Environment Canada Environnement Canada

Revised Procedures for Adjusting Salinity of Effluent Samples for Marine Sublethal Toxicity Testing Conducted under Environmental Effects Monitoring (EEM) Programs

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REVISED PROCEDURES FOR ADJUSTING SALINITY OF EFFLUENT SAMPLES FOR MARINE SUBLETHAL TOXICITY TESTING CONDUCTED UNDER ENVIRONMENTAL EFFECTS MONITORING (EEM) PROGRAMS

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1.0 *Application*

The following procedures for adjusting the salinity of samples of effluent are now recommended by Environment Canada when performing toxicity tests consistent with EEM effluent monitoring requirements for facilities discharging to the marine environment. They are intended for use with effluent samples evaluated for sublethal toxicity using:

(a) the 7-day larval survival-and-growth test with the inland silverside fish *Menidia beryllina*, performed according to Section 13 of USEPA (1994);

(b) the 7-day larval survival-and-growth test with the topsmelt fish, *Atherinops affinis*, performed according to Section 11 of USEPA (1995);

(c) the fertilization assay using echinoids (sea urchins or sand dollars), performed according to the amended (November 1997) version of EC (1992); and

(d) the sexual reproduction test using the red macroalga *Champia parvula*, performed according to Section 16 of USEPA (1994).

These procedures supersede and replace the earlier procedure recommended by Environment Canada (EC, 1997) for salinity adjustment of effluent samples intended for marine sublethal toxicity tests under the EEM program.

2.0 *Rationale*

A recently-published study (Jonczyk *et al.*, 2001) has shown that, using Environment Canada's echinoid fertilization assay (EC, 1992), the sublethal toxicity of a number of samples of industrial effluent did not differ when their salinity was adjusted by either the direct addition of dry salt or hypersaline brine (HSB) to the samples. This study also showed that sample toxicity was similar when either artificial seawater or natural seawater was used as the control/dilution water in the test. These findings, together with a desire to enable testing of the sublethal toxicity of effluent samples at concentrations up to and including 100% (i.e., undiluted effluent), has resulted in a revisiting of Environment Canada's existing guidance for adjusting salinity for marine sublethal toxicity tests performed under Environmental Effects Monitoring (EEM) programs (EC, 1997).

Following are revised procedures for salinity adjustment, now recommended by Environment Canada for this purpose. These revised procedures offer additional options for adjusting the salinity of effluent samples as well as the associated control/dilution water used for the sublethal toxicity test. Depending on the options chosen, sample toxicity may now be determined at concentrations up to and including 100%.[1] Also, these revised procedures now offer the option of using artificial seawater (prepared using artificial HSB or by the addition of dry salt directly to deionized or other water) as the control/dilution water.[2]

3.0 *Test Salinity*

The purpose of this guidance is to provide direction on the adjustment of salinity in effluent to be assessed for potential sublethal effects on marine organisms under an EEM program. For any or all of the above biological test methods identified for effluent testing and referenced herein (see Sections 1.0 and 7.0 of this guidance), a test salinity of 30 ± 2‰ is to be used. Therefore, all test solutions must be within the range of 28 to 32‰ throughout the test.[3]

4.0 *Adjusting Salinity of Effluent Samples*

The salinity of each effluent sample used in any of the toxicity tests identified herein in Section 1.0 must be adjusted to 30 ± 2‰ (i.e., the test salinity; see Section 3.0) before test concentrations are prepared. Two approaches are available for this purpose: (1) sample salinity may be adjusted by the direct addition of dry salt to the effluent; or (2) sample salinity may be adjusted by the addition of hypersaline brine (HSB). In each instance, sample salinity should be adjusted as soon as possible

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[1] Using the procedure for adjusting sample salinity recommended in EC (1997), i.e., the addition of a quantity of 90 ± 1‰ HSB to the effluent sufficient to raise sample salinity to 30 ± 2‰, the highest effluent concentration that could be included in a multi-concentration toxicity test would be ~70% if the salinity of the sample were 0‰.

[2] The earlier guidance (EC, 1997) recommended that HSB should be prepared using a source of uncontaminated natural seawater, and that this (natural) HSB should be used both as the control/dilution water and for adjusting sample salinity.

[3] Two of the four marine toxicity tests under consideration here (i.e., the fertilization assay with echinoids according to EC 1992; and the *Champia* sexual reproduction test according to Section 16 of USEPA 1994) specify that the test salinity is to be 30 ± 2‰. The other two biological test methods (i.e., the two larval survival-and-growth tests using inland silverside fish or topsmelt fish) indicate that test salinity must be within the range of 5 to 32‰ (see Section 13 in USEPA, 1994) or 5 to 34‰ (see Section 11 in USEPA, 1995), with all replicate solutions within ± 2‰ of the selected test salinity. For purposes of the EEM program, and in order to establish a standard approach for adjusting the salinity of effluent samples that could be applied to each of the four marine toxicity tests, we have chosen a test salinity of 30 ± 2‰ for each of the four toxicity tests applied as part of this program.

following the receipt of the effluent in the laboratory.[4] The sample must not be warmed to the test temperature before this salinity adjustment. Rather, the temperature during salinity adjustment should approximate either that of the sample when received, or, in instances where the effluent is stored overnight (in which case it must be held in a refrigerator at 4 ± 2°C), that of the effluent upon its removal from the refrigerator.

If the first approach is chosen, either a mixture of commercially-available dry ocean salts[5] or reagent-grade salts[6] may be added to the undiluted effluent sample, in a quantity sufficient to raise sample salinity to 30 ± 2‰ (i.e., the acceptable range of salinity for each test; see Section 3.0). Any sample (or subsample) to which dry salts are added directly must be aged for a period of 16 to 24 hours before use (or, in the case of a static-renewal test, first use[4]) in a toxicity test.[7] To age the sample, the required quantity of salt must be added while stirring the effluent; thereafter, the salinity-adjusted (30 ± 2‰) sample must be held for 16 to 24 h at 4 ± 2°C in the dark and within a sealed container with minimal air space (and without any aeration) (Jonczyk *et al.*, 2001). Sample

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[4] Toxicity tests performed using larval inland silverside fish according to Section 13 in USEPA (1994), or larval topsmelt fish according to Section 11 in USEPA (1995), are static-renewal tests that require the daily renewal of each test solution throughout the 7-day test period. If either of these toxicity tests is conducted using a single sample of effluent that is held (as subsamples) at the testing laboratory in three separate containers (i.e., subsample 1 used for Days 0, 1, and 2; subsample 2 used for Days 3 and 4, and subsample 3 used for Days 5, 6, and 7), the salinity of each subsample must be adjusted just before that aliquot is readied for its first use in the test. If effluent salinity is to be adjusted by the addition of dry salts, the salts must be added just before the aging period of 16 to 24 h preceding the first use of that subsample in the toxicity test. If effluent salinity is to be adjusted by the addition of natural or artificial HSB, the brine must be added just before the first use of that subsample in the toxicity test.

[5] Commercially-available mixtures of dry sea salts include products marketed in aquarium supply stores under trade names such as Instant Ocean™, Forty Fathoms™, and HW Marinemix™ (EC, 1992; Neiheisel and Young, 1992; USEPA, 1994, 1995; Jonczyk *et al.*, 2001). Both Neiheisel and Young (1992) and Jonczyk *et al.* (2001) reported favourable results in echinoid (sea urchin) fertilization assays performed using Instant Ocean™ for adjusting sample salinity and for preparing the control/dilution water. Testing laboratories should obtain the "best quality" of commercial sea salts (e.g., Forty Fathoms™ Toxicity Test Grade) available from the supplier, and should evaluate its ability to meet the test-validity requirements in preliminary toxicity tests using such new products or batches for preparing *salt controls* (see Section 5.0).

[6] A formula for preparing artificial HSB (or artificial control/dilution water; see Section 5.0) using a mixture of reagent-grade salts dissolved in deionized water, which is referred to as "modified GP2", has been published by Spotte *et al.* (1984) and Bidwell and Spotte (1985). This formula is also available in USEPA (1994; see Table 2 in Sections 13 and 16) and USEPA (1995; see Table 2 in Section 7).

[7] Aging samples (or subsamples; see footnote 4) of salinity-adjusted effluent for a standard period of 24 hours is recommended, in instances where this duration of aging is manageable without requiring evening work. However, the aging period may be reduced to as little as 16 hours when the sample is already 2 days old upon receipt at the laboratory, and the sample arrives in the late afternoon. In such an instance, and to enable the maximum acceptable interval of 3 days between time of sampling and time of test initiation to be met (EC, 1992; USEPA, 1994, 1995), the aging period (to enable chemical equilibration of the salinity-adjusted effluent) may be limited to as little as 16 hours. In any event, the sublethal toxicity test must be started within 3 days of sample collection.

pH should be measured and recorded before salt addition and after salt addition but before aging.[8] Following this aging period, the effluent sample should be stirred, warmed to the test temperature (see EC 1992; USEPA 1994, and USEPA 1995), its pH checked and recorded[8], test concentrations prepared (see Section 5.0), and the toxicity test started.

If the second approach is chosen, sample salinity must be adjusted to the test salinity (30 ± 2‰) by the addition of the required amount of hypersaline brine (and, as necessary, deionized water). Hypersaline brine (HSB) adjusted to a final salinity of 90 ± 1‰ must be used for this purpose.[9] This can be made by concentrating an uncontaminated supply of natural seawater by freezing or evaporation; or by the addition of commercial sea salt (e.g., Instant Ocean™)[5] or an appropriate mixture of reagent-grade salts (e.g., "modified GP2"; see Spotte *et al.*, 1984; Bidwell and Spotte 1985; Table 2 in Sections 13 and 16 of USEPA 1994; or Table 2 in Section 7 of USEPA 1995), in a quantity sufficient to attain a salinity of 90 ± 1‰. Guidance for the preparation of natural HSB by freezing or evaporation is provided in Environment Canada (1992) and USEPA (1994, 1995) and has been summarized below.

To prepare natural hypersaline brine, high quality (and preferably high salinity) natural seawater should be filtered to at least 10 μm before placing it into the freezer or the evaporation chamber. If HSB is prepared by freezing, freeze at -10 to -20°C for ≥6 h, and collect the HSB under the ice when it reaches a salinity of 90 ± 1‰ (EC, 1992; USEPA, 1995). If HSB is prepared by evaporation, heat the natural seawater in a non-corrosive, nontoxic container at ≤40°C while aerating it, until the desired salinity (i.e., 90 ± 1‰) is achieved. Regardless of which technique is used (i.e., freezing or evaporation), the salinity of the brine should be monitored during its

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[8] The intent of these measurements is to determine and record the extent to which the salt addition changes sample pH. When considered alongside the pH of the effluent at the time that test solutions are prepared (see below), an understanding is gained of the effects of the salt addition and the subsequent aging on sample pH. This information is useful in determining the effects of these manipulations on sample integrity, and when interpreting the results of the toxicity test. It is well known that changes in sample pH can have a marked influence on sample toxicity. Conceivably, certain salt formulations used to adjust sample pH could alter its pH from that which would result if natural HSB were used to adjust sample salinity or upon the mixing of the effluent in natural seawater. A comparison of pH values measured upon the addition of salt to the effluent and following the subsequent period of 16-24 h for aging also provides insight into the changes in sample chemistry during this period.

[9] Although USEPA (1994, 1995) specify that solutions of hypersaline brine can be prepared at a range of salinities up to and including 100‰, Environment Canada has chosen a final salinity of 90 ± 1‰ for HSB (natural or artificial) to avoid overshoots which could result in problems due to salt precipitation. During its preparation, the salinity of HSB may increase to as high as 100‰, although exceeding this limit must be avoided and the final salinity must be adjusted to within the range of 90 ± 1‰ by the addition of the appropriate volumes of deionized water, natural seawater, or HSB with a lower salinity. For a test salinity of 30‰, the use of HSB with a final salinity of 90 ± 1‰ effectively reduces the highest test concentration from 70% effluent (if a 100‰ solution of hypersaline brine were used) to 67% (using a 90‰ solution of hypersaline brine) (EC, 1992; USEPA, 1994).

preparation, and must not exceed100‰ while being prepared.[9] After the required salinity for HSB (i.e., 90 ± 1‰) is achieved, the natural HSB should be passed through a filter with pore size ≤1 μm (USEPA, 1994, 1995) and poured into containers (e.g., 20-L collapsible polyethylene or polycarbonate water jugs).

Each container should be filled, capped, and labelled with the salinity of the HSB and the date the brine was generated. Freshly-prepared natural HSB may be used to adjust sample salinity (or as the control/dilution water; see Section 5.0) as soon as it has been prepared, or it may be stored in the dark at 4°C until required. Experience has shown that storage of up to several months under these conditions is acceptable (USEPA, 1994, 1995).

Any artificial hypersaline brine prepared using commercial sea salt (e.g., Instant Ocean™) or reagent-grade salts (e.g., "modified GP2") must be filtered (≤1 μm), and then aerated vigorously for a minimum of 24 h before use. Thereafter, unused portions should be capped and stored in the dark at 4 ± 2°C until used. Hypersaline brine is usually of acceptable quality even after several months in storage (USEPA, 1995).

If dry salt is added directly to the effluent sample according to the first approach described herein, the sublethal toxicity test can and should include a test concentration of 100% effluent. Using the second approach described herein, i.e., the addition of hypersaline brine (natural or artificial) with a salinity of 90 ± 1‰ to the undiluted sample, the maximum concentration of effluent that can be prepared and tested at a salinity of 30‰ is 67% v/v if the effluent has an initial salinity of 0‰ (EC, 1992). Accordingly, it is recommended that any sublethal toxicity test performed with effluent adjusted to a salinity of 30 ± 2‰ using HSB (natural or artificial) should include a test concentration of 67% effluent (i.e., the highest test concentration achievable using HSB) as well as other (lower) concentrations.

5.0 *Control/Dilution Water*

Control/dilution water (i.e., seawater used for the set of *dilution-water control* solutions included in the toxicity test and as the dilution water used to prepare each effluent concentration included in the test) must be adjusted to the test salinity (i.e., 30 ± 2‰) before its use. One of any of the following approaches may be used to prepare the control/dilution water: (1) artificial seawater is made up to the test salinity by adding the appropriate amount of commercially-available dry ocean salts (e.g., Instant Ocean™) or reagent-grade salts (e.g., modified GP2) to deionized water; (2) appropriate quantities of natural or artificial hypersaline brine and deionized water are mixed together; (3) a supply of uncontaminated natural seawater with a salinity of 30 ± 2‰ is used; or (4) a supply of uncontaminated natural seawater with a lower (i.e., <28‰) or higher (i.e., >32‰) salinity is mixed with the appropriate amount of dry ocean salts, reagent-grade salts, natural HSB, artificial HSB, or

deionized water sufficient to adjust its salinity to within the test range. A given batch of dilution water (natural or artificial) should not be used for more than 14 days following preparation, during which time its container should be kept covered and the contents protected from light (USEPA, 1994, 1995). During prolonged (>1 day) storage, natural or artificial seawater prepared for use as dilution water should be refrigerated (4 ± 2°C) to minimize microbial growth.

Any control/dilution water prepared by the addition of commercially-available dry ocean salts (e.g., Instant Ocean™) or reagent-grade salts (e.g., modified GP2) to deionized or other water (e.g., natural seawater with a salinity <28‰) must be mixed thoroughly during the salt addition. Thereafter, the mixture (with a salinity of 30 ± 2‰) must be aerated vigorously for a minimum of 24 h (USEPA, 1994, 1995) before its use.

Any control/dilution water prepared by the addition of natural HSB to deionized or other water (e.g., natural seawater with a salinity <28‰) must be made up following the directions in Section 4.0 for the preparation and storage of natural HSB. The mixture (with a salinity of 30 ± 2‰) may be used to prepare the controls and the test concentrations once the required test salinity and test temperature (see EC, 1992; USEPA, 1994; and USEPA, 1995) has been achieved; and after aerating the test water to achieve a dissolved oxygen concentration within the range of 90 to 100% saturation.

Any control/dilution water prepared by the addition of artificial HSB to deionized or other water (e.g., natural seawater with a salinity <28‰) must be made up following the directions in Section 4.0 for the preparation and storage of artificial HSB. Any artificial HSB used for this purpose must be filtered (≤1 μm), and then aerated vigorously for a minimum of 24 h before use.

Any control/dilution water prepared using natural seawater (salinity 30 ± 2‰) alone should be filtered, aerated, and stored as necessary, using the guidance provided in the respective test method document (EC, 1992; USEPA, 1994, 1995). Before use, the quantity (including a surplus) of natural seawater to be used as control/dilution water in the sublethal toxicity test must be adjusted to the test temperature, and aerated to within 90 to 100% of the saturation value for dissolved oxygen at that test temperature and salinity.[10]

Each toxicity test identified in Section 1.0 must include one or more complete sets of replicate control solutions. For any test whereby commercially-available dry ocean salts (e.g., Instant Ocean™) or reagent-grade salts (e.g., "modified GP2") is added directly to the effluent sample to adjust its salinity to 30 ± 2‰ (see Section 4.0), a set of replicate control solutions (i.e., "*salt controls*") must be included in the test which is prepared using the same source, batch, and

[10] Saturation values for dissolved oxygen (DO) are dependent on both the temperature and the salinity of the water (or effluent). A standard table of DO values versus temperature and salinity should be consulted.

concentration of dry salts as that added to the effluent sample. Similarly, for any test whereby natural or artificial hypersaline brine is added to the effluent sample to adjust its salinity (see Section 4.0), a set of replicate control solutions (i.e., "*HSB controls*") must be included in the test which is prepared using the same source, batch, and concentration of HSB as that added to the effluent sample. The salinity of these replicate control solutions must be 30 ± 2‰ when they are prepared.

For any test whereby the dilution water differs from the control water described in the preceding paragraph[11], a second set of control solutions (i.e., "*dilution-water controls*") must be prepared using a portion of the same batch of dilution water as that used to prepare the test concentrations of the effluent sample. The salinity of this second set of replicate control solutions must be 30 ± 2‰ when they are prepared.

The results for each set of *salt controls*, *HSB controls*, or *dilution-water controls* used in a toxicity test must be examined to determine if they independently meet the test-specific criterion or criteria for test validity (see EC 1992, Section 4.5.1; USEPA 1994, Sections 13.12 and 16.11; and USEPA 1995, Section 11.12). In instances where two sets of control solutions are used (i.e., *salt controls* or HSB *controls* as well as *dilution-water controls*), the results for the toxicity test are considered to be valid and acceptable only if each set of control solutions independently met the respective validity requirement(s).[12]

For any toxicity test which includes a set of *salt controls* or a set of *HSB controls*, as well as a set of *dilution-water controls*, and where responses in both controls have met the test-specific validity criterion or criteria of the test, the following requirement applies. The results for the two sets of control solutions must be pooled before calculating any statistical endpoints involving comparisons

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[11] Examples of situations where the dilution water differs from the control water described in the preceding paragraph include the following situations: (a) dry salts are used to adjust the salinity of the effluent sample, and a supply of uncontaminated natural seawater with a salinity of 30 ± 2‰ is used as the dilution water; (b) dry salts are used to adjust the salinity of the effluent sample, and a mixture of natural seawater and HSB (artificial or natural) with a final salinity of 30 ± 2‰ is used as the dilution water; (c) dry salts are used to adjust the salinity of the effluent sample, and a mixture of deionized water and HSB (artificial or natural) with a final salinity of 30 ± 2‰ is used as the dilution water; (d) HSB (artificial or natural) is used to adjust the salinity of the effluent sample, and a supply of uncontaminated natural seawater with a salinity of 30 ± 2‰ is used as the dilution water; and (e) HSB (natural or artificial) is used to adjust the salinity of the effluent sample, and a mixture of natural seawater and HSB (artificial or natural) with a final salinity of 30 ± 2‰ is used as the dilution water. In each of these (or any other) instances where the dilution water differs from the control water described in the preceding paragraph, a second set of control solutions must be prepared using 100% dilution water (salinity, 30 ± 2‰) only.

[12] For the results of any toxicity test which includes two sets of controls (i.e., a *salt control* and a *dilution-water control*, or an *HSB* control and a *dilution-water control*) to be considered as valid and acceptable, both controls must independently meet the criterion or criteria for test validity. Pooling control data for these two sets of controls before judging if the test results are valid or not is an unacceptable approach, since this pooling could in some instances mask an unacceptable set of control results.

of the findings for each set of test concentrations versus those for control solutions.[13]

6.0 *Expressing, Applying, and Reporting Test Concentrations*

Expressing, applying, and reporting test concentrations (% v/v) used in a sublethal toxicity test whereby effluent salinity is adjusted by the addition of dry salts (Section 4.0) is straight forward and routine, in that the 100% and lower effluent concentrations used in the toxicity test are not materially altered by the addition of the dry salts. For a test whereby effluent salinity is adjusted by the addition of an aqueous solution of natural or artificial hypersaline brine, this is less straightforward since the addition of the brine to the effluent dilutes the sample (and its test concentration) in direct proportion to the volume of brine added.[14]

In any instance where effluent salinity is adjusted by the addition of artificial or natural HSB (see Section 4.0), laboratory personnel must keep in mind that the concentration of the effluent sample has been reduced from full strength by the addition of hypersaline brine for salinity adjustment. Accordingly, any subsequent dilutions of the effluent sample using dilution water must be multiplied by this required dilution with HSB, before expressing, applying, and reporting the test concentrations.[14] Actual test concentrations (based on these calculations) should also be included when (or if) test concentrations are identified on test chambers, and when entering observations and results for each test concentration on bench sheets and estimating endpoint results (e.g., LC50 and/or ICp).

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[13] For a test which involves both a *salt control* or *HSB control* and a *dilution-water control,* pooling the data for the two sets of control solutions makes sense from a biological standpoint, since the test organisms held in a range of concentrations of the effluent sample would be exposed to each of these test solutions. The *salt control* or *HSB control* best represents the 100% concentration as well as other concentrations >50% effluent. The *dilution-water control* best represents test concentrations <50% (although it is represented by any concentration involving dilution of the effluent). Simple pooling of the data for the two sets of control solutions integrates effectively the combined influence of the salt or HSB added to the effluent sample to adjust salinity, as well as the influence of the (differing) dilution water used in the test.

[14] Following the adjustment of the salinity of a (freshwater) sample of effluent to 30‰ using 90‰ HSB, the concentration of effluent in this sample will be 67% (see Section 4, including footnote 9). Accordingly, each subsequent dilution of this salinity-adjusted sample must be multiplied by the sample's salinity-adjusted concentration to derive the test concentrations. For example, test concentrations for a dilution series involving 100%, 50%, 25%, 12.5%, 6.25%, and 3.1% of the salinity-adjusted sample of effluent would be 67%, 33.5%, 16.8%, 8.4%, 4.2%, and 2.1%, respectively, of the original effluent sample. These latter concentrations represent the ones to be reported.

7.0 *Acknowledgements*

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