Accepted Manuscript

Claire Coutris, Ailbhe L. Macken, Andrew R. Collins, Naouale El Yamani, Steven J. Brooks. Marine ecotoxicity of nitramines, transformation products of amine-based carbon capture technology. Science of The Total Environment. Volumes 527–528, 2015, pages 211-219.

The article has been published in final form by Elsevier at

http://dx.doi.org/10.1016/j.scitotenv.2015.04.119

Marine ecotoxicity of nitramines, transformation products of amine-based carbon capture technology

Running title: Aquatic ecotoxicity of nitramines

Claire Coutris*[1,2], Ailbhe L Macken[1], Andrew R Collins[3], Naouale El Yamani[3], Steven J Brooks[1]

[1] Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, 0349 Oslo, Norway

[2] Department of Soil Quality and Climate, Bioforsk, Høgskoleveien 7, 1430 Ås, Norway

[3] Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, P.O.1046 Blindern, 0316 Oslo, Norway

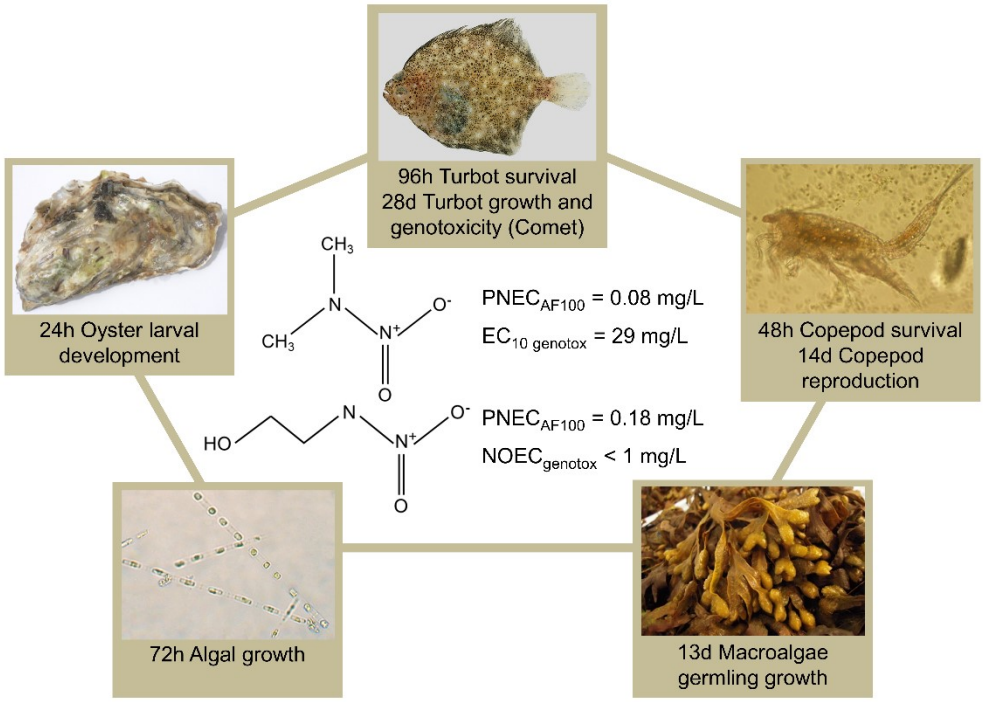
Abstract

In the context of reducing CO_2 emissions to the atmosphere, chemical absorption with amines is emerging as the most advanced technology for post combustion CO_2 capture from exhaust gases of fossil fuel power plants. Despite amine solvent recycling during the capture process, degradation products are formed and released into the environment, among them aliphatic nitramines, for which the environmental impact is unknown. In this study, we determined the acute and chronic toxicity of two nitramines identified as important transformation products of amine-based carbon capture, dimethylnitramine and ethanolnitramine, using a multi-trophic suite of bioassays. The results were then used to produce the first environmental risk assessment for the marine ecosystem. In addition, the *in vivo* genotoxicity of nitramines was studied by adapting the comet assay to cells from experimentally exposed fish. Overall, based on the whole organism bioassays, the toxicity of both nitramines was considered to be low. The most sensitive response to both compounds was found in oysters, and dimethylnitramine was consistently more toxic than ethanolnitramine in all bioassays. The Predicted No Effect Concentrations for dimethylnitramine and ethanolnitramine were 0.08 and 0.18 mg/L, respectively. The genotoxicity assessment revealed contrasting results to the whole organism bioassays, with ethanolnitramine found to be more genotoxic than dimethylnitramine by three orders of magnitude. At the lowest ethanolnitramine concentration (1 mg/L), 84 % DNA damage was observed, whereas 100 mg/L dimethylnitramine was required to cause 37 % DNA damage. The mechanisms of genotoxicity were also shown to differ between the two compounds, with oxidation of the DNA bases responsible for over 90 % of the genotoxicity of dimethylnitramine, whereas DNA strand breaks and alkali-labile sites were responsible for over 90 % of the genotoxicity of ethanolnitramine. Fish exposed to > 3 mg/L ethanolnitramine had virtually no DNA left in their red blood cells.

Highlights

- The environmental risk posed by nitramines, CO_2 capture by-products, was unknown.
- A multi-trophic suite of bioassays was used to assess ecotoxicity and genotoxicity.
- Nitramine toxicity through necrosis was considered low.
- The first risk assessment for dimethylnitramine and ethanolnitramine was produced.
- Ethanolnitramine induced massive DNA damage in turbot.

Graphical abstract



Keywords

Environmental risk assessment, post combustion CO_2 capture, 2-(nitroamino)ethanol, single cell gel electrophoresis

1. Introduction

The capture and storage of carbon dioxide (CO_2) from the exhaust gases of fossil fuel power stations is an important technology for reducing CO_2 emissions to the atmosphere. Approximately 43% of the global CO_2 emissions in 2011 were attributed to the generation of electricity from fossil fuel power stations (IEA, 2013). Chemical absorption with amines is emerging as the most advanced mitigation technology for post combustion capture of CO_2 from fossil fuel power stations (Reynolds et al., 2012). The exhaust gas from the power station is bubbled through an amine solution in the absorber unit, producing a CO_2-saturated amine solution. In the stripper unit, heat separates CO_2 and amines, resulting in pure CO_2 ready to be stored on one side, and amine solution, recycled and sent back to the absorber unit, on the other side. The main advantage of post combustion CO_2 capture is that the technology can be incorporated into existing power plants, avoiding the need to build new facilities. In addition, the technology benefits from almost two decades of full-scale experience for removal of CO_2 from natural gas (Lackner, 2009; Reynolds et al., 2012). However, the environmental impacts of replacing CO_2 emissions with the discharge of amine solvents and their chemical transformation products, as by-products of the capturing process, are largely unknown.

There is increasing public and environmental concern with regard to two main groups of amine transformation products, nitrosamine and nitramine contamination in air and drinking water supplies downstream of amine-based CO_2 capture plants (Reynolds et al., 2012). A few *in vitro* studies showed that nitramines could be carcinogenic and mutagenic (Fjellsbø et al., 2014; Wagner et al., 2014). Recent theoretical modelling and controlled laboratory experiments reported the occurrence of nitramines as transformation products of amines in the carbon capture process within the discharge effluent (Bråten et al., 2008; Nielsen et al., 2009). Two of the nitramine compounds that were identified included dimethylnitramine (CAS No. 4164-28-7) and ethanolnitramine (CAS No. 74386-82-6). However, despite the likelihood of these compounds increasing in the environment, with the potential to cause environmental harm, no environmental

toxicity data for these compounds currently exist. Due to the location of some CO_2 capture and storage plants along the coastline, as well as their tendency to partition to the water phase, amines and their transformation products are likely to end up in the marine environment. Therefore, an ecotoxicity assessment performed on marine organisms is needed in order to provide an appropriate assessment of the environmental risk.

In addition to organismal toxicity, there is a real concern that nitrosamines and nitramines can cause genotoxic effects (Fjellsbø et al., 2014; Frei et al., 1984, 1986; Wagner et al., 2012). However, although nitramines are considered not as potent as nitrosamines in terms of their genotoxic potential, the greater persistence of nitramines in the environment increases their likelihood to cause environmental harm (Låg et al., 2011). In the present study, the comet assay was used in fish exposed to sub-lethal concentrations of the two nitramine compounds to assess their potential *in vivo* genotoxicity. The comet assay detects DNA strand breaks and alkali-labile sites (i.e. apurinic and apyrimidinic sites or AP sites), which arise from the loss of a damaged base. In normal cells, strands breaks and AP sites are not the only kind of damage. Oxidized bases are present in at least as great a number and can be readily detected with the comet assay, by incorporating an additional step involving formamidopyrimidine DNA glycosylase (FPG, Collins et al., 2008).

The first aim of the study was to determine the acute and chronic ecotoxicity of dimethylnitramine and ethanolnitramine using a suite of standardized and non-standardized tests on marine species belonging to several trophic groups. The ecotoxicity data were then used to assess the environmental risk of the two nitramine compounds in the marine environment. The second aim was to determine the potential *in vivo* genotoxicity of these two compounds by measuring the frequency of DNA damage in fish blood.

2. Materials and methods

102 2.1. Nitramines

103 Dimethylnitramine (CAS No. 4164-28-7, purity >98%) and ethanolnitramine (CAS No. 74386-82-
104 6, purity 98%) were purchased from Chiron AS (Norway). Stock solutions at 5 g/L were prepared
105 in ultrapure water (Milli-Q, Millipore, USA) and stored at 4 °C until use. Aged filtered (0.45 µm)
106 seawater collected from a depth of 60 m from the Outer Oslo fjord, Norway, was used as a
107 negative control and for the preparation of dilution series. In all bioassays, test solutions were
108 prepared on the first day of testing (and, in chronic tests, on days where exposure media had to
109 be renewed), by diluting stock solutions with appropriate amounts of filtered seawater to produce
110 the required concentration series.

111

112 2.2. Bioassays

113 A bioassay battery consisting of three acute toxicity tests (24 h oyster larval development, 48 h
114 copepod mortality and 96 h turbot mortality), a sub-chronic toxicity test (72 h algal growth), and
115 three chronic toxicity tests (13 d macroalgae germling growth, 14 d copepod reproduction and 28
116 d turbot growth) was applied for both nitramines.

117

118 2.2.1. Oyster larval development

119 The toxicity of nitramines to the developing embryos of the Pacific oyster *Crassostrea gigas* was
120 assessed based on the standard protocol ASTM E724 (ASTM, 1994). For both nitramines, the
121 concentration series tested was 0, 2, 4, 9, 21, 45 and 100 mg/L. Zinc sulphate ($ZnSO_4.7H_2O$,
122 CAS No.7446-20-0) was used as a positive control.

123 Oysters were obtained in spawning condition from Guernsey Sea Farms Ltd, Guernsey, UK.
124 Separate male and female gamete suspensions were made by stripping the gonads and placing
125 them in filtered seawater. Prior to fertilization, egg density was adjusted to 3000 ± 300 eggs/mL
126 and sperm mobility was confirmed by microscopic examination at ×400 magnification. For
127 fertilization, 10 mL of the sperm suspension was added to 1 L of the egg suspension. The
128 fertilized embryos were allowed to develop into trocophore larvae (2 h after fertilization) before

129 they were placed in the test vessels. The test was performed in 12 well microplates with four
130 replicate vessels for each test concentration and eight replicate vessels for controls. The number
131 of fertilized embryos added to each replicate vessel was approximately 50 per mL. The vessels
132 were incubated in the dark for 24 ± 2 h at 24 ± 1 °C. The test was terminated and the embryos
133 fixed with the addition of 200 µL of neutral buffered formalin. Dissolved oxygen, salinity and pH
134 were measured in the high, medium and low concentration test solutions at the start and the end
135 of the exposure period and were within accepted validity criteria (ASTM, 1994). Samples of the
136 lowest and highest test concentration solutions were taken at the start and the end of the
137 exposure period for analytical determination of nitramine concentrations.
138 The number of normal D-larvae (normally developed embryos) was counted in 1 mL of test
139 solution for each test vessel after 24 ± 2 h using an inverted microscope at ×100 magnification.
140 Normal D-larvae were considered those that possessed a completely formed shell and contained
141 cellular material. Any small differences in the shape of the shell e.g. pinch to the hinge, was still
142 counted as normal as long as it was still fully D shaped. The percentage development from
143 trocophore to veliger (D-shaped) larvae in the controls was assessed as a quality control measure
144 and was within accepted validity criteria.
145
146 2.2.2. Copepod mortality
147 In the copepod mortality test, 6 ± 2 day old *Tisbe battagliai* (first copepodid stage) were exposed
148 to nitramines for a period of 48 h based on the ISO standard procedure 14669 (ISO, 1999;
149 Environment Agency, 2007). For both nitramines, the concentration series tested was 0, 2, 4, 9,
150 21, 45 and 100 mg/L. Potassium dichromate ($K_2Cr_2O_7$, CAS No.7778-50-9) was used as a
151 positive control. The test was performed with four replicate wells for each test concentration of
152 nitramine, negative control and positive control, with each well containing 5 individuals. Test
153 organisms were maintained at 20 ± 1 °C and were not fed during the test. Samples of the lowest
154 and highest test concentration solutions were taken at the start and the end of the exposure
155 period for analytical determination of nitramine concentrations. Organisms were considered dead

when no swimming or appendage movement was observed within 10 seconds of gently agitating the test container.

2.2.3. Turbot mortality

Juvenile turbot *Scophthalmus maximus* were exposed to nitramines for a period of 96 h based on the OECD test guideline 203 (OECD, 1992). Hatchery-reared juvenile turbot were obtained from Maximus A/S, Denmark, and acclimatized at the Marine research station in Solbergstrand, Norway, for approximately 4 weeks prior to testing. For both nitramines, the concentration series tested was 0, 1, 3, 10, 30 and 100 mg/L. The experimental setup included one 20 L aquarium containing 10 fish per nitramine concentration, and two 20 L control aquaria with 10 fish in each. Individual fish weight was 2.0 ± 0.2 g (wet weight) at the beginning of the test. The test was performed at 16 ± 1 °C, with a 16 h light: 8 h dark cycle. Fish were fed a ration of 2 % body weight per day (Nutra Parr 1.5 mm, Skretting A/S, Norway). Water was aerated continuously with airstones to ensure a satisfactory dissolved oxygen concentration. Test solutions were half exchanged once during the test. Samples of the lowest and highest test concentration solutions were taken at the start and the end of the exposure period for analytical determination of nitramine concentrations. Mortality was checked within the first 2 h and then every 24 h until the end of the test.

2.2.4. Algal growth

The unicellular algae *Skeletonema costatum* were exposed for 72 h to a concentration range of nitramines (0, 18, 32, 56, 10, 180, 320 mg/L for dimethylnitramine and 0, 200, 360, 1120, 2000, 3600 mg/L for ethanolnitramine), following the ISO standard procedure 10253 (ISO, 2006). Growth medium was prepared by adding ISO 10253 stock solutions to filtered seawater. The growth of the algal inoculum (5.6×10^6 cells/L) placed on an orbital shaker in continuous cool white fluorescent light (68 ± 4 µmol/m^2/s, Philips TLD 36W/950) under constant temperature (20 ± 1 °C) was measured every 24 h for the duration of the test using a Beckman Coulter Multisizer 3

(Beckman Coulter, USA). Three replicates were used per nitramine concentration with 6 replicates for the control. Samples of the lowest and highest test concentration solutions were taken at the beginning of the exposure period for analytical determination of nitramine concentrations.

The relative growth rate (RGR) for each test concentration was calculated using the equation: $RGR = (lnN_n - lnN_0)/(t_n - t_0)$, where N_n = Cell density at time t_n, N_0 = Cell density at time zero (t_0). The percentage inhibition of growth rate as compared to the control was calculated for each concentration.

2.2.5. Macroalgae germling growth

The toxicity of nitramines to the growth of *Fucus vesiculosus* germlings was assessed based on the method described by Brooks et al. (2008). The algae were collected in the Oslo fjord (GPS 59.904 N, 10.702 E) and thoroughly rinsed with filtered seawater. The receptacles were then left for 6 h in seawater to allow for the release of eggs and sperm cells. The resulting zygote suspension was filtered through a 90 µm sieve, collected on a 25 µm sieve, and its quality and density assessed at ×20 magnification. Microscope slides were placed in a shallow tray, covered to a depth of 2 cm with seawater, and 1 mL of zygote suspension (50-100 zygotes) was placed onto each individual slide. The tray, covered with film to prevent evaporation, was left for 48 h to enable the zygotes to attach and develop into germlings. After 48 h, 5 or 6 slides with at least 6 germlings on each were placed in separate Coplin jars, one jar per concentration. Due to low abundance of germlings, the setup was reduced to control, dimethylnitramine at 100 mg/L and 200 mg/L, and ethanolnitramine at 100 mg/L and 500 mg/L. Renewal of the exposure solutions was performed on day 7. Exposure solutions were sampled at the start and at the end of the exposure period for analytical determination of nitramine concentrations. All germlings from each slide were photographed and measured on day 0, 4, 7, 10 and 13. The RGR was calculated using the equation: $RGR = (lnL_n - lnL_0)/(t_n - t_0)$, where L_n = germling length at time t_n, L_0 = germling length at time zero (t_0).

210

211 2.2.6. Copepod reproduction

212 The reproductive output of *T. battagliai* was recorded over a 14 d exposure period to nitramines.
213 Test solutions were prepared by diluting stock solutions with appropriate amounts of filtered
214 seawater to produce a concentration series of 0, 12.5, 25, 50 and 100 mg/L nitramine. The test
215 was performed in 12-well microplates with 10 individually housed organisms (replicates) per
216 concentration including controls. Tests were initiated by introducing female copepods at the start
217 of their adult reproductive period (after the appearance of the first egg sac). Test organisms were
218 maintained at 21 ± 1 °C with a 16 h light: 8 h dark cycle, and were fed a diet of *Rhodomonas*
219 *baltica* at a rate of 2 x 10^5 cells/mL at each renewal period. Exposure solutions were renewed on
220 day 2, 6, 9, and 12. At each renewal, adult females were transferred to a new set of test vessels
221 containing fresh exposure solutions and algae. The old test vessels were then poured and
222 thoroughly rinsed into counting chambers and nauplii were counted. Observation of mortality and
223 behavior were made daily. Samples of the lowest and highest test concentration solutions were
224 taken at the beginning and on day 2, 12 and 14 of the exposure period for analytical
225 determination of nitramine concentrations.

226

227 2.2.7. Turbot growth

228 The effect of nitramines on the growth of juvenile turbot *S. maximus* was determined based on
229 the standard guideline OECD 215 (OECD, 2000). Hatchery-reared juvenile turbot were obtained
230 from Maximus A/S, Denmark, and acclimatized at the Marine research station in Solbergstrand,
231 Norway, for approximately 4 weeks prior to testing. The experimental design included one 20 L
232 aquarium containing 10 fish per nitramine concentration (1, 3, 10, 30, 100 mg/L), and two 20 L
233 control aquaria with 10 fish in each. Average individual fish weight was 2.0 ± 0.2 g (wet weight) at
234 the beginning of the test. The water temperature was maintained at 16 ± 1 °C, continuously
235 aerated and semi static conditions (50 % renewal was carried out twice a week). Fish were fed a
236 ration of 2% body weight per day (Nutra Parr 1.5 mm, Skretting A/S, Norway). Samples of the

lowest and highest test concentration solutions were taken at the beginning and on day 2, 24 and 28 of the exposure period for analytical determination of nitramine concentrations. After 28 days, fish were weighed and the 'pseudo' specific growth rate (SGR) was calculated using the equation: $SGR = (lnW_n - \overline{lnW_0})/(t_n - t_0) \times 100$, where lnW_n = logarithm of the weight of an individual fish at time t_n, and $\overline{lnW_0}$ = average of the logarithms of the weights of individual fish at time zero (t_0).

2.3. Comet assay

DNA damage was analyzed in blood samples collected from the caudal vein of juvenile turbot *S. maximus* after 28 day exposure to nitramines, using a version of the comet assay (Collins, 2004; Collins and Azqueta, 2012) in which 12 mini-gels are set on each slide (Shaposhnikov et al., 2010). The experimental setup included 4 concentrations of ethanolnitramine and dimethylnitramine (1, 3, 30, 100 mg/L), with 6 fish per nitramine concentration and 12 fish per control. Cell density was optimized beforehand by preparing blood dilutions in PBS and measuring cell number with the aid of a Coulter counter. A cell density of 2.5×10^5/mL was found to give an appropriate number of cells per mini-gel.

Blood samples were diluted in PBS (1:10000) and kept on ice. For each blood sample, 15 µL of cell suspension at 2.5×10^5/mL and 70 µL of 1 % low melting point agarose in PBS at 37 °C were mixed by pipetting up and down once. One 5 µL drop of this agarose-cell suspension was placed on three microscope glass slides pre-coated with normal melting point agarose, each intended for a different treatment (with gels from 12 different samples on each slide). All slides were incubated in lysis buffer consisting of 2.5 M NaCl, 0.1 M EDTA Titriplex (CAS nr. 60-00-4), 10 mM Trizma base (CAS nr. 77-86-1), and 1 % Triton X-100, with pH adjusted to 10 with NaOH. Lysis removes membranes, soluble cell constituents, and histones, leaving DNA as nucleoids. One of the three slides, referred to as *LYS*, was simply lysed, in order to measure DNA strand breaks and alkali-labile sites.

Measurement of DNA base oxidation requires subsequent digestion with the enzyme formamidopyrimidine DNA glycosylase (FPG, provided by A. Collins, University of Oslo), which

detects oxidized purines. After lysis, the other two slides, *FPG* and *BUF,* were gently washed 3 times for 5 min in enzyme buffer at pH 8.0 (10 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 g/L BSA) and then incubated for 30 min at 37 °C in either enzyme buffer (without enzyme) (slide *BUF*), or with FPG enzyme made up in the same buffer at 1:50000 (slide *FPG*). After incubation, these two slides and the *LYS* slide were placed in a horizontal gel electrophoresis tank and DNA was allowed to unwind for 20 min in cold alkaline solution (0.3 M NaOH and 1 mM Na_2EDTA, pH>13) followed by electrophoresis for 20 min at 0.8 V/cm at 4 °C. Slides were then washed twice with cold PBS for 5 min and for 1 min in distilled water. Gels were dehydrated and DNA fixed by incubating slides in 70 % ethanol for 5 min and in absolute ethanol for a further 5 min. They were then placed on the bench at room temperature to dry.

For visualization, gels were stained in a bath with SYBR Gold (Molecular Probes) at a 1:10000 dilution of stock solution in TE buffer (10 mM Tris, 1 mM EDTA pH 7.5) as recommended by the manufacturer. Stained nucleoids (comets) were visualized using an epifluorescence microscope at ×20 magnification. The slides were coded and the entire analysis was carried out blind. Images were analyzed with Comet Assay IV software (Perspective Instruments), recording the % DNA in the tail of 50 comets per gel. The scores (% tail DNA) of gels on *BUF* slides were subtracted from the scores of *FPG* slides to give the net FPG-sensitive sites (Collins et al., 2008).

2.4. Chemical analysis

The method used to determine concentrations of dimethylnitramine and ethanolnitramine in exposure media was modified from the USEPA method 521 (USEPA, 2004). Solid phase extraction (SPE) of samples was carried out on activated charcoal columns (SPE EPA method 521 and 522, 6 mL/2 g activated charcoal, Restek, USA). To avoid peak saturation on the SPE column and the chromatograph, samples with high nominal concentrations of nitramines were diluted with ultrapure water to a nominal concentration of 2 mg/L nitramine. Columns were conditioned by successive solvent washes (3 mL dichloromethane, 9 mL methanol, 15 mL ultrapure water), then loaded with 5 mL of sample, followed by 3 mL of ultrapure water. Columns

were then eluted with 15 mL dichloromethane. Samples were dried with sodium sulfate and spiked with internal standard (2.5 µg 1,2,4-trichlorobenzene and 1.25 µg tetrachloroethane). Samples were finally reduced to 0.5 mL under a nitrogen flow and added 0.5 mL methanol.

For quality assurance, blanks and spiked samples containing 2 mg/L dimethylnitramine and ethanolnitramine, processed in the same way as the samples, and 5 standard solutions in the range 0.1 to 10 mg/L, were analyzed. The analysis was performed by gas chromatography Agilent 6890N with a 63Ni µECD detector. GC separation was performed using an Agilent J&W DB5 capillary column (30 m × 0.25 mm, 1.0 µm film). The oven temperature program was setup as follows: 60 °C held for 2 min, then ramped at 7 °C/min to 125 °C, held for 3 min, then ramped at 10 °C/min to 250 °C held for 2 min. Injection temperature was 200 °C, detector temperature was 240 °C, and carrier gas flow (hydrogen) was 3 mL/min.

2.5. Data analysis

Where toxicity was observed, calculation of toxicity parameters (Effect Concentrations EC_{10}, EC_{20} and EC_{50}) was performed using the Hill model of the Excel macro REGTOX developed by Eric Vindimian (Vindimian et al., 1983). Homogeneity of variance was checked with a Levene's test before a one-way analysis of variance (ANOVA) was used to evaluate effects of nitramines on the various biological endpoints, and differences were identified with a Holm-Sidak test. When normality and homoscedasticity assumptions were not reached, a Kruskal-Wallis analysis of variance on ranks was used, followed by a Dunn's test (SigmaPlot 12.5, Systat software).

3. Results

3.1. Chemical analysis of exposure media

The recovery in the spiked samples was 100-120 % and 90-118 % for dimethylnitramine and ethanolnitramine, respectively. Dimethylnitramine and ethanolnitramine were persistent over time under experimental exposure conditions. In addition, nitramines were also found to be remarkably stable at 4 °C, as concentrations in exposure media remained unchanged after one year.

Measured concentrations in exposure media compared well with the respective nominal concentrations and were typically within ± 10 % of the nominal concentration. The only exception was the copepod (*T. battagliai*) acute test where measured ethanolnitramine concentrations were 3 times below the nominal concentrations. In this case, the determination of toxicity values was based on measured concentrations, rather than on nominal concentrations.

3.2. Acute toxicity

3.2.1. Oyster larval development

The positive control (zinc sulphate) used for the oyster embryo bioassay confirmed that the sensitivity of the embryos was within the quality control limits for the test. Dimethylnitramine caused a significant reduction in the number of normal D larvae at 45 mg/L and completely inhibited the development of the embryos at 100 mg/L (Figure 1). Ethanolnitramine was slightly less toxic to the oyster larvae than dimethylnitramine, with a significant reduction in the number of normal D larvae at the highest exposure of 100 mg/L. The calculated ecotoxicity endpoints are summarized in Table 1. For dimethylnitramine, the no observable effect concentration (NOEC), lowest observable effect concentration (LOEC) and the concentration affecting 50 % of the population (EC_{50}) were 21, 45 and 47 mg/L, respectively. For ethanolnitramine, NOEC, LOEC and EC_{50} were 45, 100 and 107 mg/L.

3.2.2. Copepod mortality

The copepod *T. battagliai* responded as expected to the positive control (potassium dichromate), which confirmed the validity of the bioassay. No significant mortality was observed in copepods exposed for 48 h to dimethylnitramine or ethanolnitramine at concentrations up to 100 mg/L (Table 1).

3.2.3. Turbot mortality

There was no significant mortality observed in juvenile turbot *S. maximus* following a 96 h exposure to dimethylnitramine or ethanolnitramine at concentrations up to 100 mg/L (Table 1).

3.3. Sub-chronic and chronic toxicity

3.3.1. Algal growth

The growth rate of the unicellular algae *S. costatum* after 72 h exposure was significantly reduced by dimethylnitramine at concentrations ≥ 32 mg/L (Figure 2A). The calculated NOEC, LOEC and EC_{10} concentrations for dimethylnitramine were 18, 32 and 48 mg/L, respectively (Table 1). The relatively high EC_{50} concentration (591 mg/L, extrapolated value) despite a LOEC of 32 mg/L was reflective of the limited effect on growth achieved at higher exposure concentrations, with only approximately 40 % reduction in growth rate achieved at the top concentration of 320 mg/L. Ethanolnitramine, on the other hand, had no negative effect on the growth of *S. costatum*, unless very high concentrations, well above those considered environmentally relevant, were reached. The experimental NOEC and LOEC for ethanolnitramine were 2000 and 3600 mg/L, respectively, and are reported as >100 mg/L in Table 1.

3.3.2. Macroalgae germling growth

The growth of *F. vesiculosus* germlings after 13 day exposure was reduced by 45 % and 64 % at 100 mg/L and 200 mg/L dimethylnitramine, respectively, whereas ethanolnitramine had no significant effect at 100 mg/L, and induced 84 % growth reduction at 500 mg/L (Figure 2B, table 1).

3.3.3. Copepod reproduction

The number of offspring (mean ± SD) produced over a 14 day period per adult female *T. battagliai* was 124 ± 37 in the controls (Figure 2C). In the presence of dimethylnitramine, a significant decrease in reproductive output was observed at 50 mg/L with 57 ± 45 offspring. For ethanolnitramine, a significant reduction in reproductive output was achieved following exposure

371 to 100 mg/L (63 ± 33 offspring). The calculated NOEC, LOEC and EC_{50} concentrations were 25,
372 50 and 70 mg/L, respectively, for dimethylnitramine, and 50, 100 and 108 mg/L, respectively, for
373 ethanolnitramine (Table 1).
374
375 3.3.4. Turbot growth
376 No significant decrease in the growth rate of the turbot *S. maximus* was recorded following a 28
377 day exposure to 100 mg/L of dimethylnitramine or ethanolamine (Figure 2D, Table 1). Large
378 variations in growth rate were observed within groups, with mean values between 0.7-1.6 times
379 that of mean control fish for dimethylnitramine and mean values between 0.7-2.3 times the growth
380 rate of the control fish for ethanolamine. An apparent increase in turbot growth rate was observed
381 at 3 and 10 mg/L ethanolnitramine, although no statistically significant difference was found.
382
383 3.4. Genotoxicity
384 Dimethylnitramine induced DNA damage in red blood cells of juvenile turbot after 28 day
385 exposure as shown in figure 3. The percentage of tail DNA for total damage (i.e. strand-breaks,
386 alkali-labile and fpg-sensitive sites) was 11 % at 30 mg/L, and 37 % at 100 mg/L, compared to < 1
387 % in control fish. DNA damage was almost entirely due to the presence of oxidized bases (fpg-
388 sensitive sites). The estimated EC_{10}, EC_{20} and EC_{50} (with their 95% confidence interval) for total
389 DNA damage were 29 (22-36) mg/L, 55 (47-63) mg/L, and 157 (129-209) mg/L dimethylnitramine,
390 respectively (for EC_{50}, values are outside the concentration range tested).
391 All concentrations (from 1 to 100 mg/L) of ethanolnitramine induced massive DNA damage in
392 turbot blood cells (Figure 3). The magnitude of the effect was much higher than observed with
393 dimethylnitramine; comets were already close to saturation at 1 mg/L (84 % tail DNA, total
394 damage) and only ghosts were visible at 30 and 100 mg/L (virtually no DNA left in cells). In the
395 absence of data points between 0 and 1 mg/L, EC values could not be calculated. For
396 ethanolnitramine, NOEC and LOEC were <1 mg/L and ≤1 mg/L, respectively.
397

4. Discussion

The main source of nitramines in the environment has been through their use in weapons manufacturing (Ryon et al., 1984). High environmental concentrations of nitramines, hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro 1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), which are important ingredients in explosives, have been found in both ground and surface waters near to army ammunition plants. Concentrations as high as 1.9 mg/L and 0.21 mg/L have been reported for RDX and HMX respectively (Best et al., 1999; Lewin et al., 1997). Data for the environmental concentrations of other nitramine compounds not associated with ammunition plants are limited. However, chlorination of public waters has led to concerns about nitramine and nitrosamine formation. For example, dimethylnitramine was detected at median values of 64.5, 50 and 203 ng/L in outdoor pools, indoor pools, and hot tubs, respectively (Walse and Mitch, 2008), with the nitramine levels comparable to measured levels of the nitrosamine, N-nitrosodimethylamine.

The introduction of post combustion CO_2 capture plants using amines, where monoethanolamine is the mostly widely used, has the potential to contribute as a nitramine source (Da Silva and Booth, 2013). The concentration of nitramines from such facilities depends on a variety of factors, including the amine solvent used, the composition of the flue gas, and the plant operation conditions. Emission data from post combustion CO_2 capture facilities with amines are relatively scarce. This is partly due to the confidentiality issues surrounding the solvent systems used by the various companies, as well as the only relatively recent interest in CCS emissions. Emission data that are available mostly derive from pilot plants where predominantly monoethanolamine (MEA) has been used as the solvent. A recent summary of emission data from a range of pilot plants including Maasvlakte (Texas, USA), Esbjerg (Denmark), and Mongstad (Norway) have indicated low concentrations (ppb levels) of nitramines (Da Silva et al., 2013). Monitoring of the lakes and fjords within the catchment area of the test plant at Mongstad did not detect nitramines above quantification limits (0.7-1.5 ng/L) (Grung et al., 2012). Due to the recent change of

425 government in Norway, although the test facility remains operational, the full scale launch of post
426 combustion CO_2 capture with amines has been put on hold. Therefore, accumulation of
427 nitramines as degradation product of amines in CO_2 capture may not be a threat at Mongstad in
428 the short term. However, outside of Norway, the first commercial post-combustion coal fired
429 carbon capture and storage facility was started in September 2014 at the SaskPower Boundary
430 Dam power station in Estevan, Saskatchewan (Canada) (Stéphenne, 2014). Emission data for
431 nitramines from this facility were not available at the time of publication. The success of this
432 facility is likely to have a bearing on the implementation of similar power stations throughout the
433 world for tackling CO_2 emissions. In addition, with increasing pressures on nations to limit and
434 reduce their carbon footprint, such post combustion technology may be likely to become favorable
435 and economically viable in the future.
436
437 Nitramines are thought to preferentially partition to the water phase, suggesting potential
438 exposure to aquatic organisms. Based on the whole organism toxicity bioassays, which included
439 both acute (survival) and chronic (growth and reproduction) endpoints in marine species
440 belonging to several trophic levels, dimethylnitramine and ethanolnitramine were considered to
441 exhibit low levels of toxicity. Large interspecific differences in sensitivity of the two compounds
442 were observed. Among acute toxicity assays, the oyster larval development test was the most
443 sensitive to both nitramines. The reason for this may be linked to the fact that susceptibility to
444 toxicants is often inversely related to the age of exposed organisms, and oysters being exposed
445 at an earlier developmental stage (embryo/ larvae), compared to fish (juvenile) and copepod
446 (copepodite).
447
448 Dimethylnitramine was the more toxic of the two compounds, with the most sensitive LOEC found
449 in the unicellular algae *S. costatum* and the embryos of the oyster *C. gigas* at 32 mg/L and 45
450 mg/L, respectively. However, in *S. costatum* higher concentrations of dimethylnitramine did not
451 fully inhibit growth of the algae, which led to a particularly high EC_{50} of 591 mg/L. In contrast, the

most sensitive LOEC for ethanolnitramine was only 100 mg/L from both the embryos of the oyster and the copepod reproduction. These were also the only two species where ethanolnitramine toxicity was found and where EC values could be calculated.

Nitrosamines have been well studied as components of tobacco smoke and cured meats, and are known to be potent carcinogens (Shah and Karnes, 2010). Until recently, it was unknown whether the carcinogenic potency of nitramines was comparable to that of nitrosamines. However, *in vitro* mutagenicity bioassays in *Salmonella typhimurium* and acute genotoxicity in Chinese hamster ovary cells showed that nitramines were 15 times less mutagenic/genotoxic than their nitrosamine analogues (Wagner et al., 2014). Despite this, current limits for nitramines in drinking water in Norway have been set at 4 ng/L, which are based on the most potent nitrosamine, N-nitrosodimethylamine. The conservative approach taken is due to the lack of genotoxic/mutagenic and carcinogenic data available for nitramines in order to propose reliable exposure limits.

For our second goal, namely the determination of *in vivo* genotoxicity of nitramines, we adapted and applied the comet assay – a sensitive method for measuring DNA damage – to cells from experimentally exposed turbot. The genotoxicity assessment of the two compounds revealed contrasting results to the whole organism toxicity bioassays, with ethanolnitramine found to be more genotoxic than dimethylnitramine by three orders of magnitude. At the lowest ethanolnitramine concentration (1 mg/L), 84 % DNA damage was observed. In contrast, 100 mg/L dimethylnitramine was required to cause 37 % DNA damage. The mechanisms of genotoxicity were also shown to differ between the two compounds, with oxidation of the DNA bases responsible for over 90 % of the genotoxicity of dimethylnitramine, whereas DNA strand breaks and alkali-labile sites were responsible for over 90 % of the genotoxicity of ethanolnitramine. Fish exposed to > 3 mg/L ethanolnitramine had virtually no DNA left in their red blood cells.

The large difference in genotoxicity observed between the two nitramine compounds highlights the danger of inferring toxicity from structurally similar compounds for environmental risk assessment, and conversely shows the importance of compound specific assessments.

Fjellsbø et al. (2014) evaluated the genotoxicity of nitramines including dimethylnitramine and ethanolnitramine, using the bacterial reverse mutation (Ames) test, the cytokinesis block micronucleus (CBMN) assay and the comet assay. Ethanolnitramine was found to show mutagenic potential with the Ames test, was weakly genotoxic in the CBMN assay, but showed no increase in DNA strand breaks in the comet assay despite concentrations up to 1.9 g/L. The lack of genotoxic response in the comet assay is in contrast to the present study. Differences in the type and duration of exposure between the studies may explain the differences in toxicity observed. A 3 h *in vitro* exposure of human TK6 cells to 1.9 g/L ethanolnitramine failed to show a significant increase in DNA strand break frequency, in spite of the positive CBMN response (Fjellsbø et al., 2014). However, our 28 day *in vivo* exposure produced significant DNA damage at the lowest concentration tested (1 mg/L). It is possible that metabolic activation of the ethanolnitramine does not occur to a sufficient extent in a 3 h exposure, whereas the longer incubation period of the CBMN assay or our *in vivo* exposure allows activation to occur. In addition, the likely differences in specific metabolising capacity (due to variations in cytochrome P450 enzymes) between fish and humans could account for the divergence between these two reports. The differences observed between the two nitramines in the present study could be accounted for by the presence or absence of compound-specific P450 enzymes in the fish. The large differences observed between *in vivo* and *in vitro* genotoxicity highlight the need for thorough ecotoxicity evaluations for environmental risk assessment. To the authors' knowledge, this is the only long term *in vivo* exposure study where genotoxicity of nitramines has been evaluated, and more data for the different trophic groups would assist in determining the genotoxicity of ethanolnitramine to aquatic life.

To date most of the risk assessments carried out for nitrosamines and nitramines have focused on the risks to human health (De Koeijer et al., 2013; NIPH, 2009; Ravnum et al., 2014) associated with CO_2 capture, with little focus on the environmental risks. The ecotoxicology data generated through this work is essential in contributing to an environmental risk assessment. Although the studies conducted were not carried out according to Good Laboratory Practice (GLP), they were based on accepted international standards and guidelines (ISO, OECD, ASTM) and any modification to these were detailed in full. Therefore, the data generated can be considered to be of high quality and provide information on these amine derivatives for which little or no data presently exists.

One simple way of assessing the environmental risk of a compound is to calculate its risk quotient, which is the ratio between its predicted environmental concentration (PEC) and its predicted no effect concentration (PNEC) (ECHA, 2008). The PNEC is derived by dividing the most sensitive EC_{10} by the appropriate assessment factor. Considering long-term results (EC_{10}) from three species representing three trophic levels, an assessment factor of 100 was used in the present study (ECHA, 2008). The calculated PNECs (with their 95 % confidence interval) were 0.08 (0.01-0.42) mg/L for dimethylnitramine and 0.18 (0.01-0.78) mg/L for ethanolnitramine. Based on these results, environmental concentrations exceeding 0.08 mg/L dimethylnitramine and 0.18 mg/L ethanolnitramine may be expected to pose a potential risk to the aquatic marine environment. It is noted however, that the PEC/PNEC approach provides a conservative estimate of concentrations below which an unacceptable effect will most likely not occur, but where further action is necessary if exceeded.

5. Conclusions

The multi-trophic battery of bioassays encompassing multiple endpoints, acute and chronic exposures and a biomarker response (genotoxicity) add valuable data for the two nitramine compounds ethanolnitramine and dimethylnitramine, for which no ecotoxicological data exists at

present. Overall, based on the whole organism toxicity bioassays, the toxicity of dimethylnitramine and ethanolnitramine was considered to be low. The most sensitive response for both nitramines was found in the early life stages of the oyster. However, dimethylnitramine was consistently more toxic than ethanolnitramine in all bioassays. The calculated PNECs for dimethylnitramine and ethanolnitramine were 0.08 and 0.18 mg/L, respectively, suggesting that marine PECs above these calculated PNECs have the potential to pose environmental harm.

In contrast to the toxicity observed through necrosis, higher genotoxic potency was observed for the nitramines, with ethanolnitramine exhibiting significantly more genotoxicity than dimethylnitramine. Significantly elevated levels of DNA damage were observed at the lowest concentration of ethanolnitramine tested (1 mg/L). Overall, the toxicity of the two nitramine compounds through necrosis was considered to represent a low environmental risk, with potential environmental harm unlikely to occur except around ammunition sites were nitramines are known to accumulate. However, the *in vivo* genotoxicity of ethanolnitramine poses the highest environmental risk to aquatic life and further evidence to support the genotoxic observation and refine the toxicity assessment are required.

Acknowledgements

The authors are grateful to Harald Heiaas and Inger-Lise Nerland for their help during ecotoxicity testing and to Alfhild Kringstad for analytical support and chemical analyses. Funding was provided by the Norwegian Research Council (grant number 199874) with 20 % industry contribution (Shell, Statoil, Vattenfall).

552 6. References

553 ASTM. 1994. E724 Standard guide for conducting acute toxicity tests starting with embryos of four
554 species of saltwater bivalve molluscs. Annual book of ASTM standards Vol. 11.04: Pesticides,
555 resource recovery, hazardous substances and oil spill responses, waste management, biological
556 effects. Pp. 430-447.

557 Best EPH, Sprecher SL, Larson SL, Fredrickson HL, Bader DF. 1999. Environmental behavior of
558 explosives in groundwater from the Milan army ammunition plant in aquatic and wetland plant
559 treatments. Removal, mass balances and fate in groundwater of TNT and RDX. Chemosphere
560 38: 3383-3396.

561 Brooks SJ, Bolam T, Tolhurst L, Bassett J, La Roche J, Waldock M, Barry J, Thomas KV. 2008.
562 Dissolved organic carbon reduces the toxicity of copper to germlings of the macroalgae,
563 *Fucus vesiculosus*. Ecotoxicology and Environmental Safety 70: 88-98.

564 Bråten HB, Bunkan AJ, Bache-Andreassen L, Solimannejad M, Nielsen CJ. 2008. Final report on
565 a theoretical study on the atmospheric degradation of selected amines. Oslo/Kjeller (NILU
566 OR 77/2008)

567 Collins AR. 2004. The comet assay for DNA damage and repair. Molecular Biotechnology 26:
568 249-261.

569 Collins AR, Azqueta A, Brunborg G, Gaivão I, Giovannelli L, Kruszewski M, Smith CC, Stetina R.
570 2008. The comet assay: topical issues. Mutagenesis 23: 143-151.

571 Collins AR and Azqueta A. 2012. Single-cell gel electrophoresis combined with lesion-specific
572 enzymes to measure oxidative damage to DNA. Methods in Cell Biology. ISSN 0091-679X.
573 Pp. 69-92.

574 Da Silva EF and Booth A. 2013. Emissions from postcombustion CO_2 capture plants.
575 Environmental Science and Technology 47: 659-660.

576 Da Silva EF, Hoff KA, Booth A. 2013. Emissions from CO_2 capture plants; an overview. Energy
577 Procedia 37: 784-790.

578 De Koeijer G, Talstad VR, Nepstad S, Tønnessen D, Falk-Pedersen O, Maree Y, Nielsen C.
579 2013. Health risk analysis for emissions to air from CO_2 Technology Centre Mongstad.
580 International Journal of Greenhouse Gas Control 18: 200-207.
581 Environment Agency. 2007. The direct toxicity assessment of aqueous environmental samples
582 using the marine copepod *Tisbe battagliai* lethality test.
583 European Chemicals Agency (ECHA). 2008. Guidance on information requirements and chemical
584 safety assessment. Chapter R.10: Characterisation of dose [concentration]-response for
585 environment.
586 Fjellsbø LM, Verstraelen S, Kazimirova A, Van Rompay AR, Magdolenova Z, Dusinska M. 2014.
587 Genotoxic and mutagenic potential of nitramines. Environmental Research 134: 39-45.
588 Frei E, Pool BL, Plesch W, Wiessler M.1984. Biochemical and biological properties of prospective
589 N-nitrodialkylamine metabolites and their derivatives. IARC Scientific Publication 57: 491-
590 497.
591 Frei E, Pool BL, Glatt HR, Gemperlein-Mertes I, Oesch F, Schlehofer JR, Schmezer P, Weber H,
592 Wiessler M. 1986. Determination of DNA single strand breaks and selective DNA
593 amplification by N-nitrodimethylamine and analogs, and estimation of the indicator cells
594 metabolic capacities. Journal of Cancer Research and Clinical Oncology 111(2): 123-128.
595 Grung M, Ranneklev S, Garmo O, Wright RW, Myking T, Heegard E, Øyen BH, Schei FH, Blom
596 HH. 2012. Terrestrial and aquatic baseline study and monitoring programme for CO_2
597 technology centre Mongstad. NIVA report 6311-2012.
598 International Energy Agency (IEA). 2013. CO_2 emissions from fuel combustion, 2013 edition. p.158.
599 http://www.iea.org/publications/freepublications/publication/CO2EmissionsFromFuelCombustion
600 Highlights2013.pdf
601 ISO. 1999. Water Quality – Determination of acute lethal toxicity to marine copepods (Copepoda,
602 Crustacea). ISO 14669:1999.
603 ISO. 2006. Water quality – Marine algal growth inhibition test with *Skeletonema costatum* and
604 *Phaeodactylum tricornutum.* ISO 10253:2006.

605 Lackner KS. 2009. Capture of carbon dioxide from ambient air. European Physical Journal:
606 Special Topics 176: 93-106.
607 Lewin U, Wennrich L, Efer J, Engewald W. 1997. Determination of highly polar compounds in
608 water samples around former ammunition plants. Chromatographia 45: 91-8.
609 Låg, M. Lindeman B, Instanes C, Brunborg B, Schwarze P. 2011. Health effects of amines and
610 derivatives associated with CO_2 capture. The Norwegian Institute of Public Health (ISBN:
611 978-82-8082-462-2).
612 Nielsen CJ, D'Anna B, Dye C, George C, Graus M, Hansel A, Karl M, King S, Musabila M, Muller
613 M, Schmidbauer N, Stenstrøm Y, Wisthaler A. 2009. Atmospheric degradation of amines
614 (ADA). Summary report: Gas phase photo-oxidation of 2-aminoethanol (MEA). CLIMIT
615 project no. 193438.
616 Norwegian Institute of Public Health (NIPH). 2009. Health effects of different amines and possible
617 degradation products relevant for CO_2 capture. Eds Låg M, Andreassen Å, Instanes C,
618 Lindemann B. FHI rapport 2009:3.
619 OECD. 1992. Fish, acute toxicity test. OECD guideline for testing of chemicals 203.
620 OECD. 2000. Fish, juvenile growth test. OECD guideline for testing of chemicals 215.
621 Ravnum S, Rundén-Pran E, Fjellsbø, LM, Dusinska M. 2014. Human health risk assessment of
622 nitrosamines and nitramines for potential application in CO_2 capture. Regulatory Toxicology
623 and Pharmacology 69(2): 250-255.
624 Reynolds AJ, Verheyen TV, Adeloju SB, Meuleman E, Feron P. 2012. Towards commercial scale
625 postcombustion capture of CO_2 with monoethanolamine solvent: key consideration for
626 solvent management and environmental impacts. Environmental Science and Technology
627 46: 3643-3654.
628 Ryon MG, Pal BC, Talmage SS, Ross RH. 1984. Database assessment of the health and
629 environmental effects of munition production waste water ORNL-6018 (NTIS DE84-
630 016512).

631 Shah KA and Karnes HT. 2010. A review of the analysis of tobacco-specific nitrosamines in
632 biological matrices. Critical Reviews in Toxicology 40(4): 305-327.
633 Shaposhnikov S, Azqueta A, Henriksson S, Meier S, Gaivão I, Huskisson NH, Smart A,
634 Brunnborg G, Nilsson M, Collins AR. 2010. Twelve-gel slide format optimised for comet
635 assay and fluorescent in situ hybridisation. Toxicology Letters 195(1): 31-4.
636 Stéphenne K. 2014. Start-up of world's first commercial post-combustion coal fired CCS project:
637 Contribution of Shell Cansolv to SaskPower boundary dam ICCS project. Energy Procedia
638 63:6106-6110.
639 US EPA. 2004. Method 521 determination of nitrosamines in drinking water by solid phase
640 extraction and capillary column gas chromatography with large volume injection and
641 chemical ionization tandem mass spectrometry (MS/MS). National Exposure Research
642 Laboratory Office of Research and Development, EPA/600/R-05/054.
643 Vindimian E, Robaut C, Fillion G. 1983. A method for cooperative and non cooperative binding
644 studies using non linear regression analysis on a microcomputer. Journal of Applied
645 Biochemistry 5: 261-268.
646 Wagner ED, Hsu KM, Lagunas A, Mitch WA, Plewa MJ. 2012. Comparative genotoxicity of
647 nitrosamine drinking water disinfection byproducts in *Salmonella* and mammalian cells.
648 Mutation Research 741: 109-115.
649 Wagner ED, Osiol J, Mitch WA, Plewa MJ. 2014. Comparative in vitro toxicity of nitrosamines and
650 nitramines associated with amine-based carbon capture and storage. Environmental
651 Science and Technology 48: 8203-8211.
652 Walse SS and Mitch WA. 2008. Nitrosamine carcinogens also swim in chlorinated pools.
653 Environmental Science and Technology 42:1032-1037.

Table 1. Ecotoxicity parameters derived from dose-response relationships for marine species exposed to dimethylnitramine (A) and ethanolnitramine (B). NOEC: no observed effect concentration, LOEC: lowest observed effect concentration, ECx: concentration giving x% effect, na: not applicable. ECx are given with their 95% confidence interval in parentheses. *values above the highest concentration tested.

A) Dimethylnitramine						
Species	Test	**NOEC** (mg/L)	**LOEC** (mg/L)	**EC_{10}** (mg/L)	**EC_{20}** (mg/L)	**EC_{50}** (mg/L)
Crassostrea gigas	Oyster larval development 24 h	21	45	39 (22-44)	42 (28-45)	47 (42-52)
Tisbe battagliai	Copepod mortality 48 h	≥ 100	> 100	na	na	na
Scophthalmus maximus	Turbot mortality 96 h	≥ 100	> 100	na	na	na
Skeletonema costatum	Algal growth 72 h	18	32	48 (33-64)	121 (97-143)	591 (521-702)*
Fucus vesiculosus	Macroalgae germling growth 14 d	< 100	100	na	na	na
Tisbe battagliai	Copepod reproduction 14 d	25	50	8 (1-42)	18 (4-55)	70 (38-202)
Scophthalmus maximus	Turbot growth 28 d	≥ 100	> 100	na	na	na
B) Ethanolnitramine						
Species	Test	**NOEC** (mg/L)	**LOEC** (mg/L)	**EC_{10}** (mg/L)	**EC_{20}** (mg/L)	**EC_{50}** (mg/L)
Crassostrea gigas	Oyster larval development 24 h	45	100	65 (23-92)	78 (42-95)	107 (99-140)
Tisbe battagliai	Copepod mortality 48 h	≥ 100	> 100	na	na	na
Scophthalmus maximus	Turbot mortality 96 h	≥ 100	> 100	na	na	na
Skeletonema costatum	Algal growth 72 h	> 100	> 100	na	na	na
Fucus vesiculosus	Macroalgae germling growth 14 d	100	500	na	na	na
Tisbe battagliai	Copepod reproduction 14 d	50	100	18 (1-78)	35 (6-93)	108 (55-421)
Scophthalmus maximus	Turbot growth 28 d	≥ 100	> 100	na	na	na

660 **Figure legends**

661 **Figure 1**. Effects of dimethylnitramine (left) and ethanolnitramine (right) on the larval development
662 of the oyster *Crassostrea gigas* after 24 h exposure. Results are shown as the average fraction of
663 normal D larvae ± one standard deviation; statistical differences are indicated by different letters
664 ($p < 0.05$).

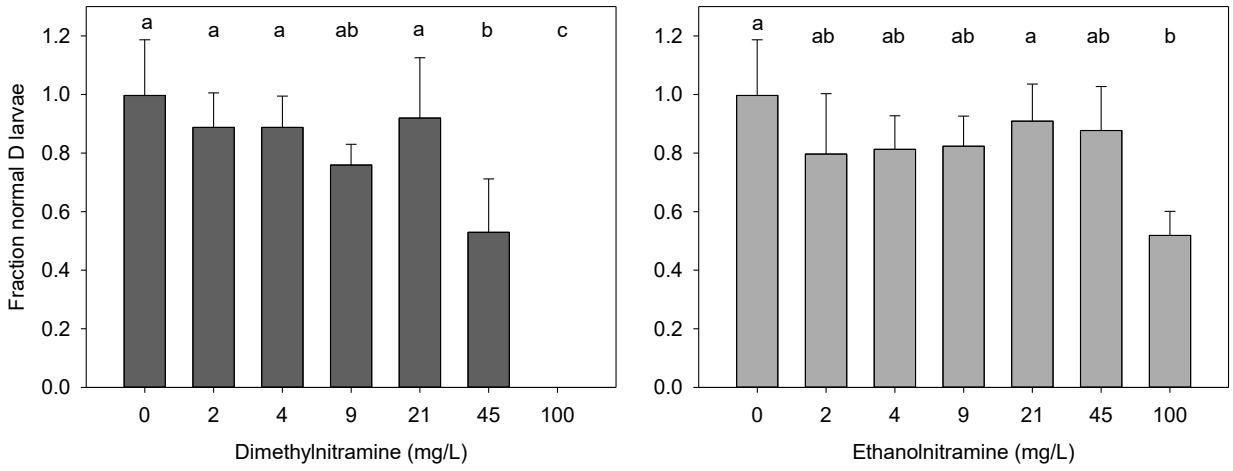
665

666 **Figure 2.** Effects of dimethylnitramine (left) and ethanolnitramine (right) on the growth of the
667 unicellular algae *Skeletonema costatum* after 72 h exposure (A); the growth of the macroalgae
668 *Fucus vesiculosus* after 13 day exposure (B); the reproductive output of the copepod *Tisbe*
669 *battagliai* over a 14 day exposure (C); the growth of juvenile turbot *Scophthalmus maximus* after
670 28 day exposure (D). Results are means ± one standard deviation; statistical differences are
671 indicated by different letters ($p < 0.05$).

672

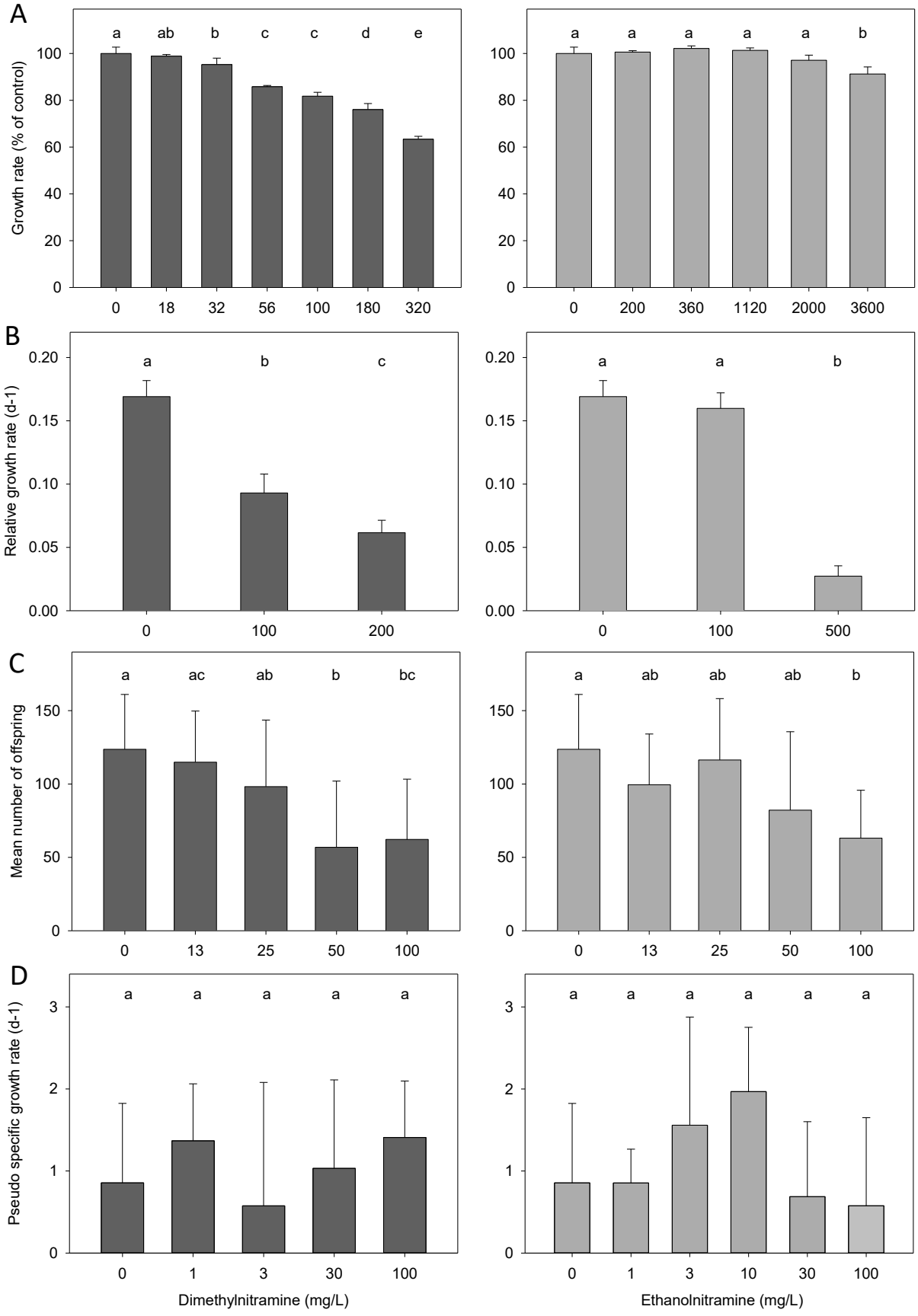
673 **Figure 3**. Effects of dimethylnitramine (left) and ethanolnitramine (right) on DNA damage in red
674 blood cells of juvenile turbot *Scophthalmus maximus* after 28 day exposure. For total damage (i.e.
675 strand-breaks, alkali-labile and fpg-sensitive sites), statistical differences are indicated by different
676 letters ($p < 0.05$).

677 **Figure 1.**



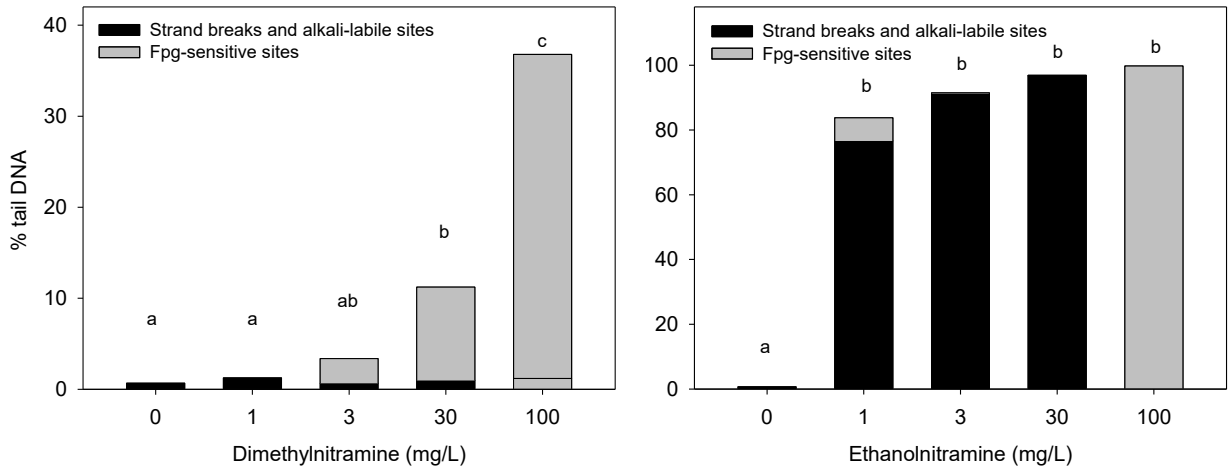
678

679 **Figure 2.**



680

681 **Figure 3.**



682