# Growth response of environmental bacteria under exposure to nitramines from $CO_2$-capture

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## Abstract

Nitramines are potentially carcinogenic by-products of amines used in post-combustion $CO_2$-capture. The influence of monoethanol (MEA)-, monomethyl (MMA)-, and dimethyl (DMA)-nitramines on the growth of environmental strains of bacteria, *Pseudomonas fluorescens* (*P. fluorescens*) and *Rhodococcus spp.* (*R. spp.*), was investigated in the laboratory. Additionally, the persistence of the nitramines in the presence of bacteria was determined. Growth of *R. spp.* was found to be sensitive to MMA-nitramine ($EC_{50}$ = 157 mg $L^{-1}$), while *P. fluorescens* growth was insensitive to all nitramines tested. Moreover, *P. fluorescens* was capable of degrading 8-10% of the nitramines during the 33 h experiments. Results from this study provide insight into important processes of bacterial response to nitramines that merit further investigation considering the ongoing implementation of $CO_2$ capture technology.



**Keywords:** Biodegradation; $CO_2$ capture; Ecotoxicity; LC-MS; Nitramine

## 1. Introduction

Technology of $CO_2$ capture offers the opportunity to reduce greenhouse gas emissions from existing large-scale point sources. Most climate models rely on global-scale implementation of the technology to limit global warming to 2 °C (and especially 1.5 °C) (IPCC, 2014). Currently, the most feasible way of capturing $CO_2$ is using amines post-combustion (Rochelle, 2009; Wang et al., 2011). However, several potentially carcinogenic nitramines may form from the amines used in post-combustion $CO_2$ capture. Formation occurs in such a way that direct introduction to the nearby environment is inevitable (Nielsen et al., 2012). A thorough risk assessment, constituting both a prediction of final exposure levels and a detailed investigation of the toxicity of the relevant nitramines, is lacking (Chen et al., 2018).

From the "benchmark" amine solvent, monoethanolamine, the following three nitramines can form: MEA-, MMA-, and DMA-nitramines. As these nitramines are small and polar ($M \geq 106.8$ g mol$^{-1}$ and $S_W \geq 176$ g L$^{-1}$) they are thought to partition readily into the aqueous phase. However, a preceding study found that MEA- and DMA-nitramines preferentially bind to soils rich in organic matter (Gundersen et al., 2017a). Surface soil horizons are typically rich in organic matter and high in biological activity, especially in boreal ecosystems. Brakstad et al. (2018) investigated the biodegradability of MEA-, MMA-, and DMA-nitramines. They found none of them to be readily biodegradable by the standards of the OECD Guideline 301, which requires 60% decay. The apparent low biodegradability may be caused by toxic effects from the nitramines.

Studies on the potential ecotoxic effect of relevant nitramines are summarized in Table 1. The most sensitive response was found in our preceding study, where a natural

oligotrophic lake-water bacterial community showed an estimated half effective concentration ($EC_{50}$) of 10 mg $L^{-1}$ MEA-nitramine (Gundersen et al., 2014). Other studies focusing on species from higher trophic levels (e.g. phytoplankton or larvae) report higher $EC_{50}$ values, ranging from 47 to > 2000 mg $L^{-1}$ (Table 1). For algae, a growth assay showed an $EC_{50}$ of 591 mg $L^{-1}$ for DMA-nitramine (Coutris et al., 2015).

The aim of this study was to explore the bacterial response to MEA-, MMA-, and DMA-nitramines exposure in pure cultures of environmental strains of *P. fluorescens* and *R. spp.* The two bacteria were selected for their high environmental relevance. They are both abundant in soils and water, and they represent the two major groups of bacteria based on cell wall structure (Gram staining). Moreover, other studies have found strains of *P. fluorescens* and *R. spp.* capable of degrading a range of different types of contaminants (Agarry & Solomon, 2008; Martinkova et al., 2009), including the cyclic nitramine explosive, known as RDX (Coleman et al., 1998). Bacterial growth was used as a response parameter. The potential for nitramine biodegradation was also assessed by determining concentrations before and after the experiments.

**Table 1: Summary of the available chronic and acute ecotoxic response expressed as the half effective concentration ($EC_{50}$, mg $L^{-1}$) or no observed concentration (NOEC, mg $L^{-1}$) of MEA-, MMA-, and DMA-nitramine. n.a. denotes not available.**

| | Test | MEA-nitramine | MMA-nitramine | DMA-nitramine |
|---|---|---|---|---|
| **Acute** | *Phytoplankton growth (Brakstad et al., 2011) | 2535 | 754 | >2000 |
| | *Vertabrate growth (Brakstad et al., 2011) | 1623 | 3314 | 2500 |

| | | | | |
|---|---|---|---|---|
| | Oyster larval development (Coutris et al., 2015) | 107 | n.a. | 47 |
| | Copepod mortality (Coutris et al., 2015) | NOEC ≥ 100 | n.a. | NOEC ≥ 100 |
| | Turbot mortality (Coutris et al., 2015) | NOEC ≥ 100 | n.a. | NOEC ≥ 100 |
| | Turbot growth (Coutris et al., 2015) | NOEC ≥ 100 | n.a. | NOEC ≥ 100 |
| | Algal growth (Coutris et al., 2015) | NOEC > 100 | n.a. | 591 |
| | Bacterial community, aerob respiration (Gundersen et al., 2014) | 4-8 | n.a. | n.a. |
| **Chronic** | Macroalgae germling growth (Coutris et al., 2015) | NOEC = 100 | n.a. | NOEC < 100 |
| | Copepod reproduction (Coutris et al., 2015) | 108 | n.a. | 70 |
| | Turbot DNA damage (Coutris et al., 2015) | Massive, NOEC < 1 | n.a. | 157 |

*Test results are from publically available reports.

## 2. Materials and Methods

### 2.1 Nitramine standard material

Standard material of 3-nitro-oxazolidon-2-one (MEA-nitramine precursor), MMA-, and DMA-nitramines at a purity of > 99 % was provided from the Norwegian University of Life sciences (NMBU), Ås, Norway (Antonsen et al., 2016).

### 2.2 Bacterial strains and sub-culturing procedure

Pure cultures of the environmental bacteria, P. *fluorescens* and *R. spp*., were provided from the University of California, Irvine, CA, USA, and were previously isolated from grassland leaf litter (Loma Ridge, CA, USA) as described by Mouginot et al. (2014).

Prior to every growth experiment, a two-step sub-culturing was performed. From pure colonies grown on lysogeny broth (LB) agar plates a loopful of bacteria was inoculated into 10/15 mL fresh liquid LB (pre-buffered capsules, Fischer Scientific, USA) in 50 mL capped Erlenmeyer flasks and incubated at 28 °C and shaken at 150 rpm. When stationary phase had been reached (determined by optical density), the cultures were diluted 1000-fold using fresh liquid LB. The second time stationary phase was reached the 1000-fold dilution was repeated, producing the ready culture to be used in the growth experiments.

### 2.3 Bacterial growth inhibition test

The bacterial growth inhibition test was conducted in accordance with the OECD Guideline Test no. 201. The bacteria were grown under the same favourable conditions described in section 2.2 while being exposed to 40, 60, 80 or 100 mg $L^{-1}$ of MEA-, MMA-, or DMA-nitramines. Negative control with tetracycline (50 mg $L^{-1}$) and blank control containing inoculum and liquid LB were included. Samples and controls were incubated

in triplicates, except for MEA-nitramine assays that employed duplicates. The experiment was run until stationary growth phase had been reached (t ≈ 33 h for *P. fluorescens* and t ≈ 43 h for *R.spp.*). Growth was quantified by cell turbidity measurements of optical density at ʎ = 600 nm ($OD_{600nm}$) using a spectrophotometer (BioTek Synergy H4, Winooski, VT, USA). The repeatability of the sample replicates was ≤ 8%, except for *P. fluorescens* exposed to 100 mg $L^{-1}$ MMA-nitramine at 12%.

At the start- and end of the experiment, sample aliquots (0.5 mL) were taken from samples and controls and kept frozen (-18 °C) until analysis of nitramine determination.

## 2.4 Determination of nitramines

The nitramines were determined using liquid chromatography-mass spectrometry (LC-MS) consisting of a Dionex Ultimate 3000 RS LC and a triple quadrupole TSQ Vantage™ MS equipped with heated electrospray ionization (Thermo Scientific, USA). The method used for MEA- and DMA-nitramine, with the exception of a few modifications, is described in Gundersen et al. (2017b). Modifications were required to deal with the severe matrix effects caused by the liquid LB and the bacterial lysate, and consisted of decreasing the injection volume (from 20 to 0.5 µL), increasing the analysis time (from 10 to 15 min), and for DMA-nitramine decreasing the water content in the mobile phase (from 90% to 80%). The method used for MMA-nitramine was similar to the one used for MEA-nitramine, but with the following specific settings: Monitored MS/MS transitions (*m*/*z*) were 75.1 → 46.0/60.0 for quantification and qualification, respectively, optimized selected reaction monitoring collision energy was 35 a.u. and the S-lens set to 20 a.u, the water content in the mobile phase was 95%, and the injection volume was 2.0 µL.

Prior to analysis, thawed samples were passed through 0.2 μm filters (regenerated cellulose, Chromacol, Thermo scientific, USA) to remove bacterial cells, and diluted 20-fold using Type II water (>1 MΩ cm at 25 °C). No loss of nitramine to the filter material was detected.

Matrix-matched five-point external calibration was used, and provided good linearity ($r^2 \geq 0.995$). The repeatability of sample triplicate readings was satisfactory (≤ 10% for MEA-nitramine, ≤ 18% for MMA-nitramine, and ≤ 7% for DMA-nitramine).

## 2.5 Data assessment

R language and environment for statistical computing and graphics (R Core Team, 2016) was used to analyse and illustrate the bacterial growth with the packages *grofit* (Kahm et al., 2010), *drfit* (Ranke, 2016), and *Hmisc* (Harrell Jr, 2016). The integral of the growth curve, including the lag- and the exponential phases, was used for the dose-response calculations.

The per cent inhibition ($\%I_i$) was calculated for each treatment concentration as follows:

$$\%I_i = \frac{(X_C - X_T)}{X_C} * 100 \quad (1)$$

Where

$X_C$ is the mean value of the blank controls

$X_T$ is the mean value of the treatment replicates

# 3. Results

## 3.1 Bacterial growth response

In Figures 1A-F the growth curves of *P. fluorescens* (left) and *R. spp* (right) during exposure to MEA- (top), MMA- (middle), and DMA-nitramines (bottom) are presented along with corresponding dose-response plots.

Remarkably, *R. spp* growth was significantly reduced by as much as 40% in the presence of MMA-nitramine compared to the blank control ($p \leq 0.05$, ANOVA). Moreover, the magnitude of the reduced growth was linearly correlated with the nitramine exposure level (Figure 1 E: $r^2 = 0.79$, $p \leq 0.05$). The $EC_{50}$ was estimated at 157 mg $L^{-1}$. No such effect was observed for *R. spp.* growth following exposure to MEA- or DMA-nitramines ($p > 0.05$). The ecotoxicity of MMA-nitramine has previously only been tested on phytoplankton and vertebrate growth (Table 1). These studies found that phytoplankton growth was more sensitive to MMA-nitramine than to the other two nitramines, with an $EC_{50}$ of 754 mg $L^{-1}$ (Brakstad et al., 2011). Growth of *P. fluorescens* was unaffected by exposure to MEA-, MMA-, and DMA-nitramine ($p \leq 0.05$, ANOVA).

The $EC_{50}$ value obtained for *R. spp.* growth by exposure to MMA-nitramine was two orders of magnitude higher than the $EC_{50}$ presented for the natural lake-water bacterial community exposed to MEA-nitramine (Gundersen et al., 2014). The reason for this difference may in part be due to the different growth conditions across the two studies: *R. spp* was grown here on rich medium, whereas the bacterial community was grown on low-nutrient medium similar to natural lake water (Gundersen et al., 2014). In the literature, several studies have found reduced toxic response from bacteria grown under optimal conditions as compared to the same type of bacteria grown under conditions mimicking

their natural habitat, e.g. see Czechowska and van der Meer (2011). Extended periods of exponential growth, such as observed here in this laboratory study, are not likely to occur in natural habitats. Additional factors that likely contribute to the observed difference in $EC_{50}$ include the different cell densities of the samples and the different strains of bacteria used in the two studies. The cause of *R. spp.* insensitivity to MEA- and DMA-nitramine is not known.

### 3.2 Nitramine stability

When exposed to *P. fluorescens,* a significant decay of all three nitramines was observed (two-tailed t-test, $p \leq 0.05$). This was not the case during growth of *R. spp.* The average decay caused by *P. fluorescens* was found to be 8 ± 5%, 9 ± 14%, and 10 ± 4% for MEA-, MMA-, and DMA-nitramine, respectively, and to be independent of initial nitramine concentration. (The relatively high uncertainty associated with MMA-nitramine is attributed to the overall poorer analytical signal for this nitramine.) In another study, Brakstad et al. (2018) found MEA-nitramine biodegradation of 27% over 28 days, and extending the experiment to 56 days resulted in almost complete loss of the nitramine. Considering the shorter duration of the experiments presented here (33 h for *P. fluorescens* and 43 h for *R. spp.*), the rate of nitramine decay by *P. fluorescens* was one order of magnitude higher than the decay obtained by Brakstad et al. (2018) over 28 days. With the assumption of continued exponential growth of *P. fluorescens,* 60% decay of the nitramines could be accomplished within 8 days. However, testing this assumption would require another experimental setup that allows for continued growth without reaching the stationary phase.

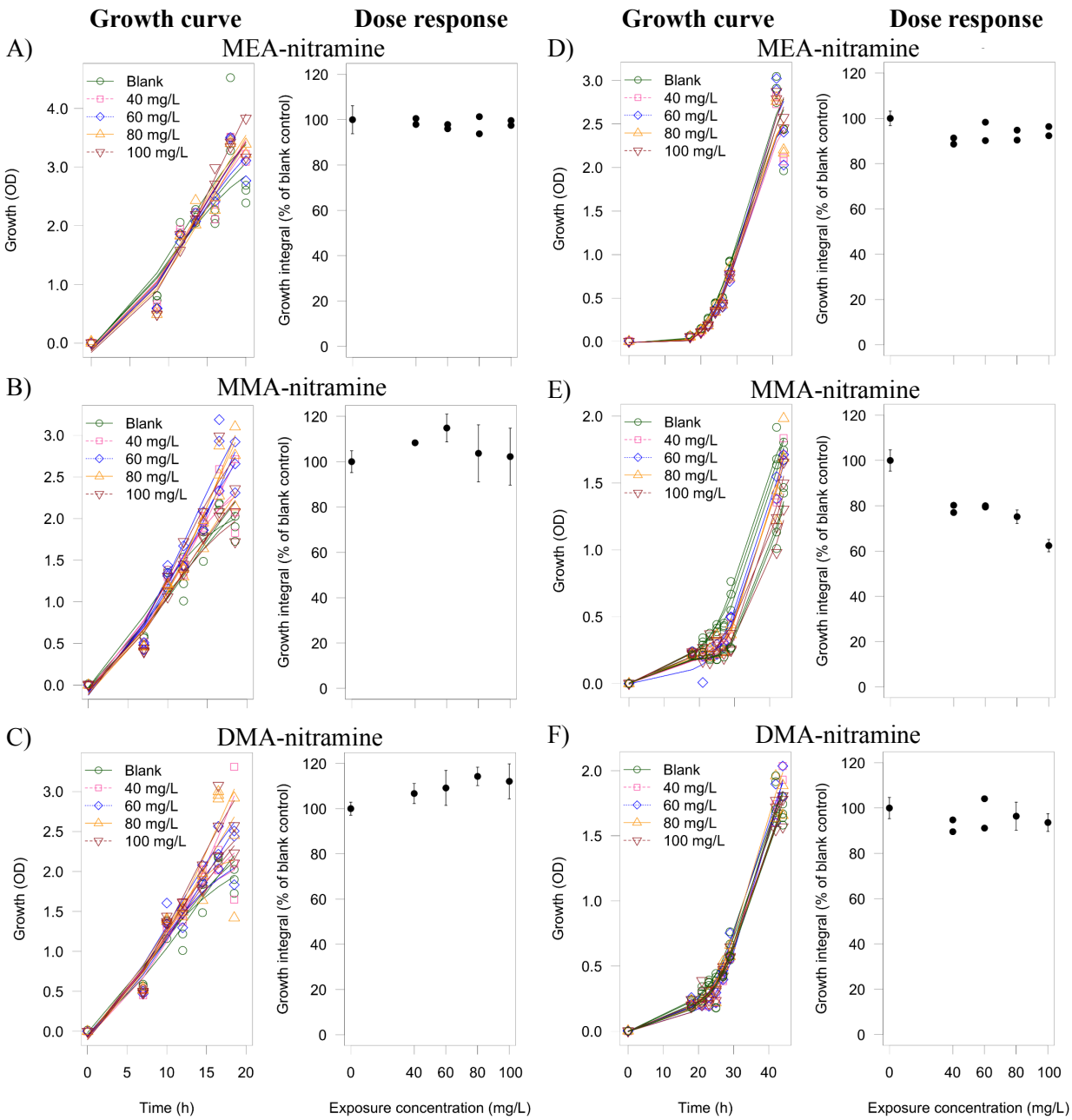


**Figure 1: Growth curves and dose-response curves for *P.* fluorescens (left side column: A, B, and C) and *R. spp.* (right side column: D, E, and F) exposed to the three nitramines (from top: MEA-, MMA-, and DMA-nitramine). Growth curves show sample replicate $OD_{600nm}$ readings by time (h). Dose-response curves were calculated from the integral of the growth curves and average values are provided with one standard deviation error bars (n = 2 for MEA-nitramine and n=3 for MMA- and DMA-nitramines).**

## 4. Concluding remarks

Environmental exposure to MEA-, MMA-, and DMA-nitramines is expected from use of the "benchmark" $CO_2$ capture amine solvent. These nitramines have been tested for ecotoxic effects on environmentally relevant *P. fluorescens* and *R. spp.* strains. Growth of *R. spp.* was inhibited by MMA-nitramine with an estimated $EC_{50}$ of 157 mg $L^{-1}$. No such effect was observed for *R. spp.* exposed to MEA- or DMA-nitramine. *P. fluorescens* was insensitive to all three nitramines. On the other hand, *P. fluorescens* was capable of degrading all the nitramines at rates of 8 - 10% during the 33 h experiment. Considering the large number of $CO_2$ capture plants needed to significantly reduce anthropogenic $CO_2$ emissions, future studies should explore responses of additional bacterial strains and communities to the potentially carcinogenic nitramines, both with regards to ecotoxicity- and biodegradation potential.

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Declaration of interest: None

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