Comparing the sensitivity of four bioassays for acrolein

A.C. Sneekes
N.H.B.M. Kaag

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Institute for Marine Resources and Ecosystem Studies

Wageningen IMARES

Client: EnvioMar GmbH
Martin Wolf
Kanalstrasse 25
44147 Dortmund
Germany

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Contents

1 Introduction

Introduction of non-indigenous species is a risk associated with discharge of ballast water from ships transporting cargo between regions. The IMO has set out a mandatory framework for ballast water management on board ships.

EnvioMar GmbH has developed a Ballast Water Treatment System (BWTS) using acrolein as active substance. The toxicity of the active substance, as well as the residual toxicity of the treated ballast water has to be assessed, according to IMO regulations on BWTS using active ingredients (G9). EnvioMar asked IMARES to conduct bioassays in order to assess the toxicity of the treated ballast water and the active ingredient, acrolein.

As a first step in the toxicity assessment, the sensitivity of four marine test species was assessed for acrolein (*Table 1*). These species are candidates for use in experiments in which the toxicity of treated ballast water is followed in time, in order to establish the most efficient treatment concentration.

Table 1 'Easy-to-use' test species used to establish relative sensitivity

Test species	**Comment**
Algae: *Phaeodactylum tricornutum*	Algae are required for approval
Crustacean: *Artemia franciscana*	Crustaceans are required for approval
Rotifer: *Brachyonus plicatilis*	Easy in use
Bacteria: *Vibrio fisheri* (Microtox)	Fastest and cheapest option. Helgoland results may be used for comparison

Based upon literature data, effect concentrations were expected in a range between 0.020 and 0.500 mg/L. Test concentrations were, therefore, established in a logarithmic range from 0.010 to 1.000 mg/L acrolein, except for the Microtox test, where a starting concentration of 0.100 mg/l was diluted 50% in each step.

2 Material & Methods

2.1 Preparation of stock solutions

A 10g/L stock solutions of acrolein were prepared following the prescriptions of the sponsor.
The stock solution was made by adding 7.7 ml diethoxypropene to 2.3 ml sulphuric acid (10%). After shaking for 30 sec and stabilising for 30 min, this was added to 100 ml milli-Q in a stirring vessel, during stirring another 280 ml milli-Q was added. The basic stock was stored in a dark bottle at 5°C until use.

2.2 Algal test: Phaeodactylum tricornutum

The test protocol is based upon OECD 201 (1984) and ISO 10253 (2006), adapted for small volume testing as described by Blaise & Vasseur (2005) and Peterson *et al.* (2005). The test species used was the marine diatom *Phaeodactylum tricornutum*. A strain of this culture was obtained from MicroBioTests (Batch: PT121007). Cultures were started at the laboratory using F2-medium with a salinity of 35‰ and incubated under the conditions of the test.

Tests were performed in clear 96-multiwell plates, with eight replicates per dilution. Two rows of blanks are included: one next to the lowest test concentration and one between the two highest test concentrations. Inhibition of the latter row indicates the influence of volatile substances.

Each test concentration was inoculated with approx. 10,000 cells/mL of the log-phase growing culture. Correction for background values (false color) was performed with four replicates per dilution. Fluorescence was measured with a multi-plate-reader and the plates were then incubated in continuous light at 18°C while shaken at 130 rpm. The development of the algal biomass, measured as fluorescence, was measured with 24 h intervals for 3 days. With each test series, a reference test was conducted, using potassiumdichromate ($K_2Cr_2O_7$) as toxicant.

The following concentrations of acrolein were tested: 0 (blank); 0.010; 0.032; 0.100; 0.316 and 1.000 mg/L.

Summary of test conditions for the algal growth inhibition test with the marine diatom *Phaeodactylum tricornutum*

Test organism	*Phaeodactylum tricornutum*
Test organism source	MicroBioTests Inc. Belgium
Test organism life stage	Log-phase growth, ca. 4 day old culture
Test duration	72 h
Test chamber	96-multiwell plate
Test solution volume	240 µL per replicate
Initial concentration	Approx. 10,000 cells/mL algae
Replicates	8
Method	Fluorescence measurements
Endpoint	Growth
Test temperature	18±2°C
Dilution water	UBW, 0.2 µm filtered
Media stock solutions	F2-medium
Photoperiod	Permanent light
Shaking	130 rpm
Test protocol	OECD 201 (1984) and ISO 10253 (2006)
Control acceptability criteria	Minimum chlorophyll-a increase by factor 16 after 72 h
Reference test	$K_2Cr_2O_7$

Statistics

Growth rates for the various test conditions were calculated by the least squares fitting exponential as described by Weisstein (2008a). The control growth rates were checked on validity ($r>0.9d^{-1}$). Percentage of inhibition was calculated using the following formula:

$$I_{\mu i} = \frac{\mu_c - \mu_i}{\mu_c} \times 100\% \quad (1)$$

Wherein: $I_{\mu i}$ = the percentage inhibition (growth rate) for each test concentration i;
μ_i = the growth rate for each test concentration i;
μ_c = the mean growth rate for the control.

Percentages of inhibition were cut-of at 100%, to correct for negative growth (mortality).

EC_{50}s were calculated as being the concentration/dilution that causes 50% growth inhibition, using a logistic formula (ISO-TS 20281, 2006). The following formula was used for calculations:

$$y = \frac{100}{1+10^{(\log EC50 - x)*Hillslope}} \quad (2)$$

Wherein: x = Concentration or dilution
y = Specific percentage inhibition mu
EC50 = effect concentration causing 50% growth inhibition
Hillslope = slope of the logistic curve

The EC50 and the Hillslope are unknown values in the formula and are determined with non-linear regression using the Gauss-Newton algorithm as described by Björck (1996), wherein the values are evaluated iterative on bases of the Jacobian matrix described by Weisstein (2008b).

The NOEC was determined as the highest test concentration in which the percentage of inhibition was not significantly reduced compared to the control (UBW). LOEC was determined as the lowest test concentration in which the percentage of inhibition was significantly reduced compared to the control (UBW). The NOEC and the LOEC were assessed by means of a T-test.

The calculated effect concentrations are corrected for dilution (ca. 4%) by added nutrient stock and algal inoculum.

2.3 Crustacean test: Artemia franciscana

The test was performed in a multiwell testplate using instar II-III larvae of the brine shrimp *Artemia franciscana*, obtained as dried cysts, conform the Standard Operational Procedure for ARTOXKIT M™ (MicroBioTests Inc. V100603).

The cysts were incubated in artificial sea water 30 h prior to the start of the tests at 25°C with continuous illumination. After 30h, most cysts were hatched and the larvae developed into instar II-III stages.

Approx. 50 larvae were transferred to a rinsing well containing test medium. From the rinsing well, 10 larvae were transferred to the three test wells per concentration/dilution for the test. The well plate was incubated at 25°C in the dark. After 24h living and dead larvae in the test wells were counted.

The following concentrations of acrolein were tested: 0 (blank); 0.010; 0.032; 0.100; 0.316 and 1.000 mg/L.

Summary of test conditions for the ARTOXKIT M wit the brine shrimp *Artemia franciscana*

Test organism	*Artemia franciscana*
Test organism source	ARTOXKIT M, MicroBioTests Inc.
Test organism life stage	Larvae <24h old
Test duration	24 h
Test chamber	24-well plate
Test solution volume	1 mL per replicate
Number of organisms/chamber	10
Replicates	3
Endpoints	Survival
Test temperature	25±2°C
Dilution water	Filtered seawater
Photoperiod	Permanent dark
Test protocol	Standard Operating Procedure MicroBioTests
Control acceptability criteria	<10% mortality

Statistics

EC_{50}s were calculated as being the concentration/dilution that causes 50% mortality, using the formula for a dose-response curve with variable slope built-in in GraphPad Prism V4.03. GraphPad Prism uses the following formula:

$$y = Bottom + \frac{Top - Bottom}{1 + 10^{(\log EC50 - x) * Hillslope}} \quad (3)$$

Wherein: x = Concentration or dilution
y = Mortality (as %)
Top = Maximum mortality (100)
Bottom = Minimum mortality (0)

The NOEC was determined as the highest test concentration in which the survival was not significantly reduced compared to the control (filtered seawater). This was assessed by means of a one-way ANOVA followed by Dunnett's multiple comparison post-test.

2.4 Rotifer test: Brachionus plicatilis

The test to be applied is commercially available as 'Testkit' at MicroBiotest Inc., Belgium under the name ROTOXKIT M and performed according to the Standard Operational Procedure provided by the manufacturer.

The cysts were incubated in artificial sea water 28-30 h prior to the start of the tests at 25°C with continuous illumination. After 28-30h, most cysts were hatched. Approx 50 larvae were transferred to a rinsing well containing test medium. From the rinsing well, 5 larvae were transferred to the six test wells per concentration/dilution for the test. The well plate was incubated at 25°C in the dark. After 24h living and dead larvae in the test wells were counted.

The following concentrations of acrolein were tested: 0 (blank); 0.010; 0.032; 0.100; 0.316 and 1.000 mg/L.

Summary of test conditions for the ROTOXKIT M wit the marine rotifer *Brachionus plicatilis*

Test organism	*Brachionus plicatilis*
Test organism source	ROTOXKIT M, MicroBioTests Inc.
Test organism life stage	Newly hatched (<2 h)
Test duration	24 h
Test chamber	multiwell plate
Test solution volume	0.3 mL per replicate
Number of organisms/chamber	5
Replicates	6
Endpoints	Survival
Test temperature	25±2°C
Dilution water	Filtered seawater
Photoperiod	Permanent dark
Test protocol	Standard Operating Procedure MicroBioTests
Control acceptability criteria	<10% mortality

Statistics

EC_{50}s were calculated as being the concentration/dilution that causes 50% mortality, using the formula for a dose-response curve with variable slope built-in in GraphPad Prism V4.03. GraphPad Prism uses the following formula:

$$y = Bottom + \frac{Top - Bottom}{1 + 10^{(\log EC50 - x) * Hillslope}} \quad (3)$$

Wherein: x = Concentration or dilution
y = Mortality (as %)
Top = Maximum mortality (100)
Bottom = Minimum mortality (0)

The NOEC was determined as the highest test concentration in which the mortality was not significantly reduced compared to the control (filtered seawater). This was assessed by means of a one-way ANOVA followed by Dunnett's multiple comparison post-test.

2.5 Bacteria test: Vibrio fischerii (Microtox)

The Microtox Basic test exposes luminescent bacteria in Reagent to aqueous samples, and measures the increase or decrease in light output by the test organisms, which is an indicator of the organism's condition. Reagent contains living luminescent bacteria that have been grown under optimal conditions, harvested, and then lyophilized (freeze-dried). The lyophilized bacteria are rehydrated with Reconstitution Solution to provide a ready-to-use suspension of organisms. The test system measures the light output of the luminescent bacteria after they have been exposed to a sample and compares it to the light output of a control (reagent blank) that contains no sample. A difference in light output (between the sample and the control) is attributed to the effect of the sample on the organisms.

The following concentrations of acrolein were tested: 0 (blank); 0.006; 0.013; 0.025; 0.050 and 0.100 mg/L.

Summary of test conditions for the Microtox® basic test

Test organism	*Vibrio fischeri*
Test organism source	Microlan, Netherlands
Test organism life stage	Lyophilized
Test duration	5,15, 30 min
Test chamber	3 mL glass cuvet
Test solution volume	1 mL
Test temperature	15±2°C
Dilution water	Diluent (2%-NaCl)
Test protocol	AZUR Environmental Basic Test (1998)
Control acceptability criteria	-
Reference toxicant	Phenol

Statistics

The EC_{10} and EC_{50} were calculated using the Microtox Omni software.

3 Results

No toxicity was found for the tests with the rotifer *Brachionus plicatilis* and the crustacean *Artemia franciscana*. The maximum concentration tested was 1 mg/L (1 ppm).

Clear concentration related effects were observed in the Microtox test and the test with the marine algae *Phaeodactylum tricornutum*. The toxicity in the Microtox test increased with increasing exposure-time. An EC50 of 0.44 mg/L was found after 5 min exposure, while after 30 min exposure, the toxicity had increased to an EC50 of 0.053 mg/L (see *Table 2*). The EC10 (approximation of the NOEC), decreased from 0.072 to 0.010 mg/L).

Table 2 Results of the Microtox test

Result		**Results for T = 5 min**		**Results for T = 15 min**		**Results for T = 30 min**	
Sample	Endpoint	Value (mg/L)	95% confidence interval	Value (mg/L)	95% confidence interval	Value (mg/L)	95% confidence interval
	EC50	0.440	(0.397-0.487 mg/L)	0.122	(0.097 - 0.155 mg/L)	0.053	(0.040 - 0.070 mg/L)
	EC10	0.072		0.024		0.010	

In the algae test, the highest toxicity was observed after 48h with an EC50 of 0.124 mg/L. After 72h exposure, the EC50 was increased to 0.203 mg/L, indicating some recovery (*Table 4*). The raw data (*Table 3*) show that recovery can be observed in all concentrations, except the highest concentration (C5). Within the time period tested, the NOEC was 0.030 mg/L.

Table 3 Results of the algae test; average raw data (after correction).

In: 17-2-2009 13:14		**0 h**	**24 h**	**48 h**	**72 h**
Concentration code	**Concentration**	***0.00***	***1.03***	***1.99***	***3.02***
C0	0.00	76	154	610	3608
C1	0.01	58	137	547	3220
C2	0.03	70	139	466	2748
C3	0.10	91	131	366	2078
C4	0.30	87	64	132	517
C5	0.96	82	53	-3	2

Table 4 Results of the algae test; toxicity endpoints.

Result		**Results for T = 48h**		**Results for T = 72h**	
Sample	**Endpoint**	**Value (mg/L)**	**95% confidence interval**	**Value (mg/L)**	**95% confidence interval**
	EC50	0.124	(0.105 - 0.146 mg/L)	0.203	(0.174 - 0.238 mg/L)
	NOEC	0.030		0.030	
	LOEC	0.095		0.095	

4 Conclusions

It can be concluded that amongst the tests selected for this study the Microtox Basic test showed the most sensitive response to acrolein. The bacteria toxicity after 15 minutes exposure was comparative to the algae toxicity after 48h exposure. However, toxicity for the bacteria after 30 minutes results in much lower values. This indicates higher toxicity. The EC_{50} value for the bacteria after 30 minutes (0.05mg/L) is comparable to the lowest values found (WHO, 2002).

It is proposed to use the Microtox test in the pre-test, in order to establish the optimal dosing concentration and remaining toxicity in time.

5 Quality Assurance

IMARES utilises an ISO 9001:2000 certified quality management system (certificate number: 08602-2004-AQ-ROT-RvA). This certificate is valid until 15 December 2009. The organisation has been certified since 27 February 2001. The certification was issued by DNV Certification B.V. Furthermore, the chemical laboratory of the Environmental Division has NEN-AND-ISO/IEC 17025:2005 accreditation for test laboratories with number L097. This accreditation is valid until 27 March 2009 and was first issued on 27 March 1997. Accreditation was granted by the Council for Accreditation, with the last inspection being held on the 5th of October 2007.

6 Literature

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Justification

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The scientific quality of this report has been peer reviewed by a colleague scientist and the head of the department of Wageningen IMARES.

Approved: E.M. Foekema
Researcher

Signature: 

Date: May 7, 2009

Approved: Drs. J.H.M. Schobben
Head of department

Signature: 

Date: may 7, 2009

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