

Confounding Factors in Bioassays with Freshwater and Marine Organisms

J. F. Postma,*¹ S. de Valk,* M. Dubbeldam,* J. L. Maas,† M. Tonkes,† C. A. Schipper,‡ and B. J. Kater‡

*AquaSense, P.O. Box 95125, 1090 HC Amsterdam, The Netherlands; †Institute for Inland Water Management and Waste Water Treatment (RIZA), P.O. Box 17, 8200 AA Lelystad, The Netherlands; and ‡National Institute for Coastal and Marine Management (RIKZ), Fieldstation Jacobahaven, Jacobaweg 2, 4493 MX Kamperland, The Netherlands

Received June 28, 2001

The use of bioassays in ecological risk assessments often raises questions about the causative factors, and insight into the possibility that confounding factors, such as pH or increased ammonia concentrations, might be responsible for the observed toxicity is needed. It was decided to develop a practical approach for the Dutch situation, in which a first screening is carried out based on provisional criteria. In collecting the required data, dozens of experiments were performed, while the scientific literature was searched for additional information. It is concluded that the provisional criteria specified are at present useful tools in interpreting results of bioassays. Depending on the outcome and the aim of the research, it might be necessary to further reduce uncertainties in the interpretation. This might require some additional experiments, using alternative controls or test procedures or altering the composition of the original sample. © 2002 Elsevier Science (USA)

Key Words: confounding factors; bioassays; freshwater; marine water.

1. INTRODUCTION

Throughout the world dozens of different toxicity tests are used to characterize the potential ecotoxicological effects of all kinds of substances. Since the implementation of these tests is often laid down by law and financial consequences might be involved, extensive attention is focused on quality assurance. This has resulted in international guidelines standardized by, for example, ISO or OECD, in which criteria for the validity of toxicity tests are established. In some guidelines these criteria also includes water quality parameters such as pH or oxygen saturation. These measures are taken to ensure (as far as possible) a constant environment and an optimal health of the organisms during the tests. If criteria are not met it might be necessary to

repeat the test, adding, for example, an artificial buffer to reduce the variation in pH. These criteria are predominately used to guarantee optimal test performance and are not based on the ecological range of the test organism.

Besides toxicity tests with pure substances, the same organisms and test facilities are used to study possible ecotoxicological effects in environmental matrices (e.g., Hill *et al.*, 1993; Traunspurger and Drews, 1996). The guidelines used for these bioassays are often based upon existing guidelines for toxicity tests and therefore specify similar ranges of variables such as oxygen or pH. In some environmental samples, violation of the criteria may have a natural cause (reduced oxygen level in some sediments, low pH values in peaty samples, etc.), while in samples from polluted areas several other important variables may also exceed the thresholds (e.g., ammonia, sulfide, salinity; e.g., Jacobs *et al.*, 1992; Meadows *et al.*, 1981; Thompson *et al.*, 1991; Wang and Chapman, 1999). These variables are often called “confounding factors,” since these variables interfere with biological effects of micropollutants. This raises several questions. First, should negative effects due to these confounding factors be treated differently from effects of micropollutants and, second (but related), are deviating values considered natural or anthropogenic? As a consequence, and depending on the aim of the research, some parameter values might be adjusted prior to and during the bioassays while others are left unchanged.

In The Netherlands bioassays are used for assessing ecological risks of waste water, surface water, and groundwater as well as sediments, soils, or dredged material (e.g., Den Besten *et al.*, 1995; Stronkhorst *et al.*, 1996; Tonkes, 1997). Many such samples do not fulfill the basic ecological demands and will be qualified as “toxic.” Although it is realized that, depending on the aim of the research, negative effects due to such confounding factors may be considered as important as the effects of micropollutants, the general approach in The Netherlands is that the influence of these confounding factors should at least be recognized. If

¹To whom correspondence should be addressed. Fax: + 31-20-5922249.
E-mail: jpostma@aquasense.com.

TABLE 1
Overview of Standardized Bioassays Used in The Netherlands

Organism		Guideline	Duration	Temperature (C°)	Use
Freshwater					
Pelagic species					
<i>Raphidocelis subcapitata</i> ^a	Alga	ISO 8692, 1989	72 h	20	e, w
<i>Brachionus calyciflorus</i>	Rotifer	Creasel, 1990a	24 h	25	e, s, w
<i>Daphnia magna</i>	Crustacean	ISO 6341, 1996	48 h	20	e, s, w
		OECD 202, 1995			
		ISO/DIS 10706, 1998	14 days ^b	20	e, s, w
<i>Thamnocephalus platyurus</i>	Crustacean	Creasel, 1992	24 h	25	e, s, w
<i>Brachydanio rerio</i>	Fish	OECD 203, 1992	96 h	23	e
		OECD 210, 1992	8 days ^b	25	e, s
Benthic species					
<i>Chironomus riparius</i>	Insect	van de Guchte <i>et al.</i> , 1993 (OECD draft)	28 days	20	s
Marine					
Pelagic species					
<i>Vibrio fischeri</i> (Microtox)	Bacteria	ISO 11348-2, 1998	30 min	15	e, s, w
<i>Phaeodactylum tricornerutum</i>	Alga	ISO 10253, 1995	72 h	20	e, w
<i>Brachionus plicatilis</i>	Rotifer	Creasel, 1990b	24 h	25	e, s
<i>Acartia tonsa</i>	Crustacean	ISO 14669, 1999	48 h	20	e
<i>Artemia salina</i>	Crustacean	Creasel, 1990c	24 h	25	e
<i>Poecilia reticulata</i>	Fish	OECD 203, 1992	96 h	23	e
Benthic species					
<i>Crassostrea gigas</i>	Oyster	ASTM E 724, 1999	48 h	20	e, s
<i>Psammechinus miliaris</i>	Urchin	Dinnel <i>et al.</i> , 1987	1 h	20	e, s, w
<i>Corophium volutator</i>	Amphipod	ASTM E 1367, 1999	10 days	15	s
<i>Echinocardium cordatum</i>	Heart urchin	Bowmer, 1993 (PARCOM)	14 days	15	s

Note. Guidelines on which test procedures are based are specified as well as test duration, temperature, and present use in The Netherlands. e, effluents; s, sediments; w, surface water.

^aFormerly *Selenastrum capricornutum*.

^bDerivatives from guidelines.

possible, the influence of confounding factors is minimized by changing pH, oxygen, or salinity values before testing, but alterations should be as small as possible. This necessitates specification of ranges for which negative effects can be excluded for each variable, organism, and exposure period. Dozens of experiments were therefore performed to determine the influence of confounding factors on routinely used bioassays, focusing on effects of different pH values, oxygen depletion, nitrite, ammonia, sulfide, and salinity.

The aim of this article is to provide a state of the art overview concerning the experimental results obtained in past years. Using these results and comparable data obtained from literature, provisional criteria are proposed.

2. MATERIALS AND METHODS

Experimental procedures. Experiments were performed with both pelagic and benthic, freshwater and marine organisms. With the exception of *Corophium volutator*, *Psammechinus miliaris*, and *Echinocardium cordatum*, which were collected in the field, all organisms were obtained from cultures. Concentration ranges tested for each combination

of confounding factor and organism were based, as far as possible, on available knowledge. Most protocols used are based on international guidelines standardized by, for example, ISO or OECD. Almost all toxicity tests were performed using a water-only exposure. In the case of the benthic organisms *Chironomus riparius*, *C. volutator*, and *E. cordatum* some of the tests were (also) performed using a sediment-water exposure system, in which case confounding factors were measured in the overlying water. Table 1 provides an overview of the organisms used as well as some additional information (guidelines, duration of the test, temperature). The methods used for each test are therefore not specified in detail. However, two deviations from the guidelines are noted, which refer to the present, standardized use of the bioassay in The Netherlands (see Table 1).

Appropriate quality assurance measures were taken for all toxicity tests performed. Most of them are directly based on the guidelines (for example, referring to the control performance). In addition, toxicity tests with reference toxicants were conducted for all organisms studied and experimental results were discarded if the reference toxicity test failed the criteria. In the case of marine organisms quality

TABLE 2
Concentration Ranges Tested in Present Experiments (Number of Concentrations in Parentheses)

Species	pH	O ₂ (% saturation)	NO ₂ ⁻ (mg/L)	NH ₄ ⁺ + NH ₃ (mg/L)	Sulfide ^a (mg/L)	Cl ⁻ (g/L)	Conductivity (μS/mm)
Freshwater							
<i>Raphidocelis subcapitata</i>	8.3-9.8 (6)		0-59 (6)	0-53 (6)		0-3.5 (6)	15-485 (6)
<i>Brachionus calyciflorus</i>		15-100 (6)		0-74 (6)		0-2.1 (6)	
<i>Daphnia magna</i> (acute)	8.3-11 (6)		0-78 (8)	0-1000 (6)		0-3.4 (7)	
<i>Daphnia magna</i> (chronic)			0-12 (6)	0-93 (6)			
<i>Thamnocephalus platyurus</i>		14-100 (6)		0-73 (6)		0-2.4 (6)	
<i>Poecilia reticulata</i>						0-11 (5)	
<i>Brachydanio rerio</i> (acute)				0-87 (6)		0-10 (6)	
<i>Brachydanio rerio</i> (chronic)	1-12 (12)	12-100 (5)	0-3000 (5)	0-50 (6)		0-10 (6)	
<i>Chironomus riparius</i>			0-67 (7)	0-1000 (6)		0-18 (7)	
Marine							
<i>Vibrio fischeri</i>	5-9 (3)	29-100 (5)	0-70 (6)	0-1000 (10)	0-12.5 (10)		0-4150 (13)
<i>Phaeodactylum tricorutum</i>	8-10 (6)		0-147 (6)	0-104 (6)		3.3-6.0 (6)	760-1380 (6)
<i>Brachionus plicatilis</i>		15-100 (6)		0-2100 (6)		0.1-0.5 (5)	17-140 (5)
<i>Acartia tonsa</i>		8-50 (5)	0-99 (6)	0-128 (8)			815-1840 (11)
<i>Artemia salina</i>		15-100 (6)		0-2160 (6)			50-250 (5)
<i>Poecilia reticulata</i>				0-91 (6)		17-23 (5)	
<i>Crassostrea gigas</i>		40-110 (5)	0-100 (5)	0-10 (5)			2250-4150 (4)
<i>Psammechinus miliaris</i>	7-9 (5)	10-100 (6)	0-320 (7)	0-180 (9)			2440-4600 (10)
<i>Corophium volutator</i>	7-9 (5)	20-100 (4)	0-965 (10)	0-310 (8)	0-50 (3)		
<i>Echinocardium cordatum</i>	7-9 (5)	20-80 (4)	0-1743 (7)	0-120 (8)	0-50 (3)		

^aConcentration expressed as total sulfide ($\Sigma[S(-II)] = [H_2S] + [HS^-]$).

assurance is described in more detail by Stronkhorst *et al.* (in press).

Acclimation of the organisms. Toxicity tests with marine or brackish organisms were normally conducted using a salinity of 32‰. In the case of chloride or salinity experiments other values were used as well. In such case, all organisms were acclimated to the values used as control prior to testing (either highest value if lowest boundary was tested or lowest value if highest boundary was tested). Therefore, toxicity tests not only reflected the ability of organisms to survive and grow at a certain salinity but also their ability to acclimate to changes in those values. An experimental setup in which organisms are acclimated to each individual salt concentration is more suitable for studying the salinity tolerance of organisms. However, bioassays performed on a routine basis normally do not include such a detailed acclimation (due to costs involved) and it was decided to study the protocols used for the standard performance of bioassays. In such cases, separate criteria should probably be specified concerning both factors. For example, the algae *Phaeodactylum tricorutum* might survive and grow well in the range between 4 and 32‰ (Sigaud and Aidar, 1993), but the maximum difference in salinity between the inoculum

and test conditions should be limited to circa 6 to 8‰ (unpublished data AquaSense). Chloride or salinity tests involving freshwater organisms were performed without prior acclimation of the organisms to increased values. In these cases, it should therefore be realized that by using such an acclimation the criteria might become more pliable.

Confounding factors. The present study focused on effects of different pH values, oxygen depletion, nitrite, ammonia, sulfide, and chloride as well as on salinity/electrical conductivity. Table 2 provides an overview of the concentration ranges tested for each individual organism and exposure period. Results are all based on measured concentrations, which were normally within reasonable limits compared to both nominal values and observed variations during the tests. If measured concentrations strongly fluctuated during the tests (for example, in the case of some sulfide experiments) an indication concerning the reliability of the criterion is added.

pH. Ranges in pH were established using HCl/NaOH (bacteria, algae, and daphnids), an artificial buffer (e.g., Trizma for *Corophium*, *Echinocardium*), or changes in the CO₂ flux (*Corophium*, *Echinocardium*). Values were regularly measured during the tests and adjusted if necessary. In the

tests with both freshwater and marine algae, pH values were measured every 3 h during the entire experiment (values were adjusted if deviation was ≥ 0.2 units compared to the nominal value). To keep the total test volume equal in all concentrations, each vessel received the same number of droplets, being HCl, NaOH, or demineralized water. pH values were measured using standard, commercially available electrodes.

Oxygen. Differences in oxygen saturation were established using N_2 . Experiments were conducted in closed vessels with, if necessary, N_2 in the air space above. Oxygen saturation values were measured using commercially available electrodes and corrected for salinity. Values were standardized at a temperature of 20°C.

Nitrite. All nitrite toxicity experiments were performed using $NaNO_2$. If necessary, pH values were adjusted using HCl or NaOH. Nitrite concentrations were measured using a spectrometer (Lasa 20, Dr. Lange). If no special notes are present, experiments were performed using standard test pH values.

Ammonia. All ammonia toxicity experiments were performed using NH_4Cl . If necessary, pH values were adjusted prior to and during the tests using HCl/NaOH, and artificial buffer (e.g., Trizma), or changes in the CO_2 flux (especially for marine tests). Ammonia concentrations were measured using a spectrometer (Lasa 20, Dr. Lange). Reported values relate to the total concentration of NH_4^+ and NH_3 unless stated differently. If necessary, the partition between NH_4^+ and NH_3 was calculated based on the actual pH, temperature, and salinity during the tests using formula such as described by the American Petroleum Institute (1981).

Sulfide. Sulfide experiments were performed with the bacteria *Vibrio fischeri*, the amphipod *C. volutator*, and the burrowing heart urchin *E. cordatum*. For the latter two organisms experimental results should be considered rough estimates due to practical difficulties encountered (e.g., maintaining sulfide concentrations on a constant level while simultaneously keeping oxygen saturation sufficient). Concentrations reported relate to the total sulfide concentration. All experiments were performed without changing the pH values (actual values around 7.5). Spectrophotometric measurements of the sulfide concentrations were based on the method described by Cline (1969).

Chloride. For all freshwater organisms, toxicity of chloride was tested by adding either NaCl or raw sea salt. Chloride concentrations were measured by titration with $AgNO_3$ according to Dutch Standard NEN 6476. In most cases results can also be used to derive criteria concerning salinity/electrical conductivity. In these cases, results are

shown only for chloride while provisional criteria are also set for the other two variables.

Salinity/electrical conductivity. In addition to the chloride test, the tolerance of the freshwater alga *Raphidocelis subcapitata* to high salinities was tested using $MgCl_2$, $CaCl_2$, and $MgSO_4$. These three mineral salts were based on the macronutrients in ISO Guideline 8692, while the other two mineral salts mentioned in this guideline (NH_4Cl and KH_2PO_4) were not used. In most cases salinity tolerance for the marine organisms is determined using dilution series based upon (natural or artificial) sea water with a salinity of 32‰ and demineralized water. In the case of the marine alga *P. tricornutum* a different experimental setup was used, resulting in two dilution series with the same electrical conductivity, while the chloride (and other macronutrients) concentrations differed. The inoculum was grown in a medium intermediate from the medium used for the two dilution series, due to which the algae for both series had to acclimate at the beginning of the tests to more or less the same osmotic shock.

Statistical analyses. Results were statistically analyzed specifying both no-observed-effect levels and $L(E)C_{50}$ values, as far as possible. If specified, statistical analyses were based on the recommendations mentioned in the guidelines. In all other cases tests and procedures were selected based on the specific variable and applied accordingly to, for example, Sokal and Rohlf (1981). Statistical analyses were performed using either ToxCalc (Tidepool, 1993) or SPSS (Norusis, 1992).

3. RESULTS

An overview of the experimental results as well as results obtained from literature is shown in Tables 3a (freshwater organisms) and 3b (marine organisms). Criteria should be set at such a level that samples, for which the criteria are not exceeded while negative effects are present, provide a strong indication that toxicants are present. The tested concentration range therefore aimed at specifying NOEC and LOEC values. Due to this choice it was not possible to estimate $L(E)_{50}$ values in all experiments. Still, $L(E)_{50}$ values provide valuable information on the slope of the dose-effect curve. Therefore, Tables 3a and 3b show not only the highest or lowest concentrations for which no significant effects were observed, but also $L(E)_{50}$ values when applicable. In cases where significant effects were already observed in the lowest concentration tested, results are considered to be preliminary and "<" is added to indicate that all concentrations tested caused significant effects.

pH. While most test organisms tolerate rather wide ranges in pH, especially the algae were shown to be extremely sensitive. Significant differences in growth rate

(differences of 5–10%) were already demonstrated when the pH value differed by not more than 0.3–0.4 units from the control (8.0 or 8.3 for the marine and freshwater species respectively). It was therefore concluded that variations in pH values should be limited to 0.2–0.3 units in relation to the control pH at the start of the experiment, although it is realized that maintaining constant pH values during the test will be difficult especially in environmental samples.

Regarding the data on pH tolerance for fish, it should be noted that these data (obtained from Alabaster and Lloyd, 1980, as is the case for the minimum oxygen levels for fish) are not specific for *Brachydanio rerio* or *Poecilia reticulata*, but concern the tolerance of fish species in general. In all other cases, presented literature is restricted to the specific species mentioned.

Oxygen. Results of most experiments indicated a minimal required oxygen level of 15–40% saturation. For the oyster *Crassostrea gigas* a maximum level is also specified, since oversaturation also affects the embryological development. With both the amphipod *C. volutator* and the heart urchin *E. cordatum* short-term experiments (72 h) were performed in which the organisms were exposed to low oxygen levels. During these experiments no increased mortality was observed, although the sensitivity of both *C. volutator* and *E. cordatum* to other stress factors such as ammonia seemed to be increased at low oxygen levels (20 and 40% saturation, respectively). Since these experiments lasted only 3 days, criteria for the semichronic bioassay (10 and 14 days, respectively) should be set at a higher level. It is however noted that exceeding those values for a short period of time will probably not cause any harm, as long as the actual levels are above 20% (*Corophium*) or 40% (*Echinocardium*).

Nitrite. In comparing freshwater and marine organisms, the latter seemed to be the least sensitive to nitrite (e.g., algae, rotifers, and crustaceans). Furthermore, for both the amphipod *C. volutator* and the heart urchin *E. cordatum* an increased sensitivity to nitrite was demonstrated at lower pH levels. In general, experiments demonstrate that nitrite measurements are less useful in many of the bioassays focusing on marine sediments, since no-observed-effect levels will seldom be exceeded.

Ammonia. As expected, effect concentrations are generally lower at higher pH levels. However, it should be noted that not all toxicity was due to NH_3 . Especially in the case of *Corophium* (and to some extent *Echinocardium*), where detailed studies were carried out at several pH values, it was concluded that NH_4^+ added to the observed toxicity at pH values less than 8.3. Part of the variation observed in ammonia toxicity to the midge *C. riparius* was due to variations in the age of the larvae tested. Results are therefore specified for different larval instars.

Sulfide. Sulfide toxicity was only tested for three marine organisms: *V. fischeri*, *C. volutator*, and *E. cordatum* and only the data for *V. fischeri* are entirely accurate. The experiments with *Corophium* and *Echinocardium* were short-term, water-only experiments (72 h) in which the sulfide concentrations strongly decreased during the first few hours of the test (due to the oxygenation). These experiments should therefore be repeated, preferably including a sediment–water exposure system.

Chloride/salinity/electrical conductivity. In the case of freshwater organisms results demonstrate that no-observed-effect levels for all organisms vary between ± 1 and 5 g $\text{Cl}^- \cdot \text{L}^{-1}$. In the case of brackish or marine organisms both low and high salinity values can cause negative effects and criteria should be specified accordingly.

In most experiments effects of increased or decreased chloride concentrations could not be distinguished from effects of an increased or decreased salinity. Results are therefore presented for only one of the variables. To distinguish effects of Cl^- from effects of salinity, additional experiments were performed with both freshwater and marine algae, in which either the NaCl concentration or the overall composition of the medium was changed. Results demonstrated that (as was to be expected) not only the absolute Cl^- concentration or conductivity, but also the relative amount of other elements such as Ca, Mg, Na, and K as well as the acclimation of the algae influenced the results.

4. DISCUSSION

Experimental results were normally in good agreement with results obtained from literature. Provisional criteria were therefore set using both the experimental data and the data obtained from literature (Tables 4a and 4b) and according to the following principles: (i) if possible, criteria are based on no-observed-effect levels; (ii) in cases where multiple tests are available, criteria are based on lowest values, unless tests differences in reliability or the original experimental data were not recovered from literature; (iii) criteria were rounded off if judged appropriate (depending on the accuracy and variation in the actual concentrations as well as the distance between NOEC and LOEC values); (iv) if test duration deviated, criteria were extrapolated based on expert judgment (in which case criteria are considered preliminary). As a consequence, samples for which the criteria are not exceeded while negative effects are present provide a strong indication that toxicants are present. The opposite is however less certain: if a criterion is exceeded this does not necessarily mean that negative effects observed are due to this factor only.

Furthermore it should be realized that even criteria set at the estimated NOEC values might not ensure complete safety for the organisms. It is possible to envisage a bioassay

TABLE 4a
Overview of Criteria Concerning Freshwater Species

Species	pH	O ₂ (% sat.)	NO ₂ ⁻ (mg/L)	NH ₄ ⁺ + NH ₃ (mg/L) (°C, pH)	Cl ⁻ (g/L)	Conductivity (µS/mm)
Freshwater pelagic						
<i>Raphidocelis subcapitata</i>	8.1–8.5 ^{p,2}	nr	<60	<25 (23, 8.0)	<1.3	<400
<i>Brachionus calyciflorus</i>	5–9	>15	<50	<70 (25, 8.0)	<1.4	<320
<i>Daphnia magna</i> (acute)	5.5–9.5	>20 ^p	<10 ^e	<60 (20, 7.5) <60 (20, 8.0) <15 (20, 8.5)	<2.8	<650
<i>Daphnia magna</i> (chronic)	6.5–9 ^p	>30 ^p	<5 ³ <2 ⁴	<30 ⁴ (20, 7.5) <15 ⁴ (20, 8.0) <5 ⁴ (20, 8.5)	<0.8	<185
<i>Thamnocephalus platyurus</i>	5–11	>20	<2	<30 ^p (25, 8.0)	<0.5	<120
<i>Poecilia reticulata</i> ¹	5–9 ^p	>60 ^p	—	<100 (23, 7.5)	<11	<2500
<i>Brachydanio rerio</i> (acute)	5–9 ^p	>60 ^p	—	<40 (23, 8.0)	<5.6	<1300
<i>Brachydanio rerio</i> (chronic)	4–9 ^p		<560	<20 > 30 (25, 8.0)	<3.2	<700
Freshwater benthic						
<i>Chironomus riparius</i>	6.6–8.5	>35 ^p	4th: <50 ^p	1–4th: <200 (20, 6.5) 1–4th: <200 (20, 7.5) 1–4th: <200 (20, 8.0) 1–4th: <40 (20, 8.5)	1st: <1.3 ^p 2nd–4th: <4.1	1st: <300 ^p 2nd–4th: <950

Note. —, no data available. ¹Organisms acclimated in freshwater; ²only applicable at $t = 0$ and based on a pH in the control of 8.3; ³mortality; ⁴reproduction. nr, not relevant. ^efirst estimate; ^ppreliminary. If no special notes are present concerning the temperature, the pH, or the salinity, experiments (e.g., concerning toxicity of ammonia) were performed using the standard experimental temperature, pH, and salinity. Criteria calculated using criteria for other variables (chloride, salinity, conductivity) are given in italics.

in which the test organisms overreact to the contaminants present, because they are simultaneously stressed by certain confounding factors, deemed “safe” based on the criteria. In such cases, however, at least part of the observed effect is due to the presence of contaminants, a conclusion that is sufficient for the use of the proposed criteria in a first tier.

Most of the criteria proposed are thought to be accurate. Some experimental results however did not fully comply with all quality criteria and indications concerning the reliability of the criteria are added. In some experiments the measured concentrations strongly varied during the tests (>50%). This was especially the case for sulfide experiments with *Corophium* and *Echinocardium* and criteria were therefore considered first estimates (denoted: ^e). Criteria are considered preliminary (denoted: ^p) if (i) variability in the measured concentrations was less than 50% but still considerable; (ii) experiments were not replicated; (iii) effects were already observed in the lowest concentrations tested; and (iv) in cases where effects were observed which were considered important although not statistically significant (e.g., EC₁₀ < no-observed-effect value). In all other cases criteria proposed are thought to be accurate.

Several of the experiments presented and criteria proposed might require a thorough discussion in relation to the available scientific literature. The purpose of this article is however more focused on presenting an overview, illustrating the way in which questions relating to confounding

factors and bioassays are handled in everyday practice in The Netherlands. This discussion will therefore address only some general aspects.

First, special attention should be paid to bioassays with algae. Not only were the composition of the medium and the duration of the acclimation important factors affecting algal growth, pH values were also of prime importance. Effects of differences in pH on algal growth have already been studied and discussed in far more detail, even considering the specific species studied here (e.g., Mayer *et al.*, 1998; Nygaard *et al.*, 1986; Nyholm and Källqvist, 1989). Nygaard *et al.* (1986), for example, stated that the growth rate of *S. capricornutum* is at maximum in the pH range 6–9. Differences in growth rate within this range (as, for example, found in the present study) might consequently be caused by the experimental procedure and attention should focus on the acclimation, a sufficient supply of CO₂ from the air, and the test duration in relation to the density of the inoculum used. Realizing that the extent to which such effects might interfere with the outcome of algal bioassays is not yet fully understood, it was decided to set rather tight criteria concerning the pH at the start of bioassays with algae, trying to reduce influences of pH values as far as possible. Criteria concerning the variation in pH values during the tests are specified in the appropriate ISO guidelines.

A comparable complex matter concerns the toxicity of ammonia. Although NH₃ is the most toxic form, present

TABLE 4b
Overview of Criteria Concerning Marine Species

Species	pH	O ₂ (% sat.)	NO ₂ ⁻ (mg/L) (pH)	NH ₄ ⁺ + NH ₃ (mg/L) (°C, pH, sal)	Sulfide ¹ (mg/L)	Cl ⁻ (g/L)	Conductivity (μS/mm)	Salinity (‰)
Marine pelagic								
<i>Vibrio fischeri</i> ²	6-8.5	>30 ^p	<70	<1000 (15, 8, 32)	<3.3	<20	<4600	18-35
<i>Phaeodacrylum tricorutum</i>	7.7-8.3 ^{p,3}	nr	<150	<60 (20, 8, 32)	—	5-20 ^{p,4}	1150-4600 ^{p,4}	9-36 ^{p,4}
<i>Brachionus plicatilis</i>	5-9	>15	<1660	<1000 ^p (25, 8, 32)	—	0.6-18	140-4150	1-32
<i>Acartia tonsa</i>	7-9	>25	<10 ^p	<30 (20, 8.0, 32)	—	5.5-22	1300-5100	10-40
<i>Artemia salina</i>	4-9	>30	<200	<600 ^p (25, 7.5, 32)	—	0.7-18	200-4150	1-32
<i>Poecilia reticulata</i>	5-9 ^p	>60 ^p	—	<70 (23, 8.0, 32)	—	<23	<5500	<41
Marine benthic								
<i>Crassostrea gigas</i>	7.5-8.5	40-110 ^p	<32 ^p	<3 (20, 8.0, 32)	<0.1 ^p	11-18	2500-4200	20-32
<i>Panmechinus miliaris</i>	7.5-8.5	>70	<15	<32 (20, 7.5, 32)	—	—	—	28-36
				<18 (20, 8.0, 32)				
				<10 (20, 8.5, 32)				
<i>Corophium volutator</i>	7-9	>50 ^p	<30 ^p (7.0)	<100 ^p (15, 7.0, 32)	<2 ^e	2-22	500-5100	4-40
			<200 ^p (8.0)	<100 ^p (15, 7.5, 32)				
			<200 ^p (9.0)	<75 (15, 8.0, 32)				
				<50 (15, 8.1, 32)				
				<45 (15, 8.3, 32)				
				<30 (15, 8.5, 32)				
				<20 (15, 8.7, 32)				
				<10 (15, 9.0, 32)				
<i>Echinocardium cordatum</i>	7.5-8.5 ^p	>60 ^p	<50 ^p (7.0)	<25 ^p (15, 7.5, 32)	<5 ^e	>16 ^p	>3500 ^p	>28 ^p
			<200 ^p (8.0)	<15 ^p (15, 8.0, 32)				
			<200 ^p (9.0)	<5 ^p (15, 8.5, 32)				

Note. —, no data available. nr, not relevant, ^efirst estimate, ^ppreliminary; ¹concentration expressed as total sulfide; ²all criteria relate to values in the actual test system, with the exception of the pH (original sample). Depending on the dilution factor, values can be corrected to be used for the original samples; ³only applicable at $t = 0$ and based on a pH in the control of 8.0; ⁴strongly dependent on acclimation. If no special notes are present concerning the temperature, the pH, or the salinity, experiments (e.g., concerning toxicity of ammonia) were performed using the standard experimental temperature, pH, and salinity. Criteria calculated using criteria for other variables (chloride, salinity, conductivity) are given in italics.

experiments as well as the literature demonstrated that NH₄⁺ can also cause toxic effects (especially if pH < 8), while differences in sensitivity might in addition be species-specific (e.g., Armstrong *et al.*, 1978; Borgmann, 1994). Criteria were therefore not set as pH-independent values for NH₃ toxicity, but instead pH-dependent threshold levels for the combined effect of NH₄⁺ and NH₃ are specified.

Looking at the salinity preference of organisms in relation to the unknown composition of environmental samples, several aspects should be mentioned. Not only the electrical conductivity of a sample (integrating the individual components) but also the individual anionic and cationic components might exert negative effects, for which the individual concentrations and the proportion in relation to other components are of vital importance (e.g., Douglas and Horne, 1997; Dwyer *et al.*, 1992). Especially in the case of waste water the anionic and cationic composition might strongly deviate from the preference of test organisms. It was therefore decided to specify criteria for the total electrical conductivity/salinity of the sample as well as for the maximum or minimal chloride concentration. Although it is realized that it is comparatively easy to construct an unpolluted

sample which fulfils both criteria and which might still be severely toxic, it is unfeasible to specify criteria for all individual components. A better way to handle possible effects of the anionic and cationic content is the construction of statistical models like those developed for several major ions (e.g., Mount *et al.*, 1997; Tietge *et al.*, 1997). These models however are not yet available for all test organisms. As this moment it is advised to characterize the anionic and cationic content in more detail. In this way a data set could be constructed as a basis to develop such statistical models in the near future.

At present the provisional criteria proposed are used on a large scale in The Netherlands as tools in interpreting the results of bioassays and trying to distinguish effects of confounding factors from effects of micropollutants. Therefore they are an essential and useful tool in ecological risk assessments, provided that its merits are well understood.

One of the most important aspects for the present goal is the interaction between the different variables. Most experiments studied the effect of one specific variable. Specified criteria should therefore be considered thresholds for direct and simple effects. Multiple stresses are not taken into

t (except for pH and ammonia), but might be imposed as an example both Cowgill and Milazzo (1990) and *et al.* (1992) demonstrated that the effect of increased concentrations depended on the hardness of the water. Other examples (among many others) concern the interaction between chloride and HCO_3^- (Hoke *et al.*, 1992), and chloride/calcium (Wickins, 1981), ammonia and ammonium (Thurston *et al.*, 1981; Tudor *et al.*, 1994), ammonia and potassium (e.g., Borgmann and Borgmann, 1994), and salinity and hardness (Dwyer *et al.*, 1992). Furthermore, all those separate variables might also influence bioavailability, and effects of toxicants.

Future experiments should therefore address these aspects and consequently the criteria proposed here might be adapted. It is however an illusion to think that such a criterion can cover all different aspects, and researchers will have to deal with uncertainties. There are several options to deal with those uncertainties. First of all, both experimental and practical experience could be exchanged between laboratories and used to further fine-tune criteria. Another option might be that individual criteria as specified here will be replaced by statistical models, taking into account several interacting variables and estimating the expected effect in a certain sample, exemplified by models to estimate the toxicity of major ions (Tietge *et al.*, 1997; Mount and Dwyer, 1997). An alternative way to reduce uncertainties would be to include an additional control in bioassays (e.g., only in a tiered approach), mimicking the anionic and cationic composition of the sample as much as possible.

In addition, it should be realized that bioassays in the field are often part of a more extended research program. In such studies chemical analyses are (among others) compared to results of bioassays to see whether they strengthen or weaken the overall results, as is the case with inventories on benthic fauna. In special cases TIE procedures are applied to further indicate the causative agent. This will reduce the risk of false-positive or -negative results as a consequence of the criteria proposed here.

5. CONCLUSIONS

It is concluded that the provisional criteria are at present useful tools in interpreting results of bioassays, especially in the first tier. Depending on the outcome and the aim of the study it might be necessary to further reduce uncertainties in the interpretation. This might require some additional experiments, using alternative controls or test procedures or adjusting the composition of the original sample.

More research is needed to further increase the reliability and interpretation of several confounding factors. In the sample, no-observed-effect levels of several variables and species should be studied in more detail. Another point of concern relates to the acclimation of the test organisms used in the bioassays, which will certainly affect the out-

come of the bioassays. Furthermore, additional research should focus on the influence of the anionic and cationic composition of the samples, especially in the case of waste water samples.

ACKNOWLEDGMENTS

Most of the research was funded by either the Institute for Inland Water Management and Waste Water Treatment or the National Institute for Coastal and Marine Management. The authors thank especially J. M. Driike, A. van Mullem, M. Schot, and E. E. M. Grootelaar for practical assistance and J. Lahr for comments on earlier versions of the manuscript.

REFERENCES

- Alabaster, J. S., and Lloyd, R. (1980). *Water Quality Criteria for Freshwater Fish*. Food and Agriculture Organisation of the United Nations, Butterworths, London, England.
- American Petroleum Institute (1981). *The Sources, Chemistry, Fate, and Effects of Ammonia in Aquatic Environments*. American Petroleum Institute, Washington DC.
- American Society for Testing and Materials E 724 (1999). Standard guide for conducting static acute toxicity tests starting with embryos of four species of saltwater bivalve molluscs. In *Annual Book of ASTM Standards, 1999*, Vol. 11.05, ASTM E 724-98, pp. 195-215.
- American Society for Testing and Materials E 1367 (1999). Standard guide for conducting 10-day static sediment toxicity tests with marine and estuarine amphipods. In *Annual Book of ASTM Standards, 1999*, Vol. 11.05, ASTM E 1367-92, pp. 733-758.
- Ankley, G. T., Katko, A., and Arthur, J. W. (1990). Identification of ammonia as an important sediment-associated toxicant in the Lower Fox River and Green Bay, Wisconsin. *Environ. Toxicol. Chem.* **9**, 313-322.
- Armstrong, D. A., Chippendale, D., Knight, A. W., and Colt, J. E. (1978). Interaction of ionized and un-ionized ammonia on short-term survival and growth of prawn larvae, *Macrobrachium rosenbergii*. *Biol. Bull.* **154**, 15-31.
- Bervoets, L., Wills, C., and Verheyen, R. (1996). Tolerance of *Chironomus riparius* larvae (Diptera: Chironomidae) to salinity. *Bull. Environ. Contam. Toxicol.* **57**, 829-835.
- Borgmann, U. (1994). Chronic toxicity of ammonia to the amphipod *Hyalella azteca*: Importance of ammonium ion and water hardness. *Environ. Pollut.* **86**, 329-335.
- Borgmann, U., and Borgmann, A. I. (1997). Control of ammonia toxicity to *Hyalella azteca* by sodium, potassium and pH. *Environ. Poll.* **95**, 325-331.
- Bowmer, C. T. (1993). *Paris Commission Sediment Reworker Ring-Test*. IMW-R 93/317.
- Brehm, J. von, and Meijering, M. P. D. (1982). On the sensitivity to low pH of some selected crustaceans (*Daphnia* and *Gammarus*, Crustacea). *Arch. Hydrobiol.* **95**, 17-27.
- Bringmann, G., and Kühn, R. (1977). Befunde der Schädwirkung Wasser-gefährdender Stoffe gegen *Daphnia magna*. *Zeitschrift Wasser Abwasser Forsch.* **10**, 161-166.
- Bulich, A. A., Greene, M. W., and Isenberg, D. L. (1981). Reliability of the bacterial luminescence assay for determination of the toxicity of pure compounds and complex effluents. In *Aquatic Toxicology and Hazard Assessment: Fourth Conference* (J. G. Pearson, R. B. Foster, and W. E. Bishop, Eds.), pp. 338-347. American Society for Testing and Materials, ASTM, STP 766.