

Paper Summary:

Toxicity of Ozonated Seawater to Marine Organisms: I. Effects of Short-Term Batch Ozonation

This paper was written for submission to Environmental Toxicology and Chemistry by members of Nutech O3, Inc.'s scientific team. The purpose of this paper was to supplement the shipboard testing on the S/T Tonsina with lab based studies. The goal of this study was to quantify oxidant loading rates and ozone toxicity to approximate the concentrations of ozone necessary to eliminate an acceptable percentage of organisms from ballast water tanks. Adult mysid shrimp, larval topsmelt, juvenile sheepshead minnows, and adult amphipods were tested.

This paper supports Nutech O3, Inc.'s belief that ozone is an effective ballast water treatment method and that TRO is the most useful measurement to determine this effectiveness.

Our results indicate that marine invertebrate and fish species can be effectively eliminated following short-term (i.e., less than 5 hours to 100% mortality) ozonation at TRO concentrations less than 1 mg/l as bromine, and that ozone-produced oxidants can accumulate and remain toxic in closed containers for at least two days. Benthic invertebrates, such as blue crabs may be relatively tolerant of ozone-produced oxidants, and so may require other control methods to prevent introductions from ballast water discharge. These organisms rarely find their way into ballast tanks due to the filters in use on the pumping systems on ships.

Although laboratory conditions differ significantly from ballast water tanks onboard ocean vessels, applying these data on a much larger scale could be useful in approximating TRO concentrations needed to remove an acceptable percentage of NIS from ballast water. Furthermore, laboratory toxicity data could be used to help evaluate how far TRO concentrations might have to be decreased prior to discharge to ensure the safety of indigenous organisms in receiving waters. Ozone-produced oxidant concentrations will decrease in ballast waters prior to discharge by natural decay processes (e.g., time, mixing, or sunlight exposure, or instantaneously if required by the addition of reductants such as sodium thiosulfate to eliminate toxicity).

APPENDIX 2

Toxicity of Ozonated Seawater to Marine Organisms
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TOXICITY OF OZONATED SEAWATER TO MARINE ORGANISMS. I:
EFFECTS OF SHORT-TERM BATCH OZONATION

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Abstract - The toxicity of ozone gas bubbled into artificial seawater (ASW) was determined for five species of marine organisms in short-term (i.e., < 5 h) batch exposures to evaluate the use of ozone for removal of non-indigenous species (NIS) from ballast water. Juvenile topsmelt and sheephead minnows (*Atherinops affinis* and *Cyprinodon variegatus*) were the most sensitive to oxidant exposure, with the mysid shrimp *Americamysis bahia* being the most sensitive invertebrate. In contrast, benthic amphipods (*Rhepoxinius abronius*, and *Leptochirus plumulosus*) were the least sensitive of all species tested. Mortality from ozone exposure occurred quickly with median lethal times ranging from 1 – 3 h for the most sensitive species, although additional mortality can occur 1 – 2 d following ozonation. Because ozone does not persist in seawater, toxicity most likely resulted from oxidation of bromide to bromine species (HOBr, OBr⁻) which persist and continue to induce mortality even after 1-2 d storage. Therefore, ozonating seawater in short-term batch exposures to generate TRO concentrations ranging from 0.3 – 1.7 mg/L as Br₂ may effectively remove significant portions of marine NIS populations, although some tolerant benthic crustaceans may require additional treatment for adequate removal. (this is 187 words, we have 200 max)

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Keywords: ozone, non-indigenous species, marine invertebrates, fish, toxicity, bromine

18 INTRODUCTION

20 The introduction of non-indigenous species (NIS) through human activities is having
severe consequences on global ecosystems, causing economic injury, and creating threats to
human health (USOTA, 1993; Vitousek, et al., 1997; Wilcove, et al., 1998; Pimentel, et al.,
22 2000). Although the negative effects of NIS in terrestrial and freshwater habitats are well
documented (e.g., Gordon 1998; Horvitz et al. 1998; Lee, 1999; Mack et al., 2000), it is evident
24 that NIS invasions have also changed coastal marine environments (e.g., Bax, et al., 2001).
Some marine species have become dominant and significantly altered ecosystem-level processes
26 (Cloern, 1996; Ruiz, et al., 1999; Grosholz, et al., 2000).

Shipping has been identified as the vector responsible for most invasions in coastal areas,
28 where organisms are carried on the hulls of ships and within ballast water tanks (Carlton, 1979;
Carlton and Geller, 1993; Cohen and Carlton, 1995; Hewitt, et al., 1999; Ruiz, et al., 2000).
30 Mid-ocean exchange of ballast water is currently the only viable management strategy available for
reducing the quantities of NIS before reaching port (NRC, 1996). However, it is generally viewed
32 as a “stop-gap” measure to reduce the risk of invasions. Structural and health-safety risks exist
when conducting ballast water exchange in bad weather and/or high seas, and data supporting the
34 effectiveness of the procedure are limited (Taylor et al., 2002; Murphy et al., 2004).

Despite efforts to develop alternatives to ballast water exchange (e.g., NRC, 1996;
36 Hallegraeff, 1998), other treatment options have yet to receive regulatory acceptance. One
method currently under evaluation is the use of ozone gas as a chemical oxidant that would kill
38 NIS prior to ballast water discharge (Cooper et al. 2002, Herwig et al., unpublished data). Ozone
has been used as a disinfectant since the late 1800s and is commonly used for drinking water
40 disinfection in Europe and, to a lesser extent, in the United States (Langlais, et al., 1991; Hoigné,

1998). While ozone itself is an effective disinfection agent owing to its strong oxidizing
42 properties, it can react with other chemical components in water that may disinfect after
becoming oxidized by ozone. As a result, ozone toxicity and disinfection is most often
44 expressed as a function of total residual oxidants (TRO), rather than as O_3 *per se* (Crececius,
1979).

46 A preliminary study testing ballast water ozonation was recently conducted onboard the *S/T*
Tonsina (Cooper et al. 2002, Herwig et al., unpublished data), a 265 m, double hull oil
48 tanker containing multiple segregated ballast water tanks with a total capacity of
41,365,000 L. Use of a prototype ozone system for 5-10 h resulted in a 71-99 % reduction
50 of selected marine phytoplankton, zooplankton, and bacteria. At the end of these ozonation
experiments, TRO concentrations exceeded 5 mg/L as Br_2 , but it was not possible to
52 quantify effective doses to any individual species with any precision in these field-scale
studies.

54 Because of the inherent challenges in performing shipboard testing of ballast water
ozonation, lab-based studies are an effective way to simulate exposure to several surrogate
56 marine species, and to verify toxicologically effective doses of TRO. Our goal was to quantify
oxidant loading rates and ozone toxicity to approximate the concentrations of ozone necessary to
58 eliminate an acceptable percentage of NIS from ballast water tanks. To accomplish this, we first
monitored oxidant formation with ozone treatment over time. Next, we attempted to simulate the
60 shipboard testing system onboard the *Tonsina* using batch-ozonation as a disinfection strategy
with five marine organisms: adult mysid shrimp (*Americamysis bahia*), larval topsmelt
62 (*Atherinops affinis*), juvenile sheepshead minnows (*Cyprinodon variegatus*), and adults of two
amphipod species (*Leptocheirus plumulosus* and *Rhepoxinius abronius*). The toxic effect of

64 short-term ozone exposure was then tested by observing post-exposure mortality of juvenile
Americamysis bahia. The persistence of ozonation byproducts was also determined by
66 conducting toxicity tests with aggressively ozonated water that had been stored from zero to 48
h.

68

MATERIALS AND METHODS

70 *Organisms*

For acute batch ozone toxicity studies, adult *Americamysis bahia*, larval *Atherinops*
72 *affinis*, juvenile *C. variegatus*, and adult *L. plumulosus* were obtained from Aquatic Biosystems
(ABS, Fort Collins, CO, USA), while adult *R. abronius* were collected in the field near
74 Anacortes, WA, and shipped overnight to the testing laboratory. Juvenile *Americamysis bahia*
(10 d) were also received from ABS for tests concerning post ozonation exposure and the
76 persistence of ozone byproducts. All organisms were in good condition before beginning testing.

78 *Testing apparatus and equipment*

All toxicity tests were conducted in glass aquaria (either 10 or 20 L) containing artificial
80 seawater (ASW; Forty Fathoms Crystal Sea and deionized water) at 28-30 ppt. Prior to testing,
aquaria were filled with ASW, placed in a water bath, and equilibrated overnight to test
82 temperature. Small pieces of nylon mesh were placed as substrate in aquaria used to conduct
toxicity tests with *L. plumulosus* and *R. abronius*.

84 Ozone was dispensed using a Model SC-10 ozone generator (Nutech 03 Inc., McLean,
VA). Total flow through the system was 2500 mL/min. Flow to each chamber was controlled
86 with an N012-10 flow meter with a glass float (Gilmont Instruments, Barrington, IL). Ozone gas

was distributed to the chambers using Kynar tubing and ozone tolerant diffusers (Aquatic
88 Ecosystems).

90 *Chemical analysis*

TRO measurements were obtained using an *N,N*-diethyl-1,4
92 phenylenediammonium/potassium iodide (DPD/KI) indicator and a Pocket Colorimeter (Hach,
Loveland, CO). This procedure was equivalent to USEPA Method 330.5 for wastewater and
94 Standard Method 4500-Cl G for drinking water. TRO concentration (mg/L) measurements were
calculated and expressed as equivalent concentrations of bromine (Br_2 , $1 \text{ mol Cl}_2 = 0.44 \text{ mol}$
96 Br_2).

98 *Rate of oxidant formation*

Three 20-L aquaria containing ASW at 28-30 ppt salinity were treated with ozone at a flow
100 rate of 61.6 ml/min over a period of 24 h. A 20-L control aquarium received compressed
air at the same flow rate. Similarly to methods used on the *Tonsina* by Cooper et al.
102 (2002), TRO measurements were obtained from all chambers at 0.5 h intervals from 0 to 6
h.

104

Ozone toxicity

106 Ozone toxicity experiments for larval *Atherinops affinis*, juvenile *C. variegatus*, and adult
R. abronius were conducted in 20-L aquaria, while experiments with adult *Americamysis bahia*
108 and adult *L. plumulosus* were performed in 10-L aquaria. All experiments used a total of five

chambers each containing ten organisms, with one chamber tested per treatment. Chambers
110 containing all organisms except *R. abronius* (15 ± 2 °C) were maintained at 23 ± 2 °C.

Total gas flow rates for 20-L chambers were 97.5, 63.2, 38.6, and 20.0 mL/min. These
112 flow rates corresponded to nominal ozone supply rates of 0.43, 0.28, 0.17, and 0.09 mg
O₃/L/min. Controls received compressed, ambient air at 97.5 mL/min (i.e., maximum flow rate).
114 Total gas flow rates for 10-L chambers were 38.6, 28.3, 20.0, and 13.1 mL/min (0.34, 0.25, 0.17,
and 0.11 mg O₃/L/min; control air flow = 38.6 mL/min). Experiments were run for a maximum
116 of five h. TRO measurements were recorded with biological observations (mortality and motility
of survivors) at 0.5-, 1-, 2-, 3-, 4-, and 5-h following test initiation. Experiments were terminated
118 within the 5-h exposure period if all organisms in a treatment died.

120 *Effects of short-term ozone exposure on longer-term survival*

Juvenile *Americamysis bahia* (10 d) were placed in five 20-L glass aquaria (19 ± 2 °C, ten
122 organisms per chamber). Total gas flow rates for 20-L chambers were 97.5, 63.2, 38.6, and 20.0
mL/min (0.43, 0.28, 0.17, and 0.09 mg O₃/L/min; control air flow = 97.5 ml/min). TRO
124 measurements were taken both before initiating ozone treatment and after 75 min of exposure.
After 90 min of exposure, surviving organisms from each chamber were removed and placed
126 into beakers of clean seawater maintained in a water bath at 19 ± 2 °C, and fed *Artemia*
franciscana (0.1 mL per beaker). The shrimp were examined at 24 h after terminating exposure
128 for mortality, and dead organisms were removed. Surviving organisms were again fed *Artemia*
franciscana, and examined again for mortality at 48 h after exposure.

130

Toxicity of residual oxidants over time

132 A 20-L glass aquarium containing ASW at 19° C was treated with ozone at 97.5 ml/min
(0.43 mg O₃/L/min) until targeted TRO values (> 4.0 mg/L) were reached (1.5 h; see results). A
134 portion of the treated water (2.5 L) was obtained for immediate use, while the remainder was
transferred from the aquarium to 20-L low-density polyethylene Cubitainers (Hedwin
136 Corporation, Laporte, IN) and stored in darkness without container headspace at 12° C. Toxicity
experiments were initiated with the ozone-treated water at 0, 24, and 48 h following this
138 exposure period.

A range of TRO concentrations were achieved by mixing the ozonated water with fresh
140 ASW. Concentrations of ozonated water used in toxicity tests were 100 % (ozonated water
only), 75 %, 50 %, 25 %, and 0 % (ASW only). Three, 300 ml replicates of each concentration
142 in 500 ml beakers were used for each test and maintained at 19 ± 2°C in a water bath. TRO was
measured for each treatment concentration. Ten juvenile *Americamysis bahia* (8 d) were used in
144 each replicate, and were fed 0.2 ml *Artemia franciscana* at test initiation. The shrimp were
examined at 24 h for mortality, and dead organisms were removed. Surviving organisms were
146 again fed *A. franciscana*, and examined again for mortality at 48 h after the beginning of the test.

148 *Data analysis*

Toxicity endpoints were expressed either as median-lethal concentrations (LC50) at
150 specific exposure times ranging from 1 – 48 h, or as median-lethal times (LT50) as a function of
ozone gas loading rates. In addition, 95 %-lethal concentrations (LC95) were calculated to
152 estimate time-specific TRO concentrations associated with nearly complete mortality. All
endpoints were calculated using the Trimmed Spearman-Kärber method (e.g. Hamilton et al.
154 1977), or by linear interpolation if acceptable trim values were exceeded. All endpoint

calculations were conducted using the Comprehensive Environmental Toxicity Information
156 System (CETIS V1.0, Tidepool Scientific Software, McKinleyville, CA). LC50 and LC95 values
for batch ozone toxicity tests were obtained from measured TRO concentrations and the total
158 number of mortalities observed at each time period following test initiation. LC50 and LC95
values for experiments testing the toxicity of residual oxidants over time were expressed as a
160 function of TRO concentrations measured immediately after test initiation.

162 RESULTS

Oxidant formation over time

164 Ozonation of ASW in glass aquaria over 5 h during the acute batch toxicity tests
indicated a gradual increase of TRO over time without saturation. An example plot of TRO
166 concentrations at each ozone flow rate as a function of time for the *L. plumulosus* tests is
presented in Figure 1. At lower flow rates (0.11 – 0.17 mg O₃/L/min), TRO concentrations
168 reached 1.9 – 3.6 mg/L, whereas concentrations reached 4.6 – 5.6 mg TRO/L at higher flow
rates. Thus, at any given exposure period, increasing ozone gas delivery rates generated
170 increasing instantaneous TRO concentrations in ASW.

172 *Effects of short-term ozone exposure on survival*

LC50 values for all organisms ranged from 0.31 to > 5.63 mg/L, with 100 % mortality of
174 each species except *L. plumulosus* occurring in less than 5 h (Table 1). The juvenile topsmelt
(*Atherinops affinis*) was the most sensitive organism tested, with LC50 values of 0.38 and 0.31
176 mg TRO/L after only 1 and 2 h of ozone exposure, respectively. Juvenile sheepshead minnows
(*C. variegatus*) were nearly as sensitive, but it took up to 4 h to reach a similar final LC50 (0.35

178 mg TRO/L). In contrast, all three invertebrates tested were significantly more tolerant of ozone
exposure, with juvenile *Americamysis bahia* reaching a lowest LC50 of 0.62 mg TRO/L at 3 h,
180 and adult *R. abronius* reaching a lowest LC50 of 0.94 mg TRO/L after 4 h. This same trend in
relative species sensitivity was also evident at 2 h (i.e., the longest exposure period with less than
182 100 % mortality for all species) with the two juvenile fish having the lowest LC50s (0.31 and
0.44 mg TRO/L), and the invertebrates *Americamysis bahia* and *R. abronius* exhibiting
184 significantly higher LC50s (1.37 and 1.72 mg TRO/L, respectively; Table 1). 95 %-lethal effect
concentrations (LC95) were approximately two to three-fold higher than LC50 values for all
186 species and time values testing (Table 2). No significant mortality was observed in the amphipod
L. plumulosus at any TRO concentrations tested up to 5.63 mg TRO/L after 5 h of batch
188 ozonation (Tables 1 and 2).

To indicate the time needed to induce significant mortality via batch ozonation, LT50
190 values were derived for the three most sensitive species (Figure 2). Similarly to the LC50 results,
juvenile topsmelt (*Atherinops affinis*) were the most sensitive to ozone exposure in ASW with
192 median lethal times ranging from 84 – 38 min at the lowest to highest ozone loading rates,
respectively. Both the mysid shrimp (*Americamysis bahia*) and sheepshead minnows (*C.*
194 *variegatus*) exhibited longer median lethal times ranging from 139 – 184 min at the lowest ozone
loading rate to 86 – 60 min at the highest ozone loading rates. LT50 data could not be derived for
196 either of the less sensitive amphipods, *R. abronius* or *L. plumulosus*.

198 *Effects of short-term ozone exposure on longer-term survival*

When juvenile mysids (*Americamysis bahia*) were removed from ozonated ASW after
200 1.5 h, only 30 – 60 % mortality had occurred at the two highest ozone loading rates (Figure 3).

However, mortality continued to occur even after organisms were transferred to clean ASW.

202 Mortality ranged from 20 – 100 % in organisms previously exposed to the highest three ozone
loading rates after 24 h, and from 60 – 100 % in organisms previously exposed to all four ozone
204 loading rates after 48 h.

206 *Toxicity of residual oxidants over time*

After 1.5 h of ozonation at 0.43 mg O₃/L/min, TRO reached 2.24 mg/L which, when
208 diluted with clean ASW, created a dilution series ranging down to 0.59 mg TRO/L at 25 %
ozonated ASW (Figure 4). Relatively little TRO loss occurred after ASW storage with a
210 maximum concentration of 2.13 mg TRO/L at 24 h, and 1.66 mg TRO/L at 48 h. As a
result, dilution series generated an acceptable range of TRO concentrations for deriving
212 median lethal effects levels in *Americamysis bahia* when measured at the time of test
initiation (Figure 4).

214 LC50 values for *Americamysis bahia* in waters tested immediately following ozone
treatment were 0.70 and 0.47 mg TRO/L at 24 h and 48 h, respectively (Table 3). For both
216 24-h and 48-h mortality data, LC50 values tended to decline slightly with increasing
storage time, but these differences were not statistically significant (i.e., 95 % confidence
218 limits all overlapped). 95 % effect concentrations exhibited similar trends with 24-h LC95s
ranging from 1.06 – 0.75 mg TRO/L, and 48-h LC95s ranging from 1.03 – 0.74 mg TRO/L
220 (Table 3).

222 **DISCUSSION**

The goal of this study was to quantify the time- and concentration-dependent toxicity of
224 ozone-produced oxidants to better understand whether ozone disinfection could be an
effective means of controlling marine NIS. Our acute batch ozone exposure experiments
226 indicated that topsmelt and sheepshead minnows (*Atherinops affinis* and *C. variegatus*)
were the most sensitive to short-term oxidant exposure, with the mysid shrimp
228 *Americamysis bahia* being the most sensitive invertebrate, and the amphipod (*L.*
plumulosus) being the least sensitive of all species tested. LC50s as low as 0.31 – 0.35 mg
230 TRO/L were observed, but the topsmelt reached this value after only 2 h of ozonation vs. 4
h for the sheepshead minnow. *Americamysis bahia* was the next most sensitive organism
232 with a 3 h LC50 of 0.62 mg TRO/L, and *R. abronius* was more tolerant with a significantly
higher 3 h LC50 of 1.37 mg TRO/L. In contrast, no mortality was observed in the second
234 amphipod, *L. plumulosus*, even after 5 h of ASW ozonation which generated up to 5.63 mg
TRO/L.

236 Mortality from ozone exposure occurred quickly with complete mortality occurring for all
but *L. plumulosus* in less than 5 h at ozone loading rates as low as 0.1 mg O₃/L/min.

238 Median lethal times also indicated rapid onset of mortality with LT50 values less than 1.5 h
for juvenile topsmelt, and ranging from 1 – 3 h for sheepshead minnows and mysid shrimp.

240 But even these short-term indicators of toxicity from ozonated ASW probably
underestimate eventual mortality levels, as indicated by mortality continuing to increase in
242 *Americamysis bahia* in 24 – 48 h after only 1.5 h of exposure to ozonated seawater.

Seawater ozonation thus can induce rapid mortality in marine organisms, but organisms can
244 still succumb 1-2 d later from sub-lethal exposures to ozonated seawater for relatively brief

periods of time. Delayed mortality has also been observed for the fast acting oxidant
246 chlorine (Brooks and Seegert 1977, Latimer et al. 1975, USEPA 1985).

248 While LC50 values are useful for characterizing relative toxicity among different species,
removal of NIS from ballast water tanks would likely require mortality rates substantially
greater than 50 %. For example, the State of Washington has set an interim ballast water
250 discharge standard of 95 % inactivation or removal of zooplankton organisms (State of
Washington, 2002), and so we derived 95 % lethal effect concentrations where possible
252 (Tables 2 and 3). Most LC95s were about 2-fold higher than their corresponding LC50s,
with LC95s of 0.59 – 1.46 mg TRO/L for the more sensitive juvenile fishes, and LC95s for
254 *Americamysis bahia* ranging from 1.14 – 1.67 mg TRO/L for short-term exposures (i.e., 2-3
h), and 0.74 – 1.06 mg TRO/L for 24 – 48 h exposures. This 2-fold difference in LC50 and
256 LC95 values denotes steep dose-response curves, which has also been observed with other
fast-acting oxidants such as chlorine (USEPA 1985). The more resistant *R. abronius*
258 exhibited less steep dose-response curves with LC95s about 3-fold higher than LC50s.
Other studies confirm that seawater ozonation can induce rapid mortality to marine
260 organisms, but comparisons of specific effect concentrations are complicated by the variety
of exposure times and oxidant measurement methods used in each study. Ozone has been
262 reported to be toxic against a number of marine organisms, including phytoplankton and
crab larvae (Toner and Brooks 1975), striped bass eggs and larvae (*Morone saxatilis*, Hall
264 et al. 1981), and white perch (*M. americana*, Block et al. 1978; Richardson et al. 1983)*. In
these studies, 100 % mortality was induced in 24 – 96 h following relatively short-term
266 exposures (less than 10 min in some cases) to ozone-produced oxidant concentrations

* While LC50 values expressed in these cases were originally reported as mg Cl₂/L, we have converted them to mg Br₂/L.

268 ranging from 0.18 – 0.44 mg TRO/L as Br₂ for most species, with mortality observed in
some crab larvae and white perch early life stages at concentrations as low as 0.04 mg
TRO/L as Br₂ (Toner and Brooks 1975, Hall et al. 1981). Continuous exposures to
270 ozonated seawater also induced LC50 values ranging from 0.03 – 0.18 mg TRO/L as Br₂ in
striped bass, depending on the life stage and exposure duration (Hall et al. 1981). Our
272 results indicate that higher oxidant concentrations may be necessary for the immediate
removal of some planktonic larvae and juvenile fish, although we did not evaluate
274 mortality at time periods longer than 5 h except for *Americamysis bahia*. We found no
other studies with marine organisms that were consistent with the high level of resistance
276 that we observed in the benthic amphipod *L. plumulosus*.

While these data confirm that ozone is toxic to marine organisms, ozone *per se* is not likely
278 to be the chemical species directly responsible for toxicity in seawater. Ozone has a very
short chemical half-life in seawater (approximately 5 seconds, Haag and Hoigné, 1984), and
280 disappears quickly following the introduction of ozone gas (Crecelius, 1979). The
presence of bromide ion (Br⁻) changes the chemistry of ozone decomposition when
282 compared to freshwater systems (Oemcke and van Leeuwen, 1998). Ozonation of seawater
oxidizes bromide ion to bromine (hypobromous acid [HOBr] and hypobromite ion [OBr⁻]
284 with a pKa of 8.8 as HOBr/OBr⁻), that may lead to bromate ion (BrO₃⁻) (Haag and Hoigne,
1983; von Gunten and Hoigné, 1992, 1996; von Gunten and Oliveras, 1997, 1998;
286 Pinkernell, et al., 2000; von Gunten and Pinkernell, 2000; Hofman and Andrews, 2001).
Additionally, the presence of organic material may result in reactions with HOBr to
288 produce bromoform (CHBr₃).

Of these three brominated species, bromine is most likely to be rapidly toxic at
290 concentrations similar to those reported in the present study. For example, standard acute
toxicity tests were conducted with bromine chloride (BrCl) for eight marine organisms
292 (including larval fish, bivalves, and crustaceans), with LC50 values ranging from 0.11 –
0.80 mg/L (Burton and Margrey 1978, Roberts and Gleeson 1978). In contrast, both
294 bromate ion and bromoform are substantially less toxic than bromine, and so are unlikely to
contribute any significant toxicity. Bromate ion LC50 values range from 30 mg/L for the
296 pacific oyster *Crassostrea gigas* to 512 mg/L for the chum salmon *Oncorhynchus keta*
(Creclius 1979), and bromoform LC50 values range from 7.1 mg/L for *C. variegatus*
298 (Ward et al. 1981) to 26 mg/L for the brown shrimp *Penaeus aztecus* (Anderson, et al.,
1979). In the studies onboard the *Tonsina*, bromate ion was never detected and bromoform
300 was found to occur at concentrations below 1 mg/L (Cooper et al., 2002, Herwig et al.,
unpublished data).

302 In seawater treated with ozone, TRO measurements are an effective means of quantifying
oxidant production. Bromine as HOBr/OBr⁻ may be the only component of TRO
304 measurements on the basis of proposed reaction pathways (e.g., Driedger et al., 2001).
Therefore HOBr/OBr⁻ can accumulate in ozonated seawater, and based on the observed
306 persistence of toxicity for at least two days in the present study, it is likely the oxidant
primarily responsible for toxicity.

308 The difference in oxidant sensitivity observed among organisms used in the present study
may be explained by the potential for ozone-produced chemicals to oxidize biological
310 membranes. Because fish gill membranes can be easily damaged (Richardson et al. 1983),
this could explain the extreme sensitivity of the larval fish relative to the invertebrates

312 tested in the present study. This is also consistent with marine fish being among the most
sensitive to chlorine in seawater (with the exception of the copepod *Acartia tonsa* and the
314 Eastern oyster *Crassostrea virginica*; USEPA 1985). By comparison, benthic crustaceans
in our study were the most tolerant of ozone exposure, perhaps because their respiratory
316 structures are not as readily exposed to ambient water. This is also consistent with the
toxicity of chlorine to marine organisms, with amphipods and crabs being among the most
318 acutely tolerant species (USEPA 1985).

320 *Conclusions*

322 Our results indicate that several marine invertebrate and fish species can be effectively
eliminated following short-term (i.e., less than 5 h) ozonation at TRO concentrations less than 1
324 mg/L as Br₂, and that ozone-produced oxidants can accumulate and remain toxic in closed
containers for at least two days. However, benthic crustaceans may be relatively tolerant of
326 ozone-produced oxidants, and so may require other control methods to prevent introductions
from ballast water discharge. Although laboratory conditions differ significantly from ballast
328 water tanks onboard ocean vessels, applying these data on a much larger scale could be useful in
approximating TRO concentrations needed to remove an acceptable percentage of NIS from
330 ballast water. For example, in companion studies we demonstrate that effective TRO
concentrations to *Americamysis bahia* are similar in several natural seawaters collected from
332 different locations (Jones et al. 2005, this issue), and that TRO concentrations of ≥ 1 mg/L were
associated with high levels of mortality in natural zooplankton assemblages in mesocosms
334 (Perrins et al, unpublished data) and actual ballast water tanks (Cooper et al. 2002, Herwig et al.,
unpublished data). Furthermore, laboratory toxicity data could be used to help evaluate how far

336 TRO concentrations might have to be decreased prior to discharge to ensure the safety of
indigenous organisms in receiving waters. Ozone-produced oxidant concentrations will decrease
338 in ballast waters prior to discharge by natural decay processes (e.g., time, mixing, or sunlight
exposure; Perrins et al. unpublished data), or by the addition of reductants such as sodium
340 thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$) to eliminate toxicity (Jones et al. 2005).

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Table 1: Median-lethal effect concentrations (LC50 in mg TRO/L as Br₂) for five marine taxa following short-term ozone exposure. Cells without values denote 100 % mortality. Data are presented according to total time of exposure.

Species	LC50 (95 % C.I.) mg TRO/L as Br ₂					
	0.5 h	1 h	2 h	3 h	4 h	5 h
<i>Americamysis bahia</i>	> 0.9	> 1.22	1.37	0.62		
<i>Atherinops affinis</i>	> 0.9	0.38	0.31	(1.29, 1.45)		
<i>Cyprinodon variegatus</i>	> 0.42	1.13	0.44	0.44	0.35	
<i>Rhepoxinius abronius</i>	> 0.48	> 1.51	1.72	1.37	0.94	
<i>Leptocheirus plumulosus</i>	> 0.65	> 1.24	> 2.93	> 3.63	> 4.21	> 5.63

Table 2: 95 %-lethal effect concentrations (LC95 in mg TRO/L as Br₂) for five marine taxa following short-term ozone exposure. Cells without values denote 100 % mortality. Data are presented according to total time of exposure.

Species	LC95 (95 % C.I.) mg TRO/L as Br ₂				
	0.5 h	1 h	2 h	3 h	5 h
<i>Americamysis bahia</i>	> 0.9	> 1.22	1.67	1.14	
<i>Atherinops affinis</i>	> 0.9	1.15	0.59		
<i>Cyprinodon variegatus</i>	> 0.42	> 1.13	0.82	1.46	0.63
<i>Rhepoxinius abronius</i>	> 0.48	> 1.51	> 2.46	2.66	2.9
<i>Leptocheirus plumulosus</i>	> 0.65	> 1.24	> 2.93	> 3.63	> 4.21
					> 5.63

Table 3. Median- and 95 %- lethal concentrations (LC50 and LC95 in mg TRO/L as Br₂) for *Americamysis bahia* exposed to freshly ozonated ASW (0 h), and ozonated ASW that had been stored for 24 and 48 h.

Storage time (h)	24-h LC50	48-h LC50	24-h LC95	48-h LC95
0	0.70 (0.63, 0.78)	0.47 (0.27, 0.90)	1.06 (1.06, 1.06)	1.03 (0.91, 1.09)
24	0.50 (0.47, 0.54)	0.43 (0.36, 0.51)	0.83 (0.83, 0.83)	0.82 (0.81, 0.82)
48	0.43 (0.38, 0.48)	0.32 (0.23, 0.43)	0.75 (0.74, 0.76)	0.74 (0.70, 0.77)

Figure 1. Observed trends in oxidation-reduction potential total residual oxidant (TRO in mg/L as Br₂) in control and all four ozone treatments. Data are from the 5-h toxicity test conducted with *L. plumulosus*.

Figure 2. Median lethal times (LT50 in min) for *Atherinops affinis*, *C. variegatus*, and *Americamysis bahia* as a function of ozone loading rates (in mg O₃/L/min). Curved lines represent best-fit power function equations for each species.

Figure 3. Percent mortality of 10-d-old *Americamysis bahia* following transfer of organisms to clean ASW after ozone exposure for 90 min. Biological observations were made at 0, 24, and 48 h following transfer of organisms.

Figure 4. Initial TRO concentrations (mg/L as Br₂) for toxicity tests using *Americamysis bahia* in ozonated seawater stored for 0, 24, and 48 h following ozonation. Treatments for each toxicity test were made in a 50 % dilution series from the same batch of ozonated water.

Figure 1.

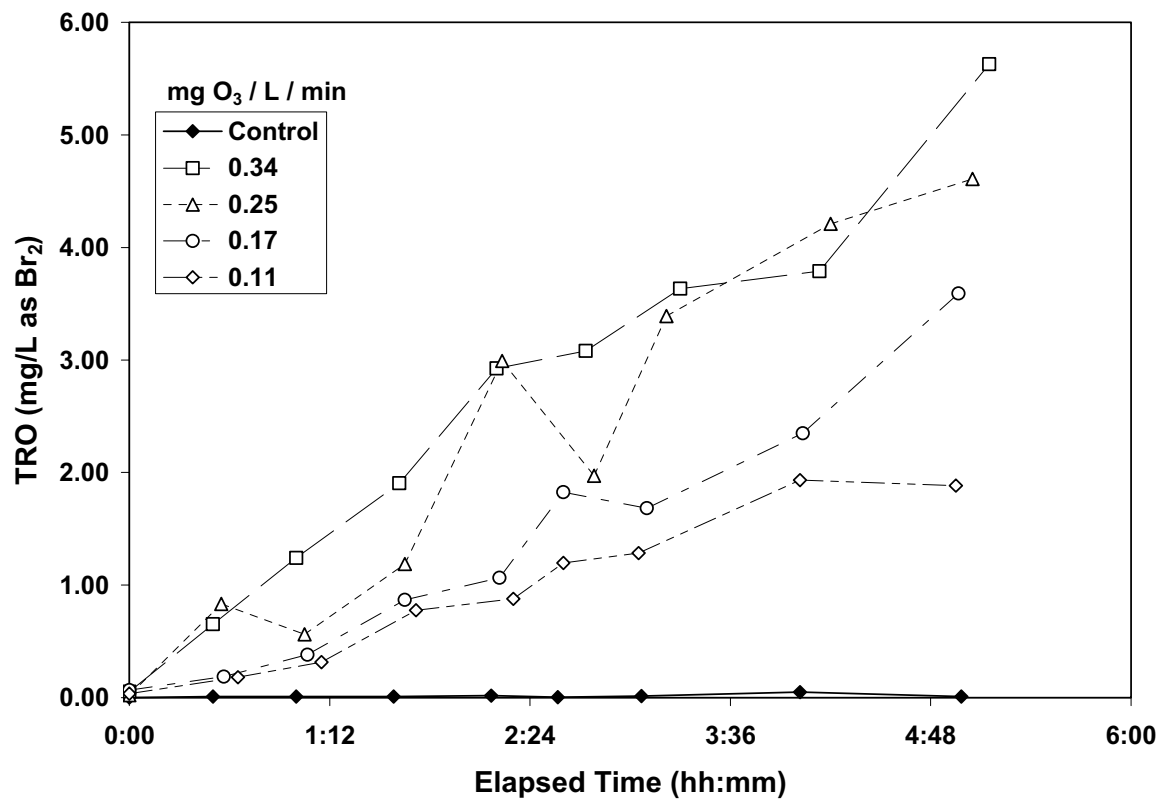


Figure 2.

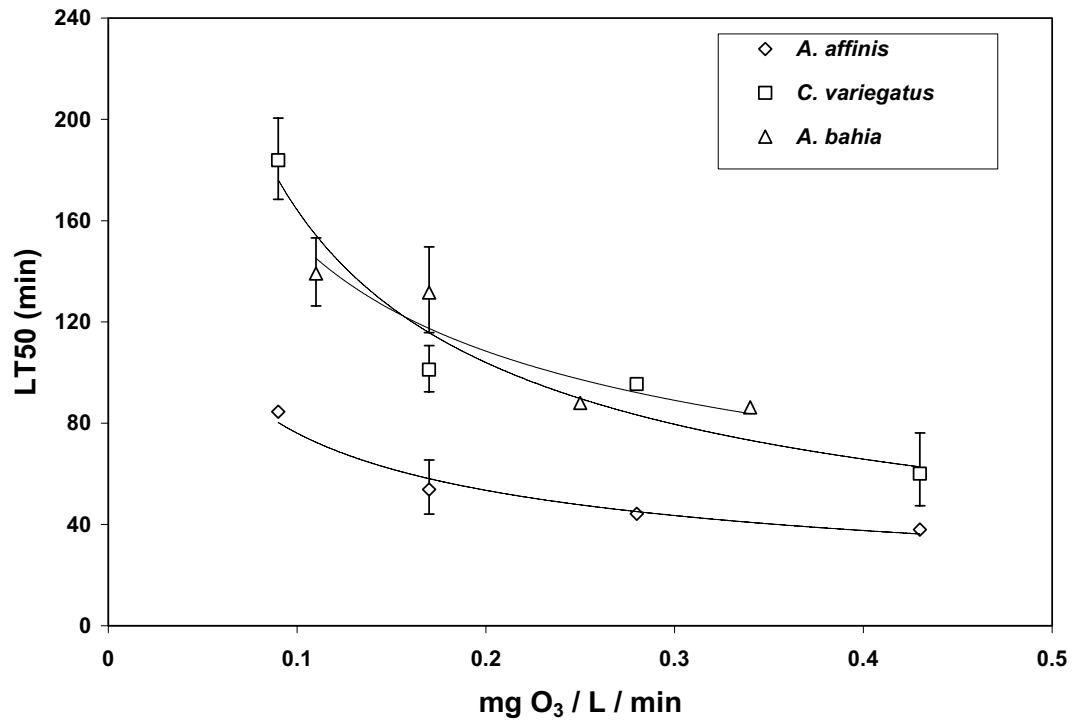


Figure 3.

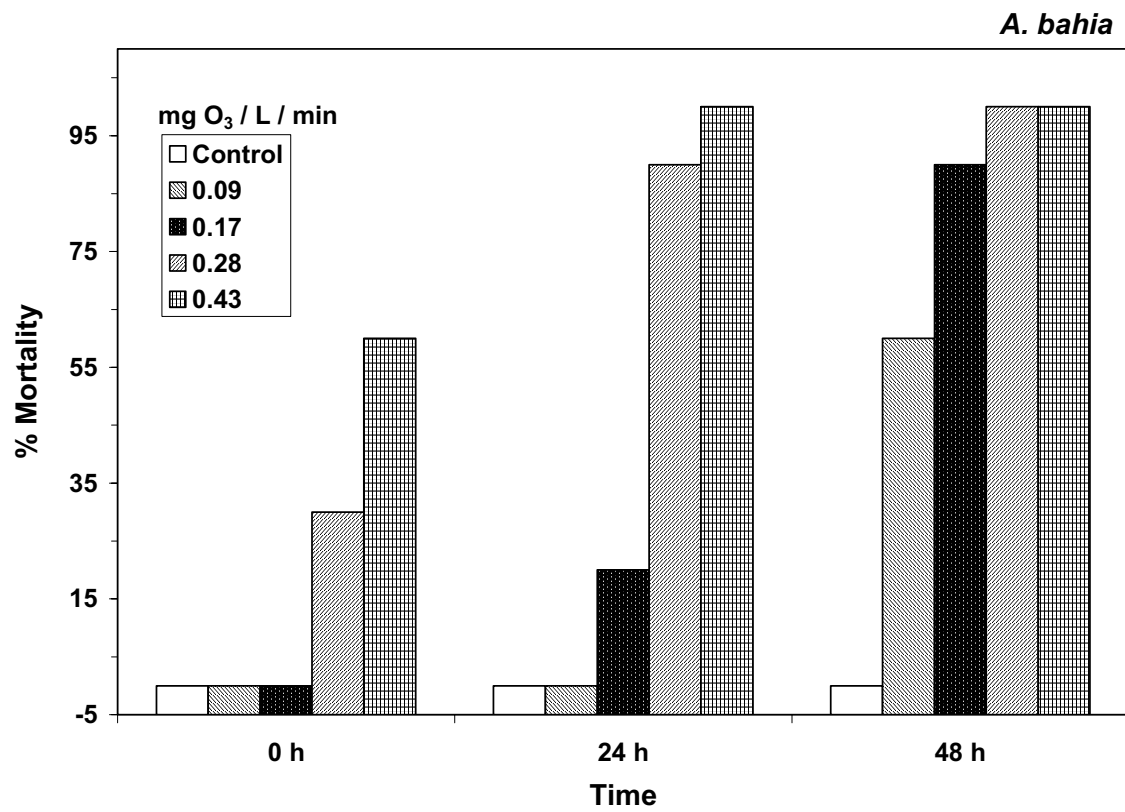


Figure 4.

