

This is an Accepted Manuscript of the following article:

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>

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It is recommended to use the published version for citation.

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

3

4 Running title: Aquatic ecotoxicity of nitramines

5

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12 Abstract

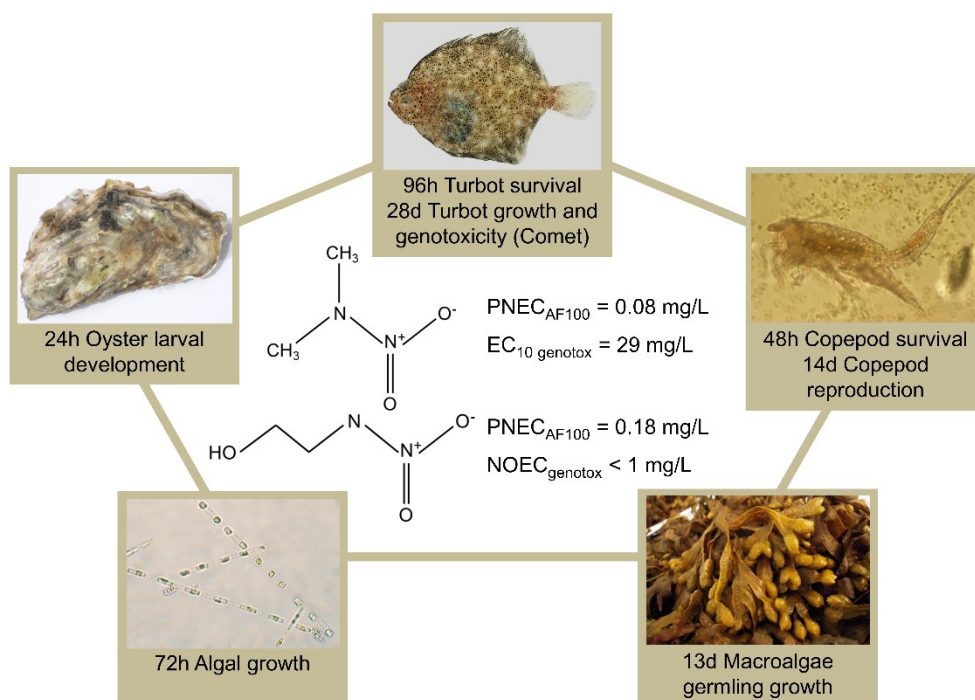
13 In the context of reducing CO₂ emissions to the atmosphere, chemical absorption with amines is
14 emerging as the most advanced technology for post combustion CO₂ capture from exhaust gases
15 of fossil fuel power plants. Despite amine solvent recycling during the capture process,
16 degradation products are formed and released into the environment, among them aliphatic
17 nitramines, for which the environmental impact is unknown. In this study, we determined the
18 acute and chronic toxicity of two nitramines identified as important transformation products of
19 amine-based carbon capture, dimethylnitramine and ethanolnitramine, using a multi-trophic suite
20 of bioassays. The results were then used to produce the first environmental risk assessment for
21 the marine ecosystem. In addition, the *in vivo* genotoxicity of nitramines was studied by adapting
22 the comet assay to cells from experimentally exposed fish. Overall, based on the whole organism
23 bioassays, the toxicity of both nitramines was considered to be low. The most sensitive response
24 to both compounds was found in oysters, and dimethylnitramine was consistently more toxic than
25 ethanolnitramine in all bioassays. The Predicted No Effect Concentrations for dimethylnitramine
26 and ethanolnitramine were 0.08 and 0.18 mg/L, respectively. The genotoxicity assessment
27 revealed contrasting results to the whole organism bioassays, with ethanolnitramine found to be
28 more genotoxic than dimethylnitramine by three orders of magnitude. At the lowest
29 ethanolnitramine concentration (1 mg/L), 84 % DNA damage was observed, whereas 100 mg/L
30 dimethylnitramine was required to cause 37 % DNA damage. The mechanisms of genotoxicity
31 were also shown to differ between the two compounds, with oxidation of the DNA bases
32 responsible for over 90 % of the genotoxicity of dimethylnitramine, whereas DNA strand breaks
33 and alkali-labile sites were responsible for over 90 % of the genotoxicity of ethanolnitramine. Fish
34 exposed to > 3 mg/L ethanolnitramine had virtually no DNA left in their red blood cells.

35 Highlights

- 36 • The environmental risk posed by nitramines, CO₂ capture by-products, was unknown.
- 37 • A multi-trophic suite of bioassays was used to assess ecotoxicity and genotoxicity.
- 38 • Nitramine toxicity through necrosis was considered low.
- 39 • The first risk assessment for dimethylnitramine and ethanolnitramine was produced.
- 40 • Ethanolnitramine induced massive DNA damage in turbot.

41

42 Graphical abstract



43

44

45 Keywords

46 Environmental risk assessment, post combustion CO₂ capture, 2-(nitroamino)ethanol, single cell
47 gel electrophoresis

48 1. Introduction

49 The capture and storage of carbon dioxide (CO₂) from the exhaust gases of fossil fuel power
50 stations is an important technology for reducing CO₂ emissions to the atmosphere. Approximately
51 43% of the global CO₂ emissions in 2011 were attributed to the generation of electricity from fossil
52 fuel power stations (IEA, 2013). Chemical absorption with amines is emerging as the most
53 advanced mitigation technology for post combustion capture of CO₂ from fossil fuel power
54 stations (Reynolds et al., 2012). The exhaust gas from the power station is bubbled through an
55 amine solution in the absorber unit, producing a CO₂-saturated amine solution. In the stripper
56 unit, heat separates CO₂ and amines, resulting in pure CO₂ ready to be stored on one side, and
57 amine solution, recycled and sent back to the absorber unit, on the other side. The main
58 advantage of post combustion CO₂ capture is that the technology can be incorporated into
59 existing power plants, avoiding the need to build new facilities. In addition, the technology benefits
60 from almost two decades of full-scale experience for removal of CO₂ from natural gas (Lackner,
61 2009; Reynolds et al., 2012). However, the environmental impacts of replacing CO₂ emissions
62 with the discharge of amine solvents and their chemical transformation products, as by-products
63 of the capturing process, are largely unknown.

64
65 There is increasing public and environmental concern with regard to two main groups of amine
66 transformation products, nitrosamine and nitramine contamination in air and drinking water
67 supplies downstream of amine-based CO₂ capture plants (Reynolds et al., 2012). A few *in vitro*
68 studies showed that nitramines could be carcinogenic and mutagenic (Fjellsbø et al., 2014;
69 Wagner et al., 2014). Recent theoretical modelling and controlled laboratory experiments reported
70 the occurrence of nitramines as transformation products of amines in the carbon capture process
71 within the discharge effluent (Bråten et al., 2008; Nielsen et al., 2009). Two of the nitramine
72 compounds that were identified included dimethylnitramine (CAS No. 4164-28-7) and
73 ethanolo nitramine (CAS No. 74386-82-6). However, despite the likelihood of these compounds
74 increasing in the environment, with the potential to cause environmental harm, no environmental

75 toxicity data for these compounds currently exist. Due to the location of some CO₂ capture and
76 storage plants along the coastline, as well as their tendency to partition to the water phase,
77 amines and their transformation products are likely to end up in the marine environment.
78 Therefore, an ecotoxicity assessment performed on marine organisms is needed in order to
79 provide an appropriate assessment of the environmental risk.

80

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

93

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

100

101 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 ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$,
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 $\times 400$ 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 μ L 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 $\times 100$ 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

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

158

159 2.2.3. Turbot mortality

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

174

175 2.2.4. Algal growth

176 The unicellular algae *Skeletonema costatum* were exposed for 72 h to a concentration range of
177 nitramines (0, 18, 32, 56, 10, 180, 320 mg/L for dimethylnitramine and 0, 200, 360, 1120, 2000,
178 3600 mg/L for ethanoloitramine), following the ISO standard procedure 10253 (ISO, 2006).
179 Growth medium was prepared by adding ISO 10253 stock solutions to filtered seawater. The
180 growth of the algal inoculum (5.6×10^6 cells/L) placed on an orbital shaker in continuous cool
181 white fluorescent light (68 ± 4 $\mu\text{mol/m}^2/\text{s}$, Philips TLD 36W/950) under constant temperature ($20 \pm$
182 1 °C) was measured every 24 h for the duration of the test using a Beckman Coulter Multisizer 3

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

187 The relative growth rate (RGR) for each test concentration was calculated using the equation:
188 $RGR = (\ln N_n - \ln N_0)/(t_n - t_0)$, where N_n = Cell density at time t_n , N_0 = Cell density at time zero
189 (t_0). The percentage inhibition of growth rate as compared to the control was calculated for each
190 concentration.

191

192 2.2.5. Macroalgae germling growth

193 The toxicity of nitramines to the growth of *Fucus vesiculosus* germlings was assessed based on
194 the method described by Brooks et al. (2008). The algae were collected in the Oslo fjord (GPS
195 59.904 N, 10.702 E) and thoroughly rinsed with filtered seawater. The receptacles were then left
196 for 6 h in seawater to allow for the release of eggs and sperm cells. The resulting zygote
197 suspension was filtered through a 90 μm sieve, collected on a 25 μm sieve, and its quality and
198 density assessed at $\times 20$ magnification. Microscope slides were placed in a shallow tray, covered
199 to a depth of 2 cm with seawater, and 1 mL of zygote suspension (50-100 zygotes) was placed
200 onto each individual slide. The tray, covered with film to prevent evaporation, was left for 48 h to
201 enable the zygotes to attach and develop into germlings. After 48 h, 5 or 6 slides with at least 6
202 germlings on each were placed in separate Coplin jars, one jar per concentration. Due to low
203 abundance of germlings, the setup was reduced to control, dimethylnitramine at 100 mg/L and
204 200 mg/L, and ethanoldinitramine at 100 mg/L and 500 mg/L. Renewal of the exposure solutions
205 was performed on day 7. Exposure solutions were sampled at the start and at the end of the
206 exposure period for analytical determination of nitramine concentrations. All germlings from each
207 slide were photographed and measured on day 0, 4, 7, 10 and 13. The RGR was calculated using
208 the equation: $RGR = (\ln L_n - \ln L_0)/(t_n - t_0)$, where L_n = germling length at time t_n , L_0 = germling
209 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 2×10^5 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

237 lowest and highest test concentration solutions were taken at the beginning and on day 2, 24 and
238 28 of the exposure period for analytical determination of nitramine concentrations. After 28 days,
239 fish were weighed and the 'pseudo' specific growth rate (SGR) was calculated using the equation:
240 $SGR = (\ln W_n - \overline{\ln W_0}) / (t_n - t_0) \times 100$, where $\ln W_n$ = logarithm of the weight of an individual fish
241 at time t_n , and $\overline{\ln W_0}$ = average of the logarithms of the weights of individual fish at time zero (t_0).

242

243 2.3. Comet assay

244 DNA damage was analyzed in blood samples collected from the caudal vein of juvenile turbot *S.*
245 *maximus* after 28 day exposure to nitramines, using a version of the comet assay (Collins, 2004;
246 Collins and Azqueta, 2012) in which 12 mini-gels are set on each slide (Shaposhnikov et al.,
247 2010). The experimental setup included 4 concentrations of ethanoldinitramine and
248 dimethylnitramine (1, 3, 30, 100 mg/L), with 6 fish per nitramine concentration and 12 fish per
249 control. Cell density was optimized beforehand by preparing blood dilutions in PBS and
250 measuring cell number with the aid of a Coulter counter. A cell density of 2.5×10^5 /mL was found
251 to give an appropriate number of cells per mini-gel.

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

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

264 detects oxidized purines. After lysis, the other two slides, *FPG* and *BUF*, were gently washed 3
265 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
266 BSA) and then incubated for 30 min at 37 °C in either enzyme buffer (without enzyme) (slide
267 *BUF*), or with FPG enzyme made up in the same buffer at 1:50000 (slide *FPG*). After incubation,
268 these two slides and the *LYS* slide were placed in a horizontal gel electrophoresis tank and DNA
269 was allowed to unwind for 20 min in cold alkaline solution (0.3 M NaOH and 1 mM Na₂EDTA,
270 pH>13) followed by electrophoresis for 20 min at 0.8 V/cm at 4 °C. Slides were then washed
271 twice with cold PBS for 5 min and for 1 min in distilled water. Gels were dehydrated and DNA
272 fixed by incubating slides in 70 % ethanol for 5 min and in absolute ethanol for a further 5 min.
273 They were then placed on the bench at room temperature to dry.

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

281

282 2.4. Chemical analysis

283 The method used to determine concentrations of dimethylnitramine and ethanoloitramine in
284 exposure media was modified from the USEPA method 521 (USEPA, 2004). Solid phase
285 extraction (SPE) of samples was carried out on activated charcoal columns (SPE EPA method
286 521 and 522, 6 mL/2 g activated charcoal, Restek, USA). To avoid peak saturation on the SPE
287 column and the chromatograph, samples with high nominal concentrations of nitramines were
288 diluted with ultrapure water to a nominal concentration of 2 mg/L nitramine. Columns were
289 conditioned by successive solvent washes (3 mL dichloromethane, 9 mL methanol, 15 mL
290 ultrapure water), then loaded with 5 mL of sample, followed by 3 mL of ultrapure water. Columns

291 were then eluted with 15 mL dichloromethane. Samples were dried with sodium sulfate and
292 spiked with internal standard (2.5 µg 1,2,4-trichlorobenzene and 1.25 µg tetrachloroethane).
293 Samples were finally reduced to 0.5 mL under a nitrogen flow and added 0.5 mL methanol.
294 For quality assurance, blanks and spiked samples containing 2 mg/L dimethylnitramine and
295 ethanolnitramine, processed in the same way as the samples, and 5 standard solutions in the
296 range 0.1 to 10 mg/L, were analyzed. The analysis was performed by gas chromatography
297 Agilent 6890N with a 63Ni µECD detector. GC separation was performed using an Agilent J&W
298 DB5 capillary column (30 m × 0.25 mm, 1.0 µm film). The oven temperature program was setup
299 as follows: 60 °C held for 2 min, then ramped at 7 °C/min to 125 °C, held for 3 min, then ramped
300 at 10 °C/min to 250 °C held for 2 min. Injection temperature was 200 °C, detector temperature
301 was 240 °C, and carrier gas flow (hydrogen) was 3 mL/min.

302

303 2.5. Data analysis

304 Where toxicity was observed, calculation of toxicity parameters (Effect Concentrations EC₁₀, EC₂₀
305 and EC₅₀) was performed using the Hill model of the Excel macro REGTOX developed by Eric
306 Vindimian (Vindimian et al., 1983). Homogeneity of variance was checked with a Levene's test
307 before a one-way analysis of variance (ANOVA) was used to evaluate effects of nitramines on the
308 various biological endpoints, and differences were identified with a Holm-Sidak test. When
309 normality and homoscedasticity assumptions were not reached, a Kruskal-Wallis analysis of
310 variance on ranks was used, followed by a Dunn's test (SigmaPlot 12.5, Systat software).

311

312 3. Results

313 3.1. Chemical analysis of exposure media

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

318 Measured concentrations in exposure media compared well with the respective nominal
319 concentrations and were typically within $\pm 10\%$ of the nominal concentration. The only exception
320 was the copepod (*T. battagliai*) acute test where measured ethanolnitramine concentrations were
321 3 times below the nominal concentrations. In this case, the determination of toxicity values was
322 based on measured concentrations, rather than on nominal concentrations.

323

324 3.2. Acute toxicity

325 3.2.1. Oyster larval development

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

336

337 3.2.2. Copepod mortality

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

342

343 3.2.3. Turbot mortality

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

346

347 3.3. Sub-chronic and chronic toxicity

348 3.3.1. Algal growth

349 The growth rate of the unicellular algae *S. costatum* after 72 h exposure was significantly reduced
350 by dimethylnitramine at concentrations ≥ 32 mg/L (Figure 2A). The calculated NOEC, LOEC and
351 EC₁₀ concentrations for dimethylnitramine were 18, 32 and 48 mg/L, respectively (Table 1). The
352 relatively high EC₅₀ concentration (591 mg/L, extrapolated value) despite a LOEC of 32 mg/L was
353 reflective of the limited effect on growth achieved at higher exposure concentrations, with only
354 approximately 40 % reduction in growth rate achieved at the top concentration of 320 mg/L.

355 Ethanolnitramine, on the other hand, had no negative effect on the growth of *S. costatum*, unless
356 very high concentrations, well above those considered environmentally relevant, were reached.
357 The experimental NOEC and LOEC for ethanolnitramine were 2000 and 3600 mg/L, respectively,
358 and are reported as >100 mg/L in Table 1.

359

360 3.3.2. Macroalgae germling growth

361 The growth of *F. vesiculosus* germlings after 13 day exposure was reduced by 45 % and 64 % at
362 100 mg/L and 200 mg/L dimethylnitramine, respectively, whereas ethanolnitramine had no
363 significant effect at 100 mg/L, and induced 84 % growth reduction at 500 mg/L (Figure 2B, table
364 1).

365

366 3.3.3. Copepod reproduction

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

371 to 100 mg/L (63 ± 33 offspring). The calculated NOEC, LOEC and EC₅₀ 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 EC₁₀, EC₂₀ and EC₅₀ (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 EC₅₀, 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

398 4. Discussion

399 The main source of nitramines in the environment has been through their use in weapons
400 manufacturing (Ryon et al., 1984). High environmental concentrations of nitramines, hexahydro-
401 1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro 1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX),
402 which are important ingredients in explosives, have been found in both ground and surface
403 waters near to army ammunition plants. Concentrations as high as 1.9 mg/L and 0.21 mg/L have
404 been reported for RDX and HMX respectively (Best et al., 1999; Lewin et al., 1997). Data for the
405 environmental concentrations of other nitramine compounds not associated with ammunition
406 plants are limited. However, chlorination of public waters has led to concerns about nitramine and
407 nitrosamine formation. For example, dimethylnitramine was detected at median values of 64.5, 50
408 and 203 ng/L in outdoor pools, indoor pools, and hot tubs, respectively (Walse and Mitch, 2008),
409 with the nitramine levels comparable to measured levels of the nitrosamine, N-
410 nitrosodimethylamine.

411
412 The introduction of post combustion CO₂ capture plants using amines, where monoethanolamine
413 is the mostly widely used, has the potential to contribute as a nitramine source (Da Silva and
414 Booth, 2013). The concentration of nitramines from such facilities depends on a variety of factors,
415 including the amine solvent used, the composition of the flue gas, and the plant operation
416 conditions. Emission data from post combustion CO₂ capture facilities with amines are relatively
417 scarce. This is partly due to the confidentiality issues surrounding the solvent systems used by
418 the various companies, as well as the only relatively recent interest in CCS emissions. Emission
419 data that are available mostly derive from pilot plants where predominantly monoethanolamine
420 (MEA) has been used as the solvent. A recent summary of emission data from a range of pilot
421 plants including Maasvlakte (Texas, USA), Esbjerg (Denmark), and Mongstad (Norway) have
422 indicated low concentrations (ppb levels) of nitramines (Da Silva et al., 2013). Monitoring of the
423 lakes and fjords within the catchment area of the test plant at Mongstad did not detect nitramines
424 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 CO₂ capture with amines has been put on hold. Therefore, accumulation of
427 nitramines as degradation product of amines in CO₂ 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 CO₂ 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 ethanoldinitramine 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 EC₅₀ of 591 mg/L. In contrast, the

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

455

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

465

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

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

480
481 Fjellsbø et al. (2014) evaluated the genotoxicity of nitramines including dimethylnitramine and
482 ethanolnitramine, using the bacterial reverse mutation (Ames) test, the cytokinesis block
483 micronucleus (CBMN) assay and the comet assay. Ethanolnitramine was found to show
484 mutagenic potential with the Ames test, was weakly genotoxic in the CBMN assay, but showed no
485 increase in DNA strand breaks in the comet assay despite concentrations up to 1.9 g/L. The lack
486 of genotoxic response in the comet assay is in contrast to the present study. Differences in the
487 type and duration of exposure between the studies may explain the differences in toxicity
488 observed. A 3 h *in vitro* exposure of human TK6 cells to 1.9 g/L ethanolnitramine failed to show a
489 significant increase in DNA strand break frequency, in spite of the positive CBMN response
490 (Fjellsbø et al., 2014). However, our 28 day *in vivo* exposure produced significant DNA damage at
491 the lowest concentration tested (1 mg/L). It is possible that metabolic activation of the
492 ethanolnitramine does not occur to a sufficient extent in a 3 h exposure, whereas the longer
493 incubation period of the CBMN assay or our *in vivo* exposure allows activation to occur. In
494 addition, the likely differences in specific metabolising capacity (due to variations in cytochrome
495 P450 enzymes) between fish and humans could account for the divergence between these two
496 reports. The differences observed between the two nitramines in the present study could be
497 accounted for by the presence or absence of compound-specific P450 enzymes in the fish. The
498 large differences observed between *in vivo* and *in vitro* genotoxicity highlight the need for
499 thorough ecotoxicity evaluations for environmental risk assessment. To the authors' knowledge,
500 this is the only long term *in vivo* exposure study where genotoxicity of nitramines has been
501 evaluated, and more data for the different trophic groups would assist in determining the
502 genotoxicity of ethanolnitramine to aquatic life.

503

504 To date most of the risk assessments carried out for nitrosamines and nitramines have focused
505 on the risks to human health (De Koeijer et al., 2013; NIPH, 2009; Ravnum et al., 2014)
506 associated with CO₂ capture, with little focus on the environmental risks. The ecotoxicology data
507 generated through this work is essential in contributing to an environmental risk assessment.
508 Although the studies conducted were not carried out according to Good Laboratory Practice
509 (GLP), they were based on accepted international standards and guidelines (ISO, OECD, ASTM)
510 and any modification to these were detailed in full. Therefore, the data generated can be
511 considered to be of high quality and provide information on these amine derivatives for which little
512 or no data presently exists.

513

514 One simple way of assessing the environmental risk of a compound is to calculate its risk
515 quotient, which is the ratio between its predicted environmental concentration (PEC) and its
516 predicted no effect concentration (PNEC) (ECHA, 2008). The PNEC is derived by dividing the
517 most sensitive EC₁₀ by the appropriate assessment factor. Considering long-term results (EC₁₀)
518 from three species representing three trophic levels, an assessment factor of 100 was used in the
519 present study (ECHA, 2008). The calculated PNECs (with their 95 % confidence interval) were
520 0.08 (0.01-0.42) mg/L for dimethylnitramine and 0.18 (0.01-0.78) mg/L for ethanolnitramine.
521 Based on these results, environmental concentrations exceeding 0.08 mg/L dimethylnitramine
522 and 0.18 mg/L ethanolnitramine may be expected to pose a potential risk to the aquatic marine
523 environment. It is noted however, that the PEC/PNEC approach provides a conservative estimate
524 of concentrations below which an unacceptable effect will most likely not occur, but where further
525 action is necessary if exceeded.

526

527 5. Conclusions

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

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

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

546

547 Acknowledgements

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

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

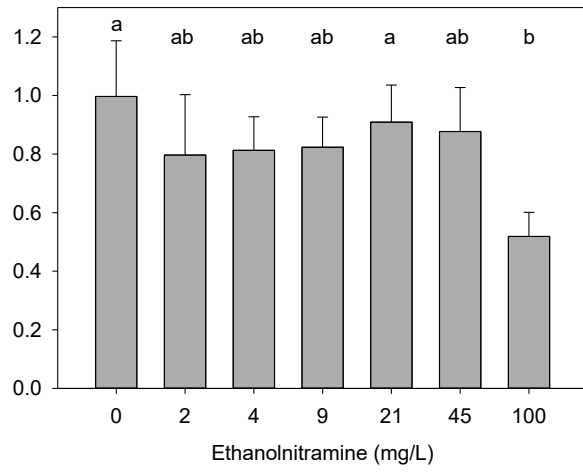
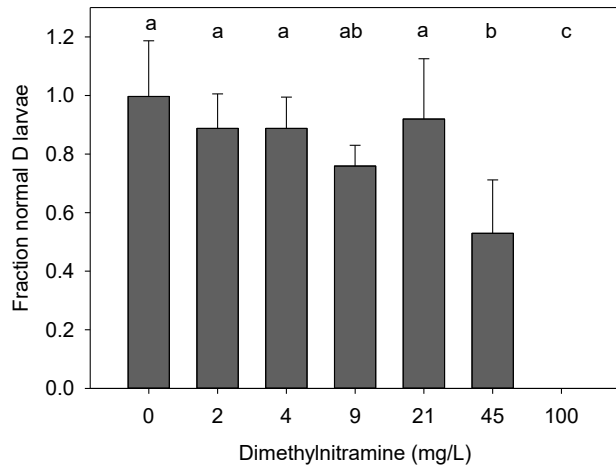
A) Dimethylnitramine						
Species	Test	NOEC (mg/L)	LOEC (mg/L)	EC ₁₀ (mg/L)	EC ₂₀ (mg/L)	EC ₅₀ (mg/L)
<i>Crassostrea gigas</i>	Oyster larval development 24 h	21	45	39 (22-44)	42 (28-45)	47 (42-52)
<i>Tisbe battagliai</i>	Copepod mortality 48 h	≥ 100	> 100	na	na	na
<i>Scophthalmus maximus</i>	Turbot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

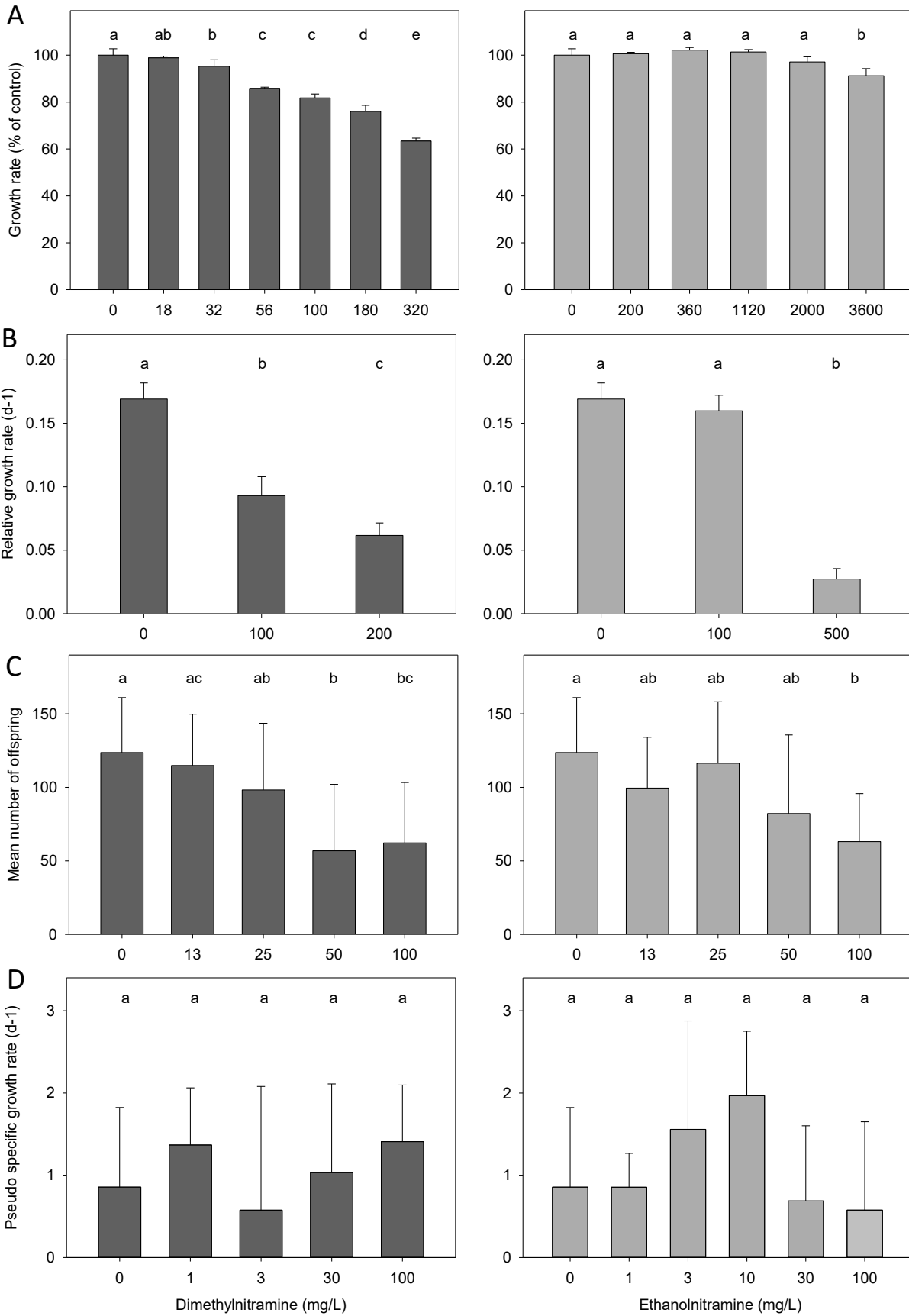
660 **Figure legends**

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 \pm one standard deviation; statistical differences are indicated by different letters
664 ($p < 0.05$).

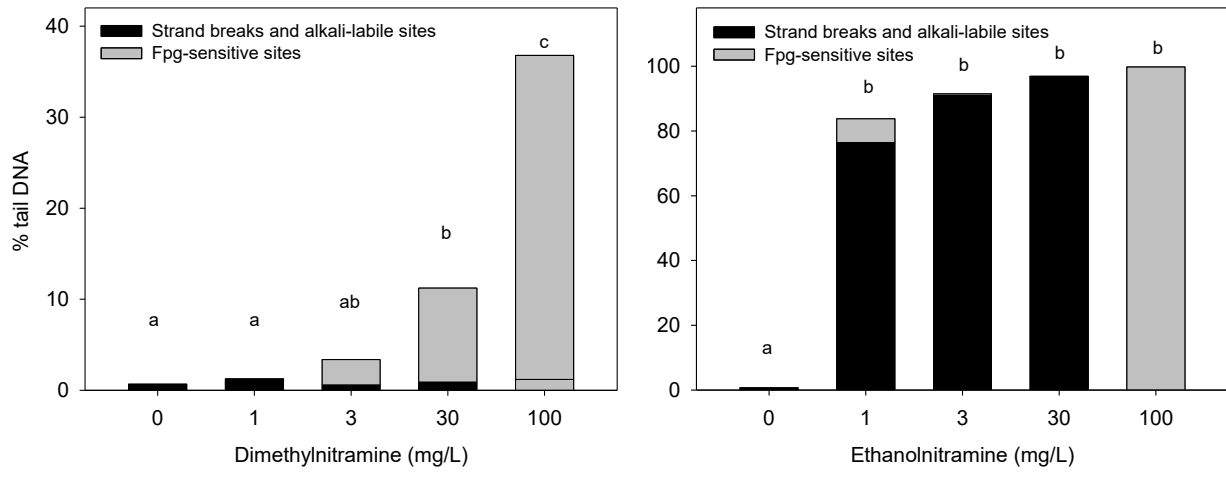
665
666 **Figure 2.** Effects of dimethylnitramine (left) and ethanolnitramine (right) on the growth of the
667 unicellular algae *Skeletonema costatum* after 72 h exposure (A); the growth of the macroalgae
668 *Fucus vesiculosus* after 13 day exposure (B); the reproductive output of the copepod *Tisbe*
669 *battagliai* over a 14 day exposure (C); the growth of juvenile turbot *Scophthalmus maximus* after
670 28 day exposure (D). Results are means \pm 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 ($p < 0.05$).





681 **Figure 3.**



682