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Bacterial response from exposure to selected aliphatic nitramines

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Abstract

Currently the most promising method for capturing anthropogenic produced carbon dioxide (CO_2) from suitable point sources is the technique using amines in Post-combustion CO_2 Capture (PCC). However, the use of amine is found associated with formation of the potent harmful and stable compound group of aliphatic nitramines. The toxic effect of these aliphatic nitramines, including species of different trophic levels, must be assessed, prior to any large-scale implementation of PCC.

In this present study bacterial response from acute exposure to the two nitramines monoethanolnitramine (MEA-NO_2) and dimethylnitramine (DMA-NO_2) have been assessed. This has been done by looking for effects in the rate of aerobic respiration by measuring the oxygen consumption using O_2 Sensor Dish®Reader system (Presens), and in community-level metabolic profiling including 31-different ecological relevant carbon substrates using the Ecoplates™ (BIOLOG). Samples of both natural bacterial community (lake-water) and pure bacterial culture have been included in this study. The nitramines have been quantified using LC-MS.

Results suggest MEA-NO_2 to inhibit rate of O_2 consumption in natural lake-water bacterial community at concentrations > 4 mg L^{-1}. No such effect was found for pure culture of *Bacillus subtilis*, neither from exposure to MEA-NO_2 nor to DMA-NO_2. Moreover, the two nitramines are found to induce slight shifts in the metabolic profile of natural lake-water bacterial communities. Challenges associated with working with highly dynamic natural bacterial communities lead to the recommendation of additional testing to be performed in order to obtain a conclusive picture of bacterial response to the nitramines. The levels of nitramines found in this study to affect bacteria are 1000 fold higher than what is currently estimated to be expected from the emissions of a PCC plant (worst-case scenario). Final environmental concentrations, following years of operation is however dependent on the effect of biogeochemical processes (e.g. soil sorption and biodegradation) taking place at any given site.

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1. Introduction

New technology is followed with the possibility of introducing new compounds, or elevated levels of compounds already present, into the environment. Bacteria living in soils and waters are among the first to be exposed to such anthropogenic emissions. These organisms play key roles in a well functioning ecosystem by mediating crucial processes such as in decomposition and in nutrient cycling, and must therefore be included as part of an environmental risk assessment when new technology is being implemented. Several species of bacteria are moreover known to degrade and/or transform a range of different molecules following their vast range of metabolic solutions [1]. Obtaining information regarding bacterial response to exposure of anthropogenic emitted molecules may thus provide insight both into possible ecotoxic effects as well as to the persistence of such compounds in the environment.

Amine based Post-combustion CO_2 Capture (PCC) is currently the most promising technology to sequester anthropogenically produced CO_2 from large-scale point sources, such as power plants and other industrial facilities [2]. The two first full-scale capture plants of this sort are already under testing in Canada (“Boundary Dam”, SaskPower) and in the USA (“Petra Nova”, Texas, USA). In addition several smaller scaled pilot plants are under operation worldwide (www.globalccsinstitute.com). Although promising for mitigating increased atmospheric CO_2 levels, the use of amines is found to lead to the formation of potential harmful compounds such as nitramines (RR’NNO_2) [3-5]. Nitramines can be formed both atmospherically from amines co-emitted with the cleaned flue gas [3], or through amine degradation inside the capture plant [6, 7]. The first pathway will result in direct introduction of nitramines to ecosystems nearby the PCC plant. The type of nitramines formed is dependent on the type of amines used in the capture process, and are generally characterized by aliphatic functional groups.

Studies documenting toxicity of these aliphatic nitramines are scarce, which is in contrast to the more well studied aromatic and cyclic nitramines associated with the use of ammunition and explosives [8]. To conduct a thorough ecotoxicity assessment, the response of organisms representing different trophic levels must be included. Brakstad et al. [9], investigating potential ecotoxic effects of the aliphatic nitramines relevant to amine-based PCC, reported low to moderate acute toxicity after exposing two different eukaryotic species, the aquatic phytoplankton *Pseudokirchneriella subcapitata* and the invertebrate *Daphnia magma*. Effective Concentration inhibiting growth by 50 % (EC_{50}) was found for the most toxic nitramine, piperazine nitramine, to be at 430 and 1031 mg L^{-1} for the aquatic phytoplankton and the invertebrate, respectively. The least toxic nitramine was found to be dimethylnitramine (DMA-NO_2). No toxic effect was found at the maximum levels of exposure (2000 and 2500 mg L^{-1}) neither for the aquatic phytoplankton nor for the invertebrate. Other studies have focused on the chronic effects of these nitramines [10, 11] from which the two nitramines DMA-NO_2 and monomethylnitramine have been listed in the Carcinogenic Potency Database (www.toxnet.nlm.nih.gov/cpdb). In a recent study, Fjellsbø et al. [12] report the three studied nitramines (DMA-NO_2, monomethylnitramine, and 2-methyl-2-(nitroamino)-1-propanol) to be classified as eye irritants. No studies are known that have investigated any acute toxic effects of bacteria after exposure to the aliphatic nitramines.

The potential a harmful compound has to exert its toxic effects in the environment is to a large extent confined by its lifetime, and its tendency to partition between different environmental compartments (e.g. soil and water). The nitramines are found persistent towards abiotic decay [13, 14], and a low biodegradability has been reported [9, 14]. Estimated Octanol-Water partitioning coefficients (K_{OW} = -0.52 to -1.51) suggest high degree of water solubility [9]. Partitioning into the water phase is known to enhance interaction with bacteria, which are dependent on aquatic surroundings for their active state [1]. The possibility of nitramine sorption to soil should however not be ruled out since selective binding to the soil is known, independent of the polarity of the molecule [15]. In fact, Mohr et al. [14] report selective sorption of the two nitramines tested (monoethanolnitramine (MEA-NO_2) and DMA-NO_2) to organic- over mineral soils. The concentrations of nitramines predicted found in surface waters from an amine based PCC plant, based on “worst-case” estimations, is in the μg L^{-1} range [16]. The final concentration following years of operation is however dependent on the effect of biogeochemical processes (e.g. sorption and biodegradation) at any given site.

Nomenclature

DMA-NO2	Dimethylnitramine
EC_{50}	Effective Concentration inhibiting growth by 50 %
K_{OW}	Octanol-Water partitioning coefficient
LB	Lysogeny Broth
LOEL	Lowest Observed Effect Level
MEA-NO_2	Monoethanonitramine
PBS	Phosphate buffered saline
PCC	Post-combustion CO_2 Capture

2. Materials and Methods

2.1. Nitramine standards and other chemicals

The nitramines included in this study are presented in Table 1. The standards were synthesized with a purity of ~ 99 % by Prof. Yngve Stenstrøm, Norwegian University of Life Sciences (NMBU, Norway). Standard solutions of the nitramines were prepared either in distilled water or in Phosphate Buffered Saline (PBS) solution. Final concentrations of both 250 and 2000 mg L^{-1} (only in distilled water) have been used. Copper sulfate ($CuSO_4$) was of origin from KEBO laboratories, and with a purity of 99.5 %. PBS concentrate (20x) of ultra-pure grade with pH 7.5 was purchased from AMRESCO®.

Table 1. Nitramines included in this study (name, abbreviation, CAS-nr, and structure).

Nitramine	Abbreviation	CAS-nr	Structure
Monoethanolnitramine	MEA-NO_2	74386-82-6	
Dimethylnitramine	DMA-NO_2	4164-28-7	

2.2. Lake-water samples

Natural lake-water samples, containing indigenous bacterial community, were collected from a oligotrophic lake ("Maridalsvannet") and a eutrophic lake ("Østensjøvannet"). Both lakes are located outside the Oslo city Centre (Norway), to the northwest and towards the east respectively. The oligotrophic lake is larger and deeper (A = 3.83 km^2, d_{max} = 45m), and serves as the drinking water source of the majority of Oslo's population. The smaller and shallower eutrophic lake (A = 0.33km^2, d_{max} = 3.2m) exhibits large and rich species diversity in both vegetation and birdlife.

New fresh samples were collected from the shore of the lakes for each individual experiment throughout the summer seasons of 2013 and 2014. Immediately upon arrival to the laboratory samples were filtrated through 2.0 µm Isopore™ membrane filters (polycarbonate, Millipore) removing organisms larger than bacteria.

2.3. Pure Bacterial Cell Cultures

Pure cultures of bacterial strains were included in this study to represent a well-defined model system for the experiment investigating bacterial respiratory response from exposure to the nitramines (Ch. 2.4). The intent was to include the different bacterial strains *Bacillus subtilis*, *Escherichia coli*, and *Micrococcus luteus,* representing major groups of bacteria (e.g. gram-positive and gram-negative), in order to reveal potentially specific interactions between the bacteria and the nitramines. Though only the first was proven suitable for this experimental setup (Ch. 2.4). *B. subtilis* is a gram-positive bacterium capable of aerobic respiration, also at low levels of oxygen. This specie has an important environmental relevance in soils and waters due to its central role in decomposition and nutrient cycling [1].

The pure bacterial strains were provided from the Department of Biosciences, UiO (lab collection, Dirk Linke). The cultures were grown in standard liquid Lysogeny Broth (LB) (5 mL) for 24 h at 37° C and at 181 rpm. Fully-grown cultures (10^8-10^9 cells mL^{-1}) were diluted hundredfold in PBS buffer. This dilution was found to be the lowest concentration of bacterial cells providing a strong and reproducible temporal signal of O_2 consumption. For each experiment a new culture was grown from the original plated culture (standard LB) which was stored at 7° C.

2.4. Respiratory response (aerobic)

The Sensor Dish®Reader system available from PreSens Precision Sensing GmbH (Regensburg, Germany) allow for online, non-invasive measurements of oxygen levels in multiple samples. The system consists of 5 mL sample glass vials each equipped with a sensor spot of oxygen-sensitive luminescent dye embedded in a polymer. The sample vials were cleaned with ethanol (10 %, v/v), rinsed thoroughly with distilled water, and dried at 50° C for 48 h prior to use. The vials are placed on top of a 24-channel Sensor Dish®Reader. A flash of light excites the dye in the sensor spot and its luminescence lifetime is subsequently measured. The luminescence lifetime is converted into oxygen levels by the software accompanying the system.

Prior to startup of the experiment, the natural lake-water bacteria sample was pre-incubated by addition of inorganic nutrients (2mM KH_2PO_4 and 20mM NH_4NO_3), in room temperature (25°) in the dark, and with agitation. This was done to ensure sufficient concentration of bacterial cells in the sample to obtain a strong signal of O_2 consumption.

The bacteria containing samples (either natural lake-water sample or pure culture) were mixed with a set volume of different concentrations of the nitramine standard solutions. In a pilot experiment with the oligotrophic lake-water sample a concentration range of 8 – 200 mg L^{-1} was selected based on the results from Brakstad et al. [9]. From the results of these initial tests (data not presented), concentrations were lowered to range from 1 to 16 mg L^{-1}. The samples containing pure bacteria culture were exposed to concentrations of nitramines that ranged from 0.2 to 30 mg L^{-1}.

The use of copper sulfate ($1x10^{-4}$ M) as bactericide for positive control was based on the application note provided by Presens precision sensing GmbH [17]. Negative control consisted of bacterial sample without addition of nitramine. Sample vials with distilled water was used to correct for random fluctuations in the measurements. Four replicates were measured unless otherwise stated. The experiments were conducted in dark to avoid growth of alga. The experiments with natural lake-water sample were conducted at room temperature (25° C), whereas for the pure culture of *B. subtilis* an elevated temperature was chosen (35° C). Measurements of O_2 levels were logged every 15 seconds for 48 h using the software provided by Presens Precision Sensing.

Measurement readings were first normalized by the average of reference wells containing only distilled water in order to correct for temporal drift in the sensor system. Normalized time series from individual non-reference wells were aggregated as means over 2400 temporal readings. Differencing the aggregated time series and reversing the sign computed the rates of O_2 consumption. The treatment effects on O_2 consumption rates were analyzed by using averages for each well as response parameters. This was done using the statistical language R [18], and with the two packages Hmisc [19] and matrixStats [20].

2.5. Metabolic community structure

Possible effect in the metabolic profile of a natural bacterial community from exposure to the nitramines was assessed using Ecoplate™ available from BIOLOG (BIOLOG Inc., Hayward, CA, USA). The Ecoplate™ comprises 31 different carbon substrates in addition to a blank. The different substrates and the blank are provided in replicates of three on each plate. Each well contains a dye (formazan), which has a colour absorbency that is dependent on the rate of respiration. The signal can thus be read photometrically. The carbon substrates are of ecological relevance and encompass the following major molecular groups: polymers (n = 4), carbohydrates (n = 7), miscellaneous (n = 3), carboxylic acids (n = 9), amino acids (n = 6), and amine/amide (n = 2).

Only the two different lake-water samples of natural bacterial community (oligotrophic and eutrophic) were used in this experiment. The samples were mixed with the nitramine standards, MEA-NO_2 and DMA-NO_2, to a final concentration of 25 mg L^{-1}. Aliquots of 100 μL were added to each of the wells. Control plates only containing lake-water were included for comparison. The Ecoplates™ were incubated in duplicates at room temperature (25°C) in the dark.

Measurements were performed periodically using a BioTek® Synergy™ Mx plate reader at wavelength 590 nm for approximately 100 h. The data was collected using Gen 5.1™ software provided by BioTek.

The absorbency readings were subtracted for the value of the blank. Corrected absorbency values were plotted against time, and the area under the curves was integrated [21]. The resulting areas derived for the total of six replicates of the 31 different substrates were assessed using the multivariate statistical method Principle Component Analysis (PCA) using the R language [18], and with the two packages pracma [22] and FactoMineR [23].

2.6. Liquid Chromatography Mass Spectrometry (LC-MS)

For quantitative measurement of MEA-NO_2 and DMA-NO_2 in samples of *B. subtilis*, LC-MS/MS was employed. The LC system used was an UltiMate3000 (Thermo Scientific, Germenering, Germany), which was connected to a TSQ Vantage triple quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). An ACE 3 C18-PFP (150 x 1.0 mm, 3 μm) reversed phase analytical column (Advances Chromatography Technologies Ltd, Aberdeen, UK) was used for isocratic separation with 1 – 2 mM acetic acid+methanol (1+1, v+v) mobile phase at a flow rate of 50 μL min^{-1}. The injection volume was 1 μL. The nitramines were ionized using heated electrospray ionization, MEA-NO_2 and DMA-NO_2 were ionized in negative and positive mode, respectively. SRM specific transitions for the nitramines were used. Quantification of the nitramines was performed using a six-point calibration curve at concentrations between 0.1 and 30 mg L^{-1}.

3. Results and Discussions

3.1. Response in (aerobic) respiration

The Sensor Dish®Reader system was used to detect toxic effects of nitramines on bacteria by assessing differences in aerobic respiration due to their exposure to the nitramines. Respiration was measured as reduction in oxygen levels in the samples over time and compared to levels in the control samples.

3.1.1. Natural Bacterial Community

Initial experiments investigating respiratory response of the natural bacterial community from an oligotrophic lake due to nitramine exposure showed complete inhibition of O_2 consumption, compared to the control sample, for the two highest exposure levels of MEA-NO_2 (100 and 200 mg L^{-1}). Reduced O_2 consumption was evident at exposure concentration as low as 8 mg L^{-1} (data not presented).

Results from the subsequent experiment, using lowered exposure levels of MEA-NO_2 (1 – 16 mg L^{-1}), are presented in Figure 1 (n =3). A clear reduction in the consumption of O_2 is evident after exposure to the nitramine, which is in agreement with the results from the initial test. The magnitude of reduction is further strongly correlated

with the exposure concentration of the MEA-NO_2 (R^2 = 0.818). EC_{50} appear to be between 4 and 8 mg L^{-1}, and a Lowest Observed Effect Level (LOEL) at between 2 and 4 mg L^{-1} MEA-NO_2.

This experimental setup, using the natural lake-water samples, was found challenging to work with due to variable response in oxygen consumption by the varying natural bacterial communities. This is speculated to result from the relatively low concentration of bacterial cells found in such surface waters. Natural bacterial communities are moreover considered to be a highly dynamic system, capable of rapidly adapting to changing external conditions (e.g. temperature, rainfall, etc.), making day-to-day variations potentially large. Water sample containing natural bacterial community from the eutrophic lake was also tested, though unfortunately this did also not result in a strong and reproducible signal of O_2 consumption.

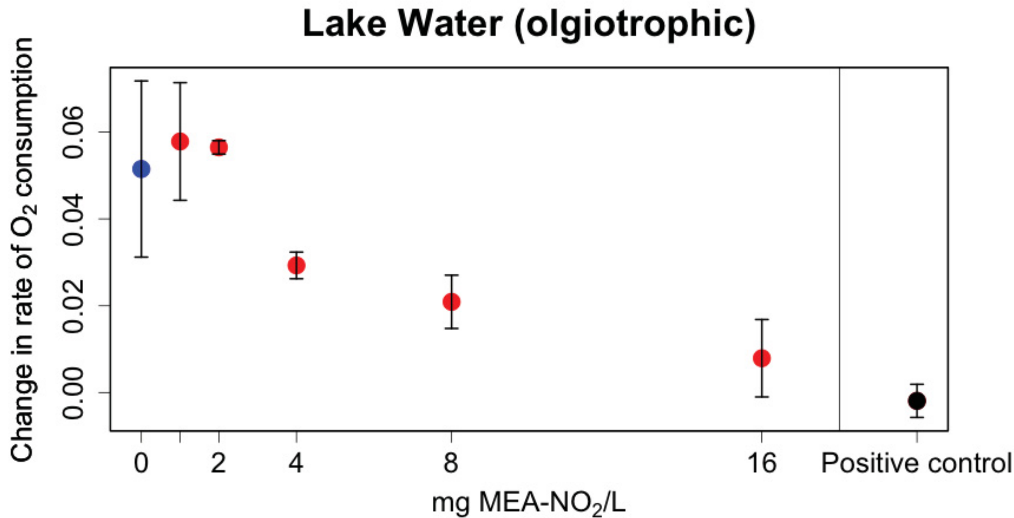

Figure 1: Average change in the rate of O_2 consumption versus exposure level of MEA-NO_2 (red) for the sample of natural bacterial community from the oligotrophic lake ("Maridalsvann", R^2 = 0.818). Negative control containing untreated lake-water (blue), and positive control containing $CuSO_4$ (black) included for comparison. Error bars represent standard deviation (n=3).

3.1.2. Pure culture of Bacillus subtilis

Results showing the average rate of O_2 consumption from *B. subtilis* exposed to MEA-NO_2 and DMA-NO_2 (0.2 – 30 mg L^{-1}) are presented in Figures 2 and 3, respectively. Bacterial samples of pure *B. subtilis* culture provided both strong and reproducible signals of O_2 consumption. No significant effect from exposure to the nitramines is found for this specie, even at a maximum exposure concentration of 30 mg L^{-1}. The concentration of bacterial cells in these cultured bacterial samples is expected to be greater than what is found in natural lake-water systems. The greater concentration of bacterial cells is likely to result in reduced sensitivity to a constant concentration of potential toxins. Thus the results between the two types of samples are not considered directly comparable.

Concentrations of the nitramines were measured before and after the experiment. This was done to assess the potential of nitramine biodegradation, while at the same time ensuring that level of exposure to the nitramines remained constant. No significant change in the concentration levels of MEA-NO_2 was found. (Data for the DMA-NO_2 was unfortunately lost.)

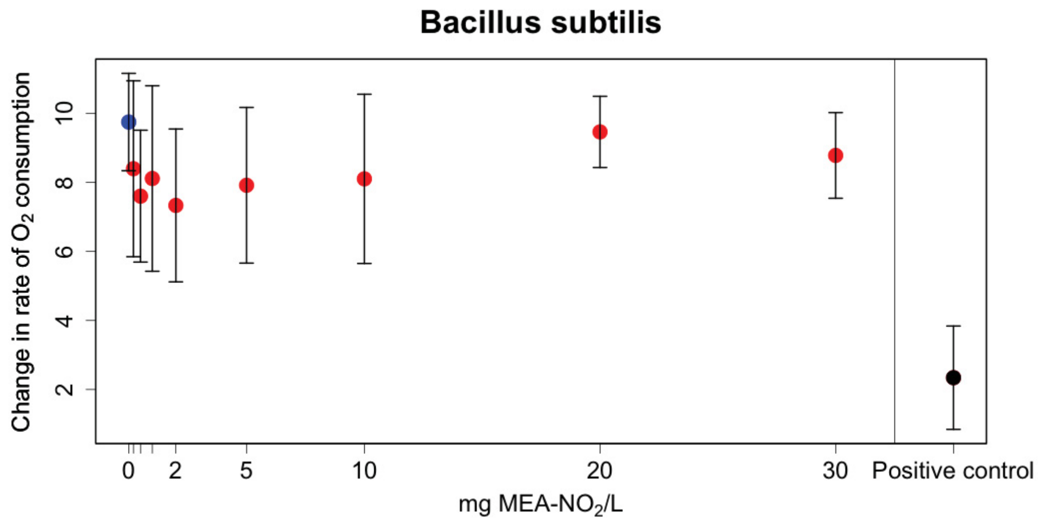

Figure 2: Average change in the rate of O_2 consumption versus exposure level of MEA-NO_2 (red) for the sample of pure culture of *B. subtilis*. Negative control containing untreated bacterial sample (blue), and positive control containing $CuSO_4$ (black) are included for comparison. Error bars represent standard deviation (n=4).

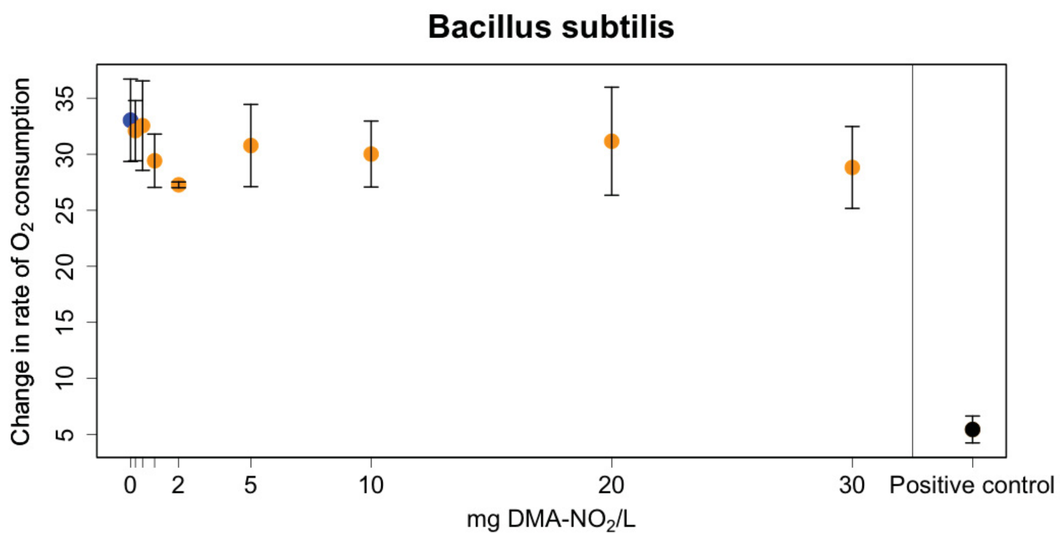

Figure: 3: Average change in the rate of O_2 consumption versus exposure level of DMA-NO_2 (orange) for the sample of pure culture of *B. subtilis*. Negative control containing untreated bacterial sample (blue), and positive control containing $CuSO_4$ (black) are included for comparison. Error bars represent standard deviation (n=4).

3.2. Metabolic community structure

Effect in the metabolic structure of the natural bacterial communities from exposure to the nitramines was assessed using the BIOLOG Ecoplate™. Differences in the utilization of the 31 different ecological relevant carbon substrates after exposure to the nitramines (MEA-NO_2 and DMA-NO_2) would thus produce insight into bacterial response to nitramine exposure on a community level. Results from the principle component analysis of the data from the oligotrophic and eutrophic bacterial lake-water samples are presented in Figures 4 and 5, respectively. The total of six parallel readings are included as individual readings (named 1-6).

The replicates of the different treatments ("MEA-NO_2", "DMA-NO_2", and "LakeWater") are not clearly spatially separated in the plane of the two main principal components of the PCA, but there appear to be a trend of separation between the untreated lake-water samples ("LakeWater") and those treated with the two nitramines (MEA-NO_2 and DMA-NO_2) along the main principal component (Dim 1), explaining only about 16 % of the total variation in the data set. This is especially evident for the samples from the eutrophic lake (Figure 5). The second principle component (Dim 2), accounting for an additional 13 – 14 % of the variation, appears to be governed by the differences between the measurement parallels. Unfortunately, it has not been possible to identify any pattern in this response, and thus no explanatory variable has been identified. The variable factor map (parameter loading) indicates that different substrates trigger the detrimental response of nitramines on the bacteria from the oligotrophic lake then from the eutrophic lake. Different substrate groups do not appear to be of any explanatory value to the toxic response variables. The amino acid, L-Serine (AA4) substrate appears to have an especially strong negative effect on the nitramine response of the bacteria from the eutrophic lake, though this substrate has poor parameter loading along the Dim1 component in the correlation circle figure of the oligotrophic lake.

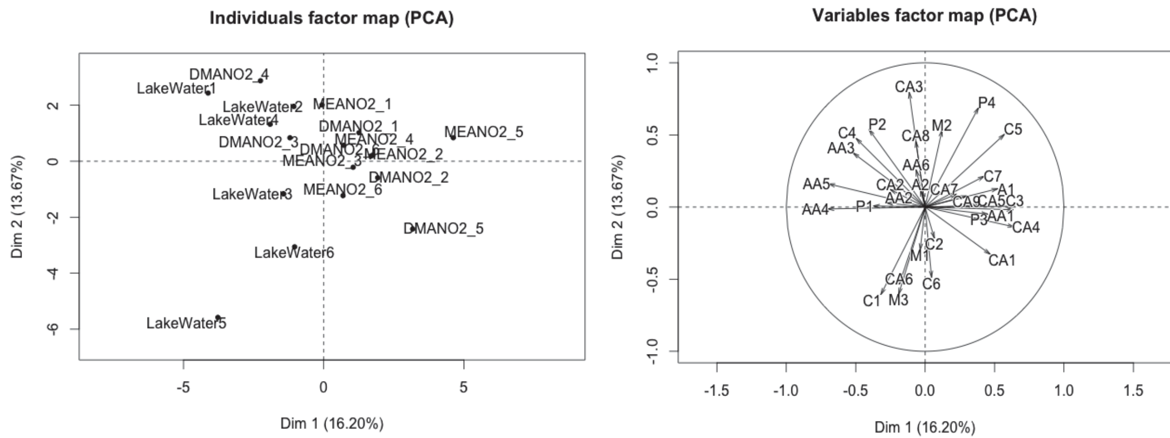

Figure 4: Principle component analysis and correlation circle for natural bacterial community from the oligotrophic lake-water with the 31 different C-substrate from the BIOLOG Ecoplate™ (n = 6). The different C-substrates are coded depending on the identity of molecular group. POLYMERS: P1 = Tween 40, P2 = Tween 80, P3 = α-Cyclodextrin, P4 = Glycogen, CARBOHYDRATES: C1 = D-Cellobiose, C2 = α-D-Lactose, C3 = β-Methyl-D-Glucoside, C4 = D-Xylose, C5 = i-Erythritol, C6 = D-Mannitol, C7 = N-Acetyl-D-Glucosamine, MISCELLANEOUS: M1 = Pyruvic Acid Methyl Ester, M2 = Glucose-1-Phosphate, M3 = D, L-a-Glycerol Phosphate, CARBOXYLIC ACIDS: CA1 = D-Glucosaminic Acid, CA2 = D-Galactonic Acid ϒ-Lactone, CA3 = D-Galacturonic Acid, CA4 = 2-Hydroxy Benzoic Acid, CA5 = 4-Hydroxy Benzoic Acid, CA6 = ϒ-Hydroxybutyric Acid, CA7 = Itaconic Acid, CA8 = α-Ketobutyric Acid, CA9 = D-Malic Acid, AMINO ACIDS: AA1 = L-Arginine, AA2 = L-Aspargine, AA3 = L-Phenylalanine, AA4 = L-Serine, AA5 = L-Threonine, AA6 = Glycyl-L-Glutamic Acid, AMINE/AMIDE: A1 = Phenylethylamine, A2 = Putrescine.

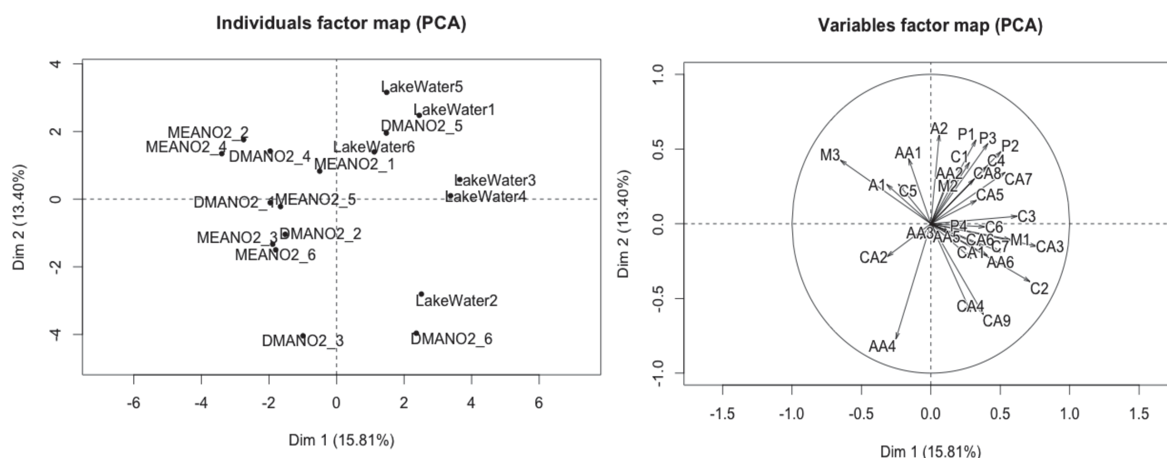

Figure 5: Principle component analysis and correlation circle for natural bacterial community from the eutrophic lake-water with the 31 different C-substrate from the BIOLOG Ecoplate™ (n = 6). The different C-substrates are coded depending on the identity of molecular group. POLYMERS: P1 = Tween 40, P2 = Tween 80, P3 = α-Cyclodextrin, P4 = Glycogen, CARBOHYDRATES: C1 = D-Cellobiose, C2 = α-D-Lactose, C3 = β-Methyl-D-Glucoside, C4 = D-Xylose, C5 = i-Erythritol, C6 = D-Mannitol, C7 = N-Acetyl-D-Glucosamine, MISCELLANEOUS: M1 = Pyruvic Acid Methyl Ester, M2 = Glucose-1-Phosphate, M3 = D, L-a-Glycerol Phosphate, CARBOXYLIC ACIDS: CA1 = D-Glucosaminic Acid, CA2 = D-Galactonic Acid ϒ-Lactone, CA3 = D-Galacturonic Acid, CA4 = 2-Hydroxy Benzoic Acid, CA5 = 4-Hydroxy Benzoic Acid, CA6 = ϒ-Hydroxybutyric Acid, CA7 = Itaconic Acid, CA8 = α-Ketobutyric Acid, CA9 = D-Malic Acid, AMINO ACIDS: AA1 = L-Arginine, AA2 = L-Aspargine, AA3 = L-Phenylalanine, AA4 = L-Serine, AA5 = L-Threonine, AA6 = Glycyl-L-Glutamic Acid, AMINE/AMIDE: A1 = Phenylethylamine, A2 = Putrescine.

4. Conclusions and Future work

This study demonstrates that the bacteria from a natural lake-water ecosystem respond to exposure of aliphatic nitramines. Reduced aerobic respiration was found for the oligotrophic lake bacterial community at concentrations > 4 mg L^{-1} MEA-NO_2, and with the magnitude of reduced respiration strongly correlated with the exposure concentration (R^2 = 0.818). No such effect was measured in samples of pure culture *B. Subtilis*, neither when exposed to MEA-NO_2 nor DMA-NO_2. This indicates a possible species-dependent response to the nitramine. The exposure levels of the MEA-NO_2 were found to be stable during the course of such experiments, ruling out the possibility of rapid biodegradation under these conditions.

Natural bacterial communities from lake-water ecosystems (oligotrophic and eutrophic) were further found to display a slight shift in metabolic profiling from exposure to the two nitramines (MEA-NO_2 and DMA-NO_2). The lack of a strong distinction in the utilization of the 31 different carbon substrates after exposure indicates that the nitramines do not exert a strong acute toxicity on these bacteria. The slight shift in the metabolic profiling was especially evident for the eutrophic bacterial community. The reason for this is unknown.

In order to obtain a conclusive picture of the bacterial response to aliphatic nitramines, more studies are needed. It is suggested to include additional environmental samples (e.g. soil), and to aim for alteration in the method of aerobic respiration to obtain a strong and reproducible signal for the lake-water bacteria. Moreover, by including different types of pure cultured bacterial strains, potential species-specific interaction may be revealed. Finally it is recommended to also consider bacterial response in anaerobic respiration from exposure to the nitramines. The nitramines may conceivably be found to influence processes in the nitrogen cycle.

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