthic O₂ and NO₃⁻ consumption and concurrent NO₂⁻ and NH₄⁺ production. Oxygen microprofiles revealed that the sediment anoxic zone rapidly rose, and anoxic niches temporarily developed within the aggregates. During 120 hours following the addition of the aggregates, denitrification of NO₃⁻ from the overlying water increased from 13.5 to 24.3 μmol N m⁻² h⁻¹, as quantified in an ¹⁵N stable isotope enrichment experiment. At the same time, the coupling between nitrification and denitrification decreased (from 33.4 to 25.9 μmol N m⁻² h⁻¹), probably due to a temporal inhibition of the nitrifying community in the sediment. At the aggregate surface, on the contrary, nitrification was very efficient in oxidizing NH₄⁺ liberated by mineralization of the aggregates. The produced NO₃⁻, however, was preferentially released into the water column (favoured by the elliptic geometry of the aggregates) and only a minor fraction (<6 %) contributed to the denitrification activity. All in all, our results indicate that the abrupt change in O₂ microdistribution caused by aggregates can decouple nitrification and denitrification both in time and space. Because of their ephemeral nature, microniches induced by settled aggregates may have been overlooked factors in regulating important processes of the benthic nitrogen cycle.

Investigation of the different routes of nitrogen reduction in denitrification beds as a function of carbon source and microbiome

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Field denitrification beds that contain polymeric plant material are increasingly used to eliminate nitrate from agricultural drainage waters. Still, knowledge regarding the microbial composition, potential synergistic effects, and the overall carbon degradation process of these systems is sparse. This study indicates that while migrating through drainages, agricultural waste waters are metabolized differently under warm and cold conditions. While fertilizers contained ammonium and nitrate, ammonium was the main nitrogen compound in the drainage waters during the warmer summer months. This is a challenge for these bed systems since they are constructed as bioreactors for the denitrification, rather than the nitrification, of nitrogen compounds. Interestingly, wood chips as a carbon source of denitrification reactors sustained the development of an archaeal community consisting of methanogenic species and members of the Thaumarchaeota phylum, while archaea were not present in bioreactors operated with wood pellets. Wood chips select for the development of a diverse microbial community that is especially suited to the removal of low nitrate concentrations. Under these conditions there seems to be a division of labor, with Clostridia inhabiting the wood chip surface and degrading the polymeric material, and Proteobacteria—primarily localized in the planktonic phase—most likely using fermentation end products as electron donors for denitrification. Interestingly, higher nitrate concentrations can lead to an activity switch from denitrification to dissimilatory reduction of nitrate to ammonium (DNRA). This switch was not significantly observed in reactors operated with wood pellets, in which a stable denitrification process occurred even at high nitrate loading rates.

Effects of nitrogen fertilization on soil N₂O reducing bacteria and their importance for mitigating N₂O emissions

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Arable soils are a major source of nitrous oxide (N₂O). The only known sink in Earth's biosphere is the reduction of N₂O to N₂ via the N₂O reductase encoded by the *nosZ* gene, identified as *nosZ* clade I and II. The *nosZI* gene is mainly found among denitrifying bacteria, whereas >50% of microorganisms with *nosZII* lack other denitrification genes. The abundance and phylogenetic diversity of *nosZII* was previously shown to correlate with a soils N₂O sink capacity. To provide a proof of principle, we manipulated 11 indigenous soil microbial communities by increasing the abundance of a non-denitrifying N₂O-reducing strain capable of growth with N₂O as the only electron acceptor. Consequently, potential N₂O emissions were lowered in 1/3 of the soils by 51% in average and the magnitude was significantly influenced by the soil pH and C/N ratio. These results provide direct evidence that non-denitrifying N₂O-reducing bacteria can contribute to lowering the net N₂O production in soil, which indicates that net N₂O emissions are ultimately determined by the relative abundance of specific bacterial taxa with and without *nosZ*. The relative abundance of these bacterial taxa is known to be influenced by fertilization. Hence, we investigated predictable effects of N fertilizer on microbial communities across 14 Swedish long-term field trials, each with fertilized and non-fertilized treatments. The 16S rRNA and *nosZ* genes were sequenced, the abundance of denitrification genes quantified and the potential denitrification and N₂O production rates determined. The genetic potential for denitrification correlated with the potential activity and high *nosZ/nir* gene ratios coincided with lower N₂O:N₂ emission ratios. The *nosZII*, but not *nosZI*, community was significantly affected by fertilization and the phylogenetic diversity of *nosZII* correlated negatively with the N₂O:N₂ emission ratio. The community data remains to be further analysed to identify factors influencing the overall bacterial and N₂O reducing communities.

Large NO and HONO emissions from biological soil crusts regulated by drying-wetting cycles and temperature

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Biological soil crusts (biocrusts), which are soil surface communities dominated by cyanobacteria, algae, lichens or bryophytes, significantly affect hydrological processes such as runoff generation and evaporation, reduce soil erosion and exert a significant effect on C and N cycling in arid and semiarid ecosystems. They can fix nitrogen from the atmosphere, which is used by the organisms, leached into deeper soil strata, or released into the atmosphere as ammonia (NH₃), nitrous oxide (N₂O) and dinitrogen (N₂) gases. Here, we show that the fixed nitrogen can also be lost as nitric oxide (NO) and nitrous acid (HONO) gases. Based on laboratory, field, and satellite measurement data, we obtained a best estimate of ≈ 1.7 Tg per year for the global emission of reactive nitrogen from biocrusts (1.1 Tg a⁻¹ of NO-N and 0.6 Tg a⁻¹ of HONO-N), corresponding to $\approx 20\%$ of global nitrogen oxide emissions from soils under natural vegetation (Weber et al., 2015). In follow-up studies, we found that during subsequent wetting and drying cycles, the NO and HONO fluxes from biocrusts increased during the 2nd and kept stable in the following cycles. Temperature treatment during a wetting and drying cycle caused NO and HONO emissions to increase at temperatures up to 45°C and to be decreased at 60°C during subsequent measurements. As NO and HONO contribute to hydroxyl radical and oxidizing capacity of the lower atmosphere, the role of biocrusts in atmospheric nitrogen cycling should be further investigated.

Reference

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Insights into sulphonium and nitrogenous osmolyte production in model marine microorganisms.

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It is vitally important for marine microorganisms to produce compatible solutes. Two of the most commonly produced marine osmolytes are the nitrogenous quaternary amine betaine and the sulphonium compound dimethylsulphoniopropionate (DMSP). These molecules are also important sources of dissolved organic N and S in the oceans. The microbial catabolism of these molecules has wide ranging environmental consequences - from signalling pathways to potential climate regulation. Despite the importance of these molecules in the marine environment very little is known about how these molecules are produced and how these synthetic processes are regulated. Here we will present our recent findings from work on model algae and bacteria. This work identifies the key genes involved in the production of betaine and DMSP. Our studies on gene regulation clearly show that both betaine and DMSP biosynthetic pathways are controlled by the availability of N amongst other environmental factors.

Selenite reduction by *Thauera selenatis*: Does nitrite reductase play a role?

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The bacterial reduction of selenium oxyanions and the biogenesis of selenium nanoparticles (SeNPs) are currently of significant interest [1]. Historically, the similarities between the biogeochemical cycles for nitrate and selenate (SeO_4^{2-}) reduction have been highlighted [2], but the fact that further reduction of selenite (SeO_3^{2-}) to selenium (Se^0) results in an insoluble respiratory product has necessitated that the mechanisms for the bacterial reduction of selenite and nitrite could be managed quite differently.

Thauera selenatis is a Gram negative α -proteobacterium that is capable of respiring selenate due to the presence of a distinct periplasmic selenate reductase (SerABC). SerABC is a molybdoenzyme that catalyses the reduction of selenate to selenite by accepting electrons from the Q-pool via a di-heme c-type cytochrome (cytc4) [3]. Selenite is not a respiratory substrate and cannot support growth under anaerobic conditions. It has been suggested that the nitrite reductase could catalyse the reduction of selenite, since a mutant deficient in nitrite reductase activity failed to generate selenium when grown on selenate. However, more recent work has established that selenite reduction is more likely to occur in the cytoplasmic compartment as this is where elemental selenium deposits are routinely observed [4].

The reduction of selenite forms SeNPs that are ultimately secreted into the surrounding medium. In *T. selenatis* the secretion of the SeNPs is accompanied by the export of a ~95 kDa protein SefA. SefA functions to stabilise the formation of the SeNPs prior to secretion; possibly providing reaction sites for creation or providing a shell to prevent subsequent Se aggregation. In the present work we have investigated the role of the SefA protein in SeNP assembly and probed the transcriptome in order to shed light on possible selenite reduction mechanisms.

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Exploring methylamine metabolism using stable isotope probing

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Stable isotope probing (SIP) is a cultivation-independent technique which can link the phylogeny and function of microorganisms in the environment. It has been used effectively to determine “which microbes are doing what” in the environment in a number of biogeochemical cycles such as the utilization of one-carbon (C1) compounds in the terrestrial and marine environment and also in the nitrogen cycle, specifically N₂ fixation. Combined with metagenomics, DNA-SIP can also be used in focussed metagenomics experiments to retrieve the genomes of uncultivated bacteria carrying out specific steps in biogeochemical cycles. Considerations of experimental design and implementation of DNA-SIP for cycling of methylotrophic compounds such as methanol and methylamine will be outlined. In the case of methylamine, this compound can be used as both a carbon and energy source by methylotrophic bacteria and as a nitrogen source by non-methylotrophs. ¹⁵N and ¹³C labelling experiments combined with DNA- and Protein-SIP can differentiate between bacteria that carry out these different modes of metabolism in coastal marine environments.

Abstracts for Posters

1. Biochemical and genetic analysis of DNA-ligases in *Paracoccus denitrificans*

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DNA ligase is involved in the final step of most cellular processes that manipulate DNA. The main role of the DNA ligase is in forming the phosphodiester bonds within the DNA backbone. DNA ligase performs this process utilizing either of two different cofactors: NAD⁺ is used by enzymes found in bacteria and ATP by enzymes from all other organisms, although the two type of enzymes can be found together in some forms of bacteria. Based on previous studies, NAD⁺-dependent-ligase is an essential enzyme for bacteria, whereas the ATP-dependent-ligase is essential in all other organisms. Analysis of the *Paracoccus denitrificans* genome led to identification of two potential genes for DNA ligases, Pden4083 for NAD⁺-dependent-ligase and Pden0412 for ATP-dependent-ligase.

Biochemically, the expressed protein from Pden4083 was purified and confirmed that it is an NAD⁺-dependent-ligase by ligation assay. On the other hand, sonication and use of protein extraction reagent were not able to solubilize recombinant protein expressed from Pden0412. So, the future work will be on expression of Pden0412 in *P. denitrificans* and protein denaturation and resolubilisation.

Genetically, although previous studies indicated that NAD⁺-dependent-ligase is an essential gene for bacteria, it has been knocked out from *P. denitrificans*, with no effect on growth in LB media. Further studies will be done to attempt deletion of both genes from *P. denitrificans* and analysis of the growth of mutant strains under different conditions.

2. A molecular genetics approach to reveal the catalytic properties of the assimilatory nitrate/nitrite reductase

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Many bacteria can use nitrate (NO_3^-) or nitrite (NO_2^-), which are readily available in agricultural soils, as the sole source of nitrogen for growth. In the soil bacterium *Paracoccus denitrificans* PD1222, NO_3^- -dependent and NO_2^- -dependent growth is observed under aerobic or anaerobic conditions when ammonium (NH_4^+) levels are low [1]. The molecular machinery for assimilatory $\text{NO}_3^-/\text{NO}_2^-$ reduction is encoded by the *nasABGHC* operon, which is under the regulatory control of the $\text{NO}_3^-/\text{NO}_2^-$ -responsive system NasS-NasT [2].

During $\text{NO}_3^-/\text{NO}_2^-$ -dependent growth, NO_3^- and NO_2^- are first imported to the cytoplasm via inner-membrane importers NasA and NasH, respectively. The NO_3^- reductase NasC and NO_2^- reductase NasBG then perform the sequential, and NADH-dependent, conversion of NO_3^- to NO_2^- and NO_2^- to NH_4^+ for incorporation into glutamate and cellular biomass [2]. NasC receives electrons from NasBG and sequence analysis reveals that it is distinct from classical NO_3^- reductases, as it contains an extended C-terminal region with the capacity to bind an additional iron-sulphur cluster [2]. Isolation of the assimilatory NO_3^- reductase and subsequent biophysical and kinetic characterisation are therefore essential to understanding the function of this system [1, 2].

In this study, a platform for overexpression of the NasC metalloenzyme has been developed by construction of an unmarked *nasC* deletion strain ($\Delta nasC$), expressing an affinity-tagged version of NasC from an inducible plasmid in *P. denitrificans*. Growth analysis reveals that the $\Delta nasC$ strain is unable to grow with NO_3^- , thus NasC is essential for assimilatory NO_3^- reduction. However, soluble cell extracts from $\Delta nasC$ cells grown with NO_2^- may retain methylviologen-dependent NO_3^- reductase activity. Preliminary data for purification and kinetic characterisation of NasC is presented, as is an extended strategy for investigation of the maturation and potential cross-talk between the different NO_3^- reductase systems present in this bacterium.

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3. Insights into sulphonium and nitrogenous osmolyte production in model marine microorganisms.

Bermejo Martinez A, Curson ARJ and Todd JD

School of Biological Sciences, University of East Anglia, Norwich Research Park, Norwich, NR4 7TJ, UK.

It is vitally important for marine microorganisms to produce compatible solutes. Two of the most commonly produced marine osmolytes are the nitrogenous quaternary amine betaine and the sulphonium compound dimethylsulphoniopropionate (DMSP). These molecules are also important sources of dissolved organic N and S in the oceans. The microbial catabolism of these molecules has wide ranging environmental consequences - from signalling pathways to potential climate regulation. Despite the importance of these molecules in the marine environment very little is known about how these molecules are produced and how these synthetic processes are regulated. Here we will present our recent findings from work on model algae and bacteria. This work identifies the key genes involved in the production of betaine and DMSP. Our studies on gene regulation clearly show that both betaine and DMSP biosynthetic pathways are controlled by the availability of N amongst other environmental factors.

4. Are small RNAs involved in the regulation of nitrogen assimilation in Haloarchaea?

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Physiological, biochemical and regulation aspects of nitrogen assimilation are well known in *Bacteria* or *Eukarya*, but they are poorly described in *Archaea* Domain. *Haloferax mediterranei* has been used as a model to study nitrogen assimilation due to its ability to grow in a defined culture media not only in the presence of inorganic nitrogen but also with amino acid as the sole nitrogen source. Over the last few years the role and transcriptional regulation of enzymes involved in the nitrogen assimilation has been studied. Recently, the global gene expression under three culture media with different nitrogen sources was analysed by microarray technique, supporting previous results which indicate the absence of ammonium as the factor responsible for the expression of genes involved in nitrate assimilation. This study permitted the identification of transcriptional regulators and changes in metabolic pathways related to the catabolism and anabolism of amino acids or nucleotides (Esclapez et al., 2015).

Small RNAs (sRNAs) are involved in many cellular and regulatory processes in all three Domains of life. Their different functions have been analysed with detail in *Bacteria* and *Eukarya*. However, few attempts have been performed to identify and analyse the role of these sRNAs in *Archaea*. In order to extend the understanding of assimilation regulation in archaea we have isolated sRNAs from cultures of *H. mediterranei* grown with ammonium or nitrate. Then, we have used high-throughput techniques to identify possible sRNAs involved in this pathway. Preliminary results show differences in the expression of putative sRNAs between the samples grown with ammonium or nitrate, so we can propose that some of the sRNAs could participate in the transcriptional regulation of nitrogen metabolism.

Esclapez J. et al (2015) J Biotechnol.193: 100.

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5. Are microbial N transformation rates in a permanent grassland soil after 17 years of elevated atmospheric CO₂ sensitive to soil temperature?

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Long-term observations (17 years) within the Giessen Free Air Carbon dioxide Enrichment (Giessen FACE) study on permanent grassland showed that the carbon fertilization caused significant changes in the ecosystem nitrogen cycle. These changes are responsible for a doubling of annual N₂O emissions under elevated atmospheric CO₂ (eCO₂) caused by increased emissions during the plant growing season. The goal of this lab study was to understand how soil temperature influences the long-term effects of eCO₂ and plant carbon input on microbial N transformations in the Giessen FACE. Therefore, a pulse labelling study with ¹⁵N tracing of ¹⁵NH₄⁺ and ¹⁵NO₃⁻ was carried out with incubated soil samples from elevated and ambient CO₂ FACE rings in climate chambers at two different temperatures (10°C and 19°C), while water filled pore space of the samples was adjusted to the same level. The various N pools in the soil (NH₄⁺, NO₃⁻, soil organic matter), N₂O emissions and simultaneous gross N transformation rates were quantified. The quantification of the gross N transformations are based on the turnover of ¹⁵NH₄⁺, ¹⁵NO₃⁻ and shall illuminate the interaction between carbon fertilization, temperature and changes in nitrogen cycle in this grassland soil. We observed an indication for higher DNRA velocity from ¹⁵N excess in NH₄⁺ and NO₃⁻ under eCO₂ and high temperature, which was supported by molecular analyses of *nrfA*, the functional marker gene for DNRA. While the soil respiration after labelling was significantly increased at 19°C compared to 10°C, N₂O emissions showed no significant differences. There were also no significant differences of N₂O emissions between soil samples from control and elevated CO₂ rings within each temperature level. As the soil temperature (within the range of 10-19°C) had no significant effects on N transformations responsible for the observed doubling of N₂O emissions under eCO₂, it seems most likely that other factors like direct carbon input by plants and/or soil moisture differences between ambient and elevated rings in the field are responsible for the observed increase in N₂O emissions under eCO₂.

6. Relationships between N₂O-reducers, N₂O emissions and agricultural practices

Luiz Horta INRA

Agriculture is the main source of terrestrial N₂O emissions- a potent greenhouse gas and the main cause of ozone depletion. The reduction of N₂O into N₂ by microorganisms carrying the nitrous oxide reductase gene (*nosZ*) is the only biological process known to eliminate this greenhouse gas. Here- we investigated how N₂O -reducers and producers were affected by agricultural practices and evaluated the consequences for N₂O emissions. We selected four sites undergoing different agricultural practices at three different locations in France. The abundance of N₂O -reducers and producers was quantified by real-time PCR- and the diversity of both *nosZ* clades was determined by 454 pyrosequencing. Potential denitrification activity- potential nitrification activity and in situ N₂O emissions were determined. To better evaluate the contribution of different factors to the in situ N₂O emissions- we partitioned these into different ranges- from low to high rates. Interestingly- the low range of in situ N₂O emissions was only related to soil pH- while the high ranges were also strongly related to the studied microbial communities. This result suggest a “base-line” N₂O emissions rate which might be more regulated by soil edaphic conditions over microorganisms- the last increasing in importance at the high emissions range. Overall we showed that the *nosZII* clade of N₂O -reducers was more sensitive to environmental factors and agricultural practices than the *nosZI* clade. Tillage increased the diversity of this clade while it did not affect the diversity of the *nosZI* clade. These results highlight the importance of understanding the ecology of the recently identified clade of N₂O -reducers for mitigating in situ N₂O emissions.

7. Covalent Modification of a Heme Ligand by a Substrate Mimic in Thiosulphate Dehydrogenase

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Cysteine-ligated hemes are present in several bacterial enzymes involved in both the nitrogen and sulphur cycles. It is predicted from sequence homology that one such enzyme is the thiosulfate dehydrogenase (TsdA) from *Campylobacter jejuni*. This enzyme allows *C.jejuni* to use tetrathionate as a terminal electron acceptor during anaerobic respiration; $S_4O_6^{2-} + 2e^- \rightarrow 2 S_2O_3^{2-}$ ($E_M = 198$ mV). Here a combination of mass spectrometry and magneto-optical spectroscopies are used to characterise *C.jejuni* TsdA. UV/visible MCD and LC-MS data are consistent with TsdA binding two *c*-type hemes. Near-infrared MCD resolved peaks at 1200 nm and 1800 nm that are indicative of His/Cys⁻ and His/Met ligated ferric hemes in the oxidised protein. A variant C138H has two *c*-type hemes but now with His/His and His/Met ligation, evidenced by peaks at 1500 nm and 1800 nm respectively. Anaerobic incubation of the wild-type enzyme with sulphite results in reduction of the His/Met ligated heme and the loss of 50% of the signal corresponding to the His/Cys⁻ ligated heme. Addition of ferricyanide returns the His/Met ligated Fe(III) heme alongside a high-spin Fe(III) heme but there is no indication of a His/Cys Fe(III) heme. LC-MS indicates covalent modification of the protein by sulphite has occurred which together with the loss of cysteine as a heme ligand suggests Cys as the site of covalent modification. In an equivalent experiment with the C138H variant, no evidence of covalent modification is detected. In addition the C138H variant shows minimal evidence for heme reduction on incubation with sulphite, suggesting the cysteine-ligated heme is key to the reactivity of the WT enzyme towards sulphite. As sulphite is an analogue of thiosulfate, the species identified in this study may represent analogues of intermediates formed in the cellular reaction with tetrathionate.

Brito et al J. Biol. Chem. 2015: 290: 9222

Grabarczyk et al J. Biol. Chem. 2015:290:9209

Kurth et al J. Am. Chem. Soc. 2015: 137:13232

8. “Transcriptomic responses during switches in respiration mode of *Paracoccus denitrificans*”

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Denitrification is one of the main causes of release of nitrous oxide in the atmosphere. Knowledge on the regulation of this process is of importance in relation to identifying the factors causing the emissions. The model organism for denitrification studies is *Paracoccus denitrificans*, which genome was sequenced in 2004.

We have designed and performed an experiments with *P. denitrificans* grown at four different conditions. The growth conditions are: aerobic, anaerobic with nitrate, semiaerobic with nitrate and semiaerobic without nitrate. Cells at these growth conditions had growth rate of 0.45 h⁻¹, 0.3 h⁻¹, 0.4 h⁻¹ respectively and semiaerobic condition without nitrate showed linear growth. The mRNA isolated at mid-log phase of these cultures was used for stranded RNA sequencing. Our aims for the sequencing approach were i) the quantification of the transcripts of P.d. at four different conditions and ii) identification of transcriptional start sites of denitrification genes.

In the anaerobic conditions with nitrate, based on the RNA sequencing data, we see a clear initiation of transcription of the denitrification genes and can locate the presumptive Transcriptional start sites for this genes. We are also able to compare between conditions and identify how many and which genes are differentially and significantly expressed in one condition compared to another. The quantitative results are presented in the Table 1.

Table1: Upregulated and downregulated genes in second condition compare to first one.

condition	upregulated genes	downregulated genes
aerobic (-NO ₃ ⁻) - denitrification (+NO ₃ ⁻)	246 ↑	45 ↓
denitrification (+NO ₃ ⁻) - semiaerobic (+NO ₃ ⁻)	50 ↑	66 ↓
semiaerobic (-NO ₃ ⁻) - aerobic (-NO ₃ ⁻)	523 ↑	357 ↓
semiaerobic (-NO ₃ ⁻) - semiaerobic (+NO ₃ ⁻)	324 ↑	427 ↓

Upregulated genes in cultures with amended nitrate included the key genes for denitrification. In addition, we noticed a set of additional gens, some of which may contribute to the adaptive responses of the cells to denitrifying conditions. Transcriptional start sites suggested by the read-mapping were close to those that were published in literature.

9. NITRIC OXIDE REDUCTASES IN HALOARCHAEA

Javier Torregrosa-Crespo, Pedro González-Torres, Vanesa Bautista, Julia M^a Esclapez, Carmen Pire, María José Bonete and Rosa María Martínez-Espinosa

Denitrification is the major mechanism by which fixed nitrogen returns to the atmosphere from soil and water and microorganisms play an important role. Denitrifying ability has been found in microorganisms belonging to Bacteria and Archaea, however, the last two reactions of this pathway have not been explored yet into detail in archaea in general, and in haloarchaea in particular.

The increasing number of genome sequencing projects offers a new way to study the genetics of the denitrifying process in haloarchaea. However, most of the available genomes are currently drafts and in most of the cases, annotation is wrong. Due to these reasons, in silico analysis becomes a difficult task.

This work summarises for the first time a bioinformatics analysis focused on 180 haloarchaeal genomes (including finished genomes and drafts). *nor* gene sequences as well as the organization of *nor* gene clusters have been analysed into detail. The results obtained reveal that the predominant Nor-like protein in denitrifying haloarchaea is q-Nor type, and halocyanin may acts as physiological electron carrier in this reaction.

References:

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10. Investigating the nitrite oxidation system of *Nitrospira moscoviensis*

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Nitrite-oxidizing bacteria (NOB) catalyze the second step of nitrification and play a significant role in the nitrogen cycle. Members of the genus *Nitrospira* appear to be the most diverse and widespread NOB, and have been found to be predominant in wastewater treatment systems. Despite their importance, *Nitrospira* are scarcely studied and we still lack detailed knowledge about their energy metabolism.

Genome analyses of *Nitrospira* revealed an unusual nitrite oxidoreductase (NXR) that is more closely related to the enzyme of anaerobic ammonium-oxidizing bacteria than to the *Nitrobacter* NXR. Furthermore, they lack a canonical haem-copper oxygen reductase, which however is indispensable for energy conservation. The NXR of *N. moscoviensis* was shown to be membrane-associated, with the catalytic alpha and beta subunits facing the periplasm. No *Nitrobacter*-like gamma subunit anchoring the complex to the membrane could be identified, but several candidate genes were proposed. Alternatively, the NXR could form a supercomplex with an uncharacterized membrane-bound cyt. bd-like oxidase. Furthermore, the transfer mechanism of high-potential electrons from nitrite oxidation to the terminal oxidase is unclear, with soluble and membrane-bound cytochromes as possible electron carriers.

In this study we investigated the NXR subunit composition and aim to shed light on electron transport mechanisms. We separated and identified the NXR subunits by SDS-PAGE and MALDI-TOF, respectively. These results were corroborated by transcriptome analysis revealing the most highly transcribed genes under continuous growth conditions. In conclusion, this study provides first important insights into the nitrite oxidation machinery of *N. moscoviensis* and will help to elucidate energy conservation mechanisms of these enigmatic organisms.

11. Primer development for the specific detection of comammox *Nitrospira*

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Complete nitrification by a single microorganism – the so-called comammox process – is a newly discovered pathway within the nitrogen cycle and was shown to be catalysed by members of the genus *Nitrospira*. The process is predicted to occur under conditions of substrate limitation selecting for high growth yields, and to be especially competitive in biofilm systems. Comammox *Nitrospira* possess the full genetic complement for ammonia and nitrite oxidation. Like canonical AOB, they utilize ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase for ammonia oxidation via hydroxylamine, in addition to the nitrite oxidoreductase necessary for nitrite oxidation. Their AMO differs from its homologs in canonical ammonia-oxidizing bacteria and belongs to a distinct clade (comammox clade A) that contains sequences from various natural and man-made environments. Additionally, a second distinct AMO group (comammox clade B) was identified by database screening and metagenomics. In this study we developed and optimized PCR primer sets targeting the comammox AMO subunit A (*amoA*) of clades A and B to allow specific detection of comammox *Nitrospira* in the environment. Testing these primers on DNA extracted from diverse environmental samples indicated that they could successfully amplify the *amoA* from both comammox groups. To elucidate the distribution of the different comammox *Nitrospira*, we applied the novel primer sets to DNA derived from a rice field soil core, drinking and wastewater treatment plants, and from a biofilter attached to a recirculating aquaculture system. Presence of *Nitrospira* in PCR-positive samples was confirmed by *Nitrospira*-specific *nxB* and 16S rRNA PCR assays. Phylogenetic analysis of the obtained *amoA* sequences from both AMO clades indicated that the new primer sets represent a specific and sensitive molecular tool to identify and monitor comammox *Nitrospira* in different natural and engineered systems. Future research will focus on selective enrichment of the new comammox from the investigated environments.

12. Enriching comammox - novel key players of the nitrogen cycle

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Nitrification, the conversion of ammonium to nitrate via nitrite is a key process of the nitrogen cycle. For decades, nitrification was thought to be a two-step process catalyzed by functionally distinct clades of microorganisms. First, ammonia-oxidizing archaea and bacteria catalyze the oxidation of ammonium to nitrite, which subsequently is oxidized to nitrate by nitrite-oxidizing bacteria. However, the perception of the nitrogen cycle was changed by the recent discovery of members of the genus *Nitrospira* capable of complete ammonia oxidation to nitrate (comammox). After their first discovery, the presence of comammox microorganisms was confirmed in various natural and man-made ecosystems, indicating that comammox bacteria are widespread. These facts raise pressing questions regarding comammox physiology, biochemistry and biological interactions. However, in order to expand our understanding of these enigmatic microorganisms, high enrichments or pure cultures are required. In this study, the genomes of the comammox bacteria *Ca. N. nitrosa* and *Ca. N. nitrificans* were analyzed in order to recognize basic features of comammox physiology. Besides the full gene sets necessary for ammonia and nitrite oxidation present in both organisms, there also are distinct differences. *Ca. N. nitrificans* encodes an alternative respiratory complex III and a bd-type terminal oxidase, suggesting a potential to grow mixo- or heterotrophically with low-potential electron donors. Moreover, only *Ca. N. nitrificans* has a superoxide dismutase and a catalase, indicating that oxygen concentrations are an important factor for selectively enriching this comammox organism. Both species feature genes for resistance against antimicrobial compounds such as acriflavine, a characteristic that can be useful for their separation from concomitant heterotrophs. Based on this information, a bioreactor for the enrichment of comammox *Nitrospira* was started. The reactor was supplied with low concentrations of ammonium and oxygen and successfully promoted growth of comammox bacteria. Their presence and degree of enrichment was verified by fluorescent *in situ* hybridization as well as by PCR assays employing specific primers for the comammox 16S rRNA and *amoA* genes. In conclusion, the enrichment culture that was obtained in this study is a crucial first step in order to provide future insights into the physiology of comammox bacteria, and into their interaction with other N-cycle bacteria.

13. Purification and biochemical characterization of the two-component nitric oxide detoxification system Bjgb-Flp from *Bradyrhizobium diazoefficiens*

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Bradyrhizobium diazoefficiens (formerly *Bradyrhizobium japonicum*) is a Gram-negative soil bacterium that establishes nitrogen-fixing symbiotic associations with soybean plants. In this bacterium, a putative haemoglobin termed Bjgb is encoded within a gene cluster that also codes for proteins that constitute a novel nitrate assimilation pathway. These including the large catalytic subunit of the assimilatory nitrate reductase (NasC), a major-facilitator superfamily-type nitrate/nitrite transporter and an FAD-dependent NAD(P)H oxidoreductase (Flp) (1).

Bjgb is a single-domain hemoglobin (sdHb) homologous to the N-terminal haem-containing domain flavohaemoglobin (FHb) from *Escherichia coli* as well as the sdHbs from *Vitreoscilla stercoraria* and *Campilobacter jejuni* (2). A role for Bjgb and Flp in protecting *B. diazoefficiens* cells from nitrosative stress in free-living conditions has recently been demonstrated (1), and the two proteins are proposed to form a nitric oxide (NO) detoxification system analogous to FHb, where Bjgb might be reduced by electrons supplied by Flp following oxidation of NAD(P)H. However, the capacity of Bjgb-Flp for NO detoxification and the end-products of NO turnover under aerobic and anaerobic conditions are not known.

In this work, we describe the construction of an overexpression system for Bjgb-Flp, as well as the purification of the *B. diazoefficiens* Bjgb and Flp proteins. The molecular structure of the Bjgb protein has been determined by X-ray crystallography. Further biophysical characterization of Bjgb by UV-visible absorbance spectroscopy has revealed the active site *b*-type haem cofactor has the ability to bind NO and that the coordination environment of the heme-iron is influenced by Flp.

References

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14. Nitrate reduction in *Shewanella oneidensis*

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S. oneidensis harbors electron transport chains to a wide variety of different terminal electron acceptors including nitrate and nitrite. This periplasmic electron transfer network mainly consists of different *c*-type cytochromes. The molecular details of routing electrons within this network remain unknown so far. The dissimilatory nitrate reduction to ammonium (DNRA) is separated in two reactions. Nitrate is reduced to nitrite via the NapAB complex whereas nitrite is reduced via the NrfA protein. Nitrite is not reduced before nitrate is depleted to a low threshold concentration. Therefore, as a typical nitrate reduction pattern, *S. oneidensis* shows a two-step reduction of nitrate by accumulating nitrite in the medium which is then reduced to ammonia as soon as nitrate is completely reduced. Deletion of the two major periplasmic *c*-type cytochromes STC and FccA leads to a different phenotype: nitrite accumulation during DNRA is replaced by a simultaneous nitrite reduction. Moreover, at low salt concentrations, nitrate reduction is completely inhibited. Since nitrite reductase NrfA is directly coupled to CymA - the membrane-bound periplasmic electron hub in *S. oneidensis* - simultaneous reduction of nitrite via NrfA seems likely. A nitrate inhibition of this reaction could be observed in the wild-type but this inhibition could be bypassed either by deletion of STC and FccA or by deletion of NapB, the heme-containing protein of the nitrate reducing NapAB complex. Here, we show data of the different nitrate reduction patterns depending on the genotype and show further interesting salt-dependent influences on the periplasmic electron flow to the nitrate and nitrite reductases NapAB and NrfA.

15. Structural changes in the active site of thiocyanate dehydrogenase with various copper ions content

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Thiocyanate dehydrogenase (TcDH) from *Thioalkalivibrio paradoxus* ARh1 catalyses oxidation of thiocyanate (SCN⁻) with formation of cyanate (CNO⁻) and sulfur and electrons transfer to an acceptor molecule. TcDH is a copper-dependent enzyme. Three x-ray structures with different copper content in the active site were solved. The structures have one (structure I, 1.7 Å), three (structure II, 1.9 Å) and five (structure III, 2.3 Å) copper ions. Crystals for structure I were obtained by crystallizing TcDH as isolated. Crystals for structure II were obtained by crystallizing TcDH incubated with Cu(II) salts. Crystals for structure III were obtained by soaking crystals II in Cu(I) solution. TcDH structure is formed by 7 bladed β -propeller. The active site is located in the central cavity of the propeller. Entrance to the active site is possible only from one side of β -propeller, the other is blocked by C-terminal of the polypeptide chain. Two TcDH dimers with strong intermolecular contacts forms a tetramer in crystal. All three structures are similar. The differences are observed in the active site. In structure I the only copper ion is coordinated by His381, His206, Asp314 residues and H₂O molecule and has occupancy 0.5. In structure II the first copper ion is located in the same place with full occupancy. The second ion also has full occupancy and is coordinated by Lys103, His135, His528 residues and H₂O molecule. The third ion is located in a channel, leading to the active site and has occupancy 0.2. It is coordinated by His482, Asp480 and an H₂O molecule. Two more copper sites were found in the structure III. The fourth copper ion is coordinated by His437, His482, H₂O, the fifth copper - by His136 and three H₂O molecules. The first four copper ions had tetrahedral coordination. The fifth ion has flat square coordination. Such active site configuration has not been described previously. The work is supported by RSCF grant 14-24-00172

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