| Tropical Urchin Fertilization Toxicity Test Method Review Comments from Formal Reviewers | | | | | |
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| **Location** | **Reviewer** | **Comment** | **Suggested changes made?** | **Response** | **Comments** |
| 18.01.01 | SB | First sentence is awkward. Recommend breaking into two. | Y | EC | |
| 18.05.21 | BP | Are cubitainers still widely used? In the world of ambient toxicity testing we have tried to move away from plastic containers for samples because of issues of sorption. | Y | EC | Qualified container use with test acceptability. |
| 18.05.21 | SB | Is this indicating that if samples are placed in cubitainers previously used for NaOH or glutaraldehyde it causes these problems? The over explanation makes this a little confusing. | Y | EC | |
| 18.05.24 | BP | Are there any problems with residual de-ionized water in the test containers? Perhaps final rinses should be with seawater. We rinse our containers with filtered seawater and them a good shake to remove residual seawater. The shaking is accomplished by placing an "egg crate" screen over the rack of vials and inverting them. | Y | EC | |
| 18.05.24 | SB | Alternative test chambers should be allowed as long as they meet test design and control performance criteria. | Y | EC | |
| 18.05.28 | AR | p.5: 18.5.28 as well as other locations in the manuscript list Sedgewick Rafter chambers for all egg counts. Regular microscope slides work just as well, are cheaper, easier to clean, etc. Modify to include microscope slides or SR Chamber. | N | CFD = VAR | |
| 18.06.09 | AR | p. 6. Cutting off pipette tips can lead to significant errors. This recommendation seems very problematic. Are there tips that are made that are approximately 1 mm in diameter? Personally, I have not had any trouble with typical pipette tips, as along as I avoid those that are specifically narrow at the tip. | N | CFD = VAR | |
| 18.06.13 | BP | Please add "as per instruction of instrument manufacturer" so technicians don't think they need a U.S. EPA-approved dissolved oxygen electrode. | N | CFD = CON | |
| 18.06.15 | SB | Mention use of formalin as a substitute for glutaraldehyde. | N | CFD = VAR | Phrase to explain why formaldehyde is not acceptable included in 18.10.11.2.2 |
| 18.06.27 | SB | Need line space after for consistency. | N | Sentence is referring to Section 18.6.28 is part of the text, not the next section | |
| 18.06.28.02 | BP | Is heat preparation of brine still widely used? We always had problems with it. It seems so much more equipment-intensive than freezing, and freezing is so much more elegant. Have there been any problems with heat preparation, and, if so, should the procedure be deleted? | N | CFD = CON | |
| 18.06.28.02 | AR | p. 15. What is GP2? | N | GP2 medium is an artificial seawater for culture or maintenance of marine organisms. Spotte et al. (1984) defines it in greater detail. | |
| 18.06.28.07.01 | BP | Are there instances when only the highest concentrations of effluent would be adjusted with brine if the lowest concentrations were within the salinity range of the urchin? Or is it required to have some brine in every concentration? | Y | EC | |
| 18.06.28.07.01 | SB | This reads as if a brine control is merely "recommended" when samples have been salinity adjusted. This should be a requirement, not a recommendation. Is more strongly suggested in 18.6.28.10. Finally listed as a requirement in 18.10.1.1. More consistent wording throughout these sections. | Y | EC | |
| 18.06.28.08.02 | BP | I recommend adjusting the pH with very high quality acid and base (certified, optima, etc.). We usually use 1N strength in our TIE adjustments. | Y | EC | |
| 18.06.28.09.01 | BP | Replace "shake" with "invert several times". | Y | EC | |
| 18.06.28.09.02 | BP | Replace "shake" with "invert several times". | Y | EC | |
| 18.06.29.02 | AR | p. 19. You might recommend PBRC as a primary contact – in the future they might only refer to another group, so this helps to ensure the longevity of the document. | Y | EC | |
| 18.06.29.02 | AR | p. 19. Also, should it be recommended that testing soon after collection is recommended as gamete viability could decrease with time?? | N | See comments 18.10.03.05.01 | |

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| 18.06.29.02 | BP | I thought scientists used the metric system. | Y | EC | |
| 18.06.29.02 | SB | Don't understand the statement about collecting animals from different areas for each round of tests due to gamete variability. Is returning animals to the wild really recommended? No concern about laboratory borne disease? | Y | EC | Wording changed to availability. Since animals are not held in seawater systems at laboratories, there is minimal likelihood of disease. However, recommendation to return organisms to the wild was deleted to be prudent & added language about spawning onsite & returning organisms. |
| 18.10.02.02.01 | AR | p. 20. I recognize that using a 0.5 factor makes the serial dilutions easier, but I question how environmentally realistic that is. In reality the difference between 1 and 5% or even 5-10% in terms of natural input dilutions may be difficult to appreciate biologically. More importantly, even a 1% dilution probably represents a very limited portion of the ecosystem area. | N | CFD = CON | |
| 18.10.02.02.02 | AR | Therefore, I recommend the following changes to 18.10.2.2.2. "If the protocols under 18.10.2.2.1 show significant toxicity at all water sample dilutions, then the assay should be repeated using a range of lower concentrations, e.g. such as 10%, 1%, 0.1%, 0.01%, 0.001%." These dilutions are likely to reflect the kinds of diluted concentrations encountered over broader areas of marine habitats, and provides a sensitive test of potential toxicity. | N | CFD = CON | |
| 18.10.02.04.03 | SB | Yeah!; a reference toxicant dilution series that makes sense and uses volumes of stock that have a logical connection to the final concentrations. A similar change should be made for the other methods in the West Coast Manual. | N | OK-NO ACTION | |
| 18.10.02.04.04 | SB | Then right back to the old system. Is it possible to make the SDS stock 100 mg/L (I don't know the solubility), then use 0.38, 0.75, 1.5, 3.1 an 6.3 ml of stock? At least as shown the series is based on 50% dilutions and the stock volume is twice the concentration value; better than the previous urchin methods. But why not use a similar format as described for copper? | Y | EC | |
| 18.10.02.04.05 | BP | The reference toxicant tests should be run concurrently with the effluent, and with the same batch of gametes. I believe this is required of other protocols using wild-caught organisms. | Y | EC | See Section 4.7 of West Coast manual (USEPA, 1995). |
| 18.10.03.01.01 | BP | Change "Allow to come to temperature" to "Allow for temperature equilibration". | Y | EC | |
| 18.10.03.01.06 | AR | p. 22. It might be useful to provide a statement about how long after injection that spawning tends to be initiated. | N | Spawning initiation variable, but Section 18.10.3.1.9 states that 30 minutes is the maximum | |
| 18.10.03.01.07 | BP | If the males are going to have their sperm collected with a pipette, are they still placed on beakers of water? Are the males allowed to spawn for a while in the water before they have their sperm collected? | Y | EC | |
| 18.10.03.01.08 | AR | Move the statement about the color of the gametes to the first sentence. | Y | EC | |
| 18.10.03.02.01 | AR | I was also a little confused here about why you would turn the males upside down rather than letting the "dry sperm" collect in a dish. By pipetting from the surface of the urchin, you run the risk of also collecting surface junk, bacteria, etc.?? | N | CFD = CON | |
| 18.10.03.03.01 | AR | p. 24. Recommend the following modification. "….. on a microscope slide; let sit for few minutes to allow sperm capacitation. Then examine the sperm for motility…." I am not sure why a well slide is recommended here, and can actually impede good focus at a higher microscope power. | N | CFD=CON, BUT ADDED "E.G." | |
| 18.10.03.04.02 | SB | The minimum volume of semen stated is not sufficient to conduct the test procedure. Carefully review the trial and test methods and make adjustments so that the minimum amount of sperm needed is feasible and sufficient to conduct the test with a reasonable margin of error. | Y | EC | |
| 18.10.03.05.01 | BP | The three hour time limit seems reasonable for a technician who has practiced the method. | Y | EC | Changed to 4 hours to allow time for trials & to be consistent with the purple sea urchin fertilization method (USEPA, 1995). |

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| 18.10.04.01.01 | AR | p. 25. Recommend the following modification: "Discard any egg samples that have a … ……..hermaphroditism, and also any with protozoa. | Y | EC | |
| 18.10.04.03.01 | AR | p. 25. Recommend that smaller volumes of egg suspensions be allowed, e.g. perhaps 0.25 or 0.5 ml volumes. Remember the Sedgewick Rafter chambers are not gridded, so counting approximately 200 eggs in a full 1 ml volume is more error prone than counting 100 or 50 eggs. | N | CFD = VAR | |
| 18.10.04.03.01 | BP | We have never used aeration to mix eggs because the eggs never get fully suspended. I recommend the use of the perforated plunger. Please add the word "perforated" before "plunger" to ensure other types of plungers are not used. | Y | EC | |
| 18.10.04.03.01 | SB | Remove the word "then" from first sentence. | Y | EC | |
| 18.10.04.03.03 | AR | p. 26. Especially important here is that counts of multiple subsamples should be averaged to generate the final estimate for egg density. | N | CFD = CON | |
| 18.10.05.01 | BP | Please add the following text: "a range-finder sperm density trial must be conducted with every test to ensure an optimum sperm control". | Y | EC | |
| 18.10.05.01 | SB | This section needs some writing attention; rewording and some missing words. Combine last sentence with next section. | Y | EC | |
| 18.10.05.02 | BP | Replace "is a cut off" with "threshold is". | Y | EC | |
| 18.10.05.05 | SB | I needed to read this section 3 times and break out my calculator before I understood the point of all of the manipulations and equations. I suggest that the first sub-section contain a brief description of the goal (making and measuring a 2000x dilution of sperm) and that the following procedure will get you there. | Y | EC | |
| 18.10.05.05.01 | BP | There should be more specific guidance for the collection of baseline data. Is it accurate to have a single regression line with $R^2$>0.95, or should data from several sperm batches be pooled? Should the lab produce a regression line from three batches? | Y | EC | |
| 18.10.05.07.02 | SB | Not having worked with these animals before, I don't know what volume of sperm they normally get, but the fixed volume method seems a little dangerous. In the example given (typical?), they wind up needing 1.25 ml of sperm. Seems like a lot and during collection instructions 0.5 ml was called out as a minimum. Using the fixed semen volume, at least you know the minimum you will need going in. | Y | EC | |
| 18.10.05.07.02.04 | AR | p. 30. Why is 18.10.5.7.2.4. separate from 18.10.5.7.2.3? This is an example of the description provided in 18.10.5.7.2.3. It would be more consistent with previous sections to combine these. | Y | EC | |
| 18.10.06 | BP | Overall Comment: I think it is very important to conduct the sperm density trial and wouldn't think of conducting a purple urchin fertilization test without first figuring out the optimal sperm to egg ratio. The instructions for performing this test are adequate. | N | OK-NO ACTION | |
| 18.10.06 | SB | The trial fertilization section is generally satisfactory, but there are some serious errors in Table 5 and inconsistencies regarding the need for replication that should be corrected. The heading for the last column of Table 5 is wrong, the data represent sperm counts, not concentrations. The volume units should be mentioned for Table 4. | Y | EC | |
| 18.10.06.04 | SB | 2 replicates should be tested for each sperm concentration. Maybe they don't each need to be counted, but a lot of time and effort is going into this trial and timewise, there is little room for error getting it completed within the 3 hour window. An extra replicate seems like a good insurance policy to guard against spills or oddball data. | Y | EC | |
| 18.10.06.06 | AR | p. 33 (and 39). Why glutaraldehyde instead of buffered formalin? It is certainly a lot more expensive. | N | ADDRESSED EARLIER; see explanation in 18.10.11.2.2 | |

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| 18.10.06.09 | SB | I hope these examples are not very real world. For this example, if one scaled up the volumes to get a reasonable sperm stock to work with say 40 ml, one would need 2.5 ml of sperm. | Y | EC | *T. gratilla* produces copious amounts of sperm compared to other urchin species, so volume of sperm is not usually limiting. However, since it is unlikely that the sperm concentrations will be in the range of $10^9$, the table was limited to sperm concentrations in the range of $10^{10}$ and the example was changed in 18.10.5.7.2.2 and 18.10.6.9. Loading 0.1 ml of sperm/test tube into 100 test tubes = only 10 mL of sperm stock. That would require less than 1 mL of concentrated sperm if volumes needed to be increased. |
| 18.10.07.02.01 | SB | Change "A new solution" to "A new dilution". I think there needs to be a little tightening of the terminology for the sperm throughout the protocol. Pooled sperm/semen, sperm stock and semen suspension are used interchangeably throughout, but one could interpret them as meaning different things. Sperm stock implies to me that it might be some mixture of sperm and water. Personally I would stick with pooled sperm or semen. At the bottom of this subsection it states that the test needs to start 90 minutes after initial sperm collection; everywhere else says 3 hrs. | Y | EC | Changes made throughout to consolidate terminology to pooled sperm, sperm stock, and sperm suspension. Sperm stock implies concentrated sperm, pooled sperm indicates that concentrated sperm from different individuals were combined, and sperm suspension indicates that sperm and seawater are mixed to create a sperm dilution. |
| 18.10.07.02.03 | BP | Isn't it required to have the containers randomized? If so, then it is unnecessary to try to inject sperm into the first replicate of each concentration. | Y | EC | |
| 18.10.07.03.02 | BP | Recommend using the perforated plunger. | Y | EC | Also removed reference to use of multiple pipettor. |
| 18.10.07.03.04 | SB | I would move this section up to where test set-up is discussed. Suggested revisions for clarity are noted on the document. | Y | EC | Revisions in wording made and moved to section 18.6.28.10.2 |
| 18.10.10.01.01 | AR | p. 39. 18.10.10.1.1 and 18.10.10.1.2 seem to conflict with 18.10.8.2??? | Y | EC; however, the last sentence in 18.10.10.1.2 was not removed to be consistent with other methods. A should statement does not imply that the lab must do this. | |
| 18.10.11.02.02 | AR | (and 39). Why glutaraldehyde instead of buffered formalin? It is certainly a lot more expensive. | N | ADDRESSED EARLIER; see explanation in 18.10.11.2.2 | |
| 18.10.11.02.03 | SB | Any reason not to offer formalin as an alternative? Its addition is consistent with the West Coast manual. | N | ADDRESSED EARLIER; see explanation in 18.10.11.2.2 | |
| 18.10.11.03.01 | BP | Capping should be required because of gluteraldehyde. "Otherwise covered" could be misinterpreted (Parafilm, plastic sheet, cardboard). | N | As is allowed provides flexibility | |

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| 18.10.11.03.01 | SB | Any data regarding the need for reading the test within 72 hrs and in one sitting? Seems rather onerous. The flexibility in counting due to preservation is one of the advantages of this type of test. | Y | EC | Lab experience shows that fertilization membranes can collapse with time; time changed to 48 hours to be consistent with the purple sea urchin fertilization method (USEPA, 1995) |
| 18.10.11.03.02 | AR | p. 40. This reads a little confusing. 100x could be misconstrued to be under oil. I assume you mean a 10X eyepiece times 10X objective. | N | CFD = CON | |
| 18.10.11.03.05 | AR | Here again, a Sedgewick Rafter is not needed; a regular microscope slide works just as well. Volume doesn't matter; the goal is to count 100 eggs from a well mixed preparation. | Y | EC | |
| 18.10.11.04.02 | AR | p. 42. Delete because this seems overly stringent. You have egg blanks to check for pre- fertilization issues. It is possible that some pollutant preparations may actually stimulate more rapid initiation of cleavage, but that still passes the fertilization test. Unless, your egg blanks fail, there is no real reason to use a few spurious cleaving eggs to negate the whole test. | Y | EC | Addition of sentence about cleavage in egg blanks in 18.6.28.11.1 and in 18.12.1. Effluent blanks (eggs only added to the highest concentration of effluent) will be the check for cleavage or fertilization without sperm additions. |
| 18.12.01 | BP | These acceptability criteria seem reasonable, particularly with the use of the ratio pre-test. | N | OK-NO ACTION | |
| 18.12.01 | AR | I assume that (1) has a typo and should read > (not <) 70%?? | Y | EC | |
| 18.13.02.07.11 | SB | Maybe I'm being particularly dense, but I don't follow this line of reasoning. Where does 4.12% come from? | N | CFD = CON | Transformed control mean (% difference from mean, MSDu) = 1.342 (4.12%) = 0.0553 |
| 18.14.01.02.01 | AR | p. 62. I agree with Gary that overall this was not a particularly stellar interlaboratory comparison, and may reflect various problems that are readily overcome with a little training and practice. Also, CuSO4 was used for these assays as the inorganic reference toxicant, and from my personal experience, it generally is more variable than CuCl2. Therefore I do think CuCl2 is a better reference toxicant. | N | OK--NO ACTION | |
| Appendix I: | SB | I like these short cookbook sections to summarize the method. This could be more useful with just a little more info. I'd like to see, sample volume, volume of sperm dilution added, volume of egg dilution added and volume of preservative. | Y | EC | |
| Appendix II | AR | p. 68. Why is a hematocrit tube recommended for loading the hemacytometer? The slower flow rates may be more problematic, and personally Pasteur pipettes or pipettors with tips work just as well (maybe better). Make the hematocrit tube more of an option. | Y | EC | |
| Figure 1 | AR | p. 7, Figure 1, the correct spelling is hermaphrodite (not hermaphradite). | Y | EC | |
| Figure 1 – 5 | AR | p. 7 – 11, Figures 1-5 seem out of sequence here. It is a much better transition from the material from page 6 to page 12, e.g from 18.6.22 to 18.6.23. The figures need to be moved to a later position in the chapter next to where they are referred to (e.g., the first reference to Figure 1 is on page 22). | N | CFD= CON. Figures 1-5 are referenced in 18.6.2. | |
| Figure 2 | AR | p. 8. I found the decanting instructions and the calculations of the % original volume here confusing; where is this used in the assay? Ultimately, the decanting is used to concentrate the eggs, but new counts will be required; do not rely on percentages to estimate egg concentrations. | N | CFD = CON. If eggs are too dilute, calculating the amount to decant the egg stock provides an efficient procedure to get an appropriate egg stock concentration. The decanting calculation does not replace the final egg count. | |
| Figure 6 | AR | p. 23, Figure 6. Delete the word "Showing" and rephrase the legend to read " The location and orientation…". Also label the aboral and oral surfaces on the diagram. | Y | EC | |

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| Figure 7 | AR | p. 27. The legend for Figure 7 reads "Sperm count and dilution steps". However the 2nd box says "choose sperm by looking at motility and fertilization of eggs". Modify to be consistent OR consider moving Box 2 to the top and treating that as a title or "Overall Aim: To select viable sperm by looking at motility and evaluate fertilization capacity of the eggs" (or something like that. Other errors noted in Figure 7. Box 3 – make the word "count" plural, e.g. counts; The sections referred to in Boxes 5, 6, 11 are incorrect. For Box 5, I think it should be: 18.10.5.5; and for Box 6, I think it should be 18.10.5.6. | Y | EC | |
| Figure 8 | AR | p. 41. Provide information on approximate diameter of the eggs and relative distance of hyaline space. | N | Information unknown | |
| Figure 9 | AR | p. 47. Are these the only tests allowed?? If so, seems too restrictive. For example, this manuscript uses Shapiro-Wilk's test for normality, but a program like Sigma Stat uses the Kolmogorov-Smirnov test, etc. Can these be presented more as examples of types of tests? Not sure why a more general test group like ANOVA is not simply listed rather than specific ANOVA-associated analyses. | N | CFD = CON | |
| Table 12 | SB | Formatting error for the 4th observation. | Y | EC | |
| Table 4 | AR | p. 32. This table is based on the maximal sperm:egg ratio recommended (e.g. 2500:1). I would recommend providing Excel spread sheet calculations for generating a table using different concentrations. | N | Instead, provided explanation for using table for other S:E ratios in Section 18.10.6.9. The formula used in generating numbers in Table 4 is shown in Section 18.10.5.7.3.2. | |
| Table 5 | AR | Also, Modify Table 5 legend to read "…..trial sperm:egg ratios when added to 10 ml." | Y | EC | |
| Table 7 | AR | p. 43 and 44. Item numbers 9 (and 16) seems problematic. Yes, 2000 eggs per chamber, but sperm concentrations will be variable?? | Y | EC | Wording now consistent with purple sea urchin fertilization method (USEPA, 1995) |
| Stats section | SB | Is there anyone in the western United States that does the stats by hand and would need this level of detail on how to do each one? The number of trees killed to show this for each toxicity test method in the manual is frightful. | N | CFD = CON | |
| General | AR | (1) I am not sure if you are familiar with a paper that I published in 1992 working with Hawaiian sea urchins, albeit a different species.<br>(Ringwood, A.H. 1992. Comparative sensitivity of gametes and early developmental stages of a sea urchin species (*Echinometra mathaei*) and a bivalve species (*Isognomon californicum*) during metal exposures. Arch. Environ. Contam. Toxicol. 22: 288-295.)<br>I also discussed there the importance of seasonal differences in optimal sperm:egg ratios, so I certainly support this the trial fertilization component of the manual. Has anyone done a concerted evaluation of seasonal differences in this species? | Y | OK - NO ACTION | *T. gratilla* was chosen because of the potential to spawn year-round unlike other Hawaiian urchin species. |

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| General | AR | (2) Given the need for the initial density determination component, the time frame of 3 hours after injection to completion of definitive assay seems to leave very little "wobble" room. The incubation time for the sperm density trial is 65 minutes, and the final assay incubation time is 80 minutes. Conducting sperm and egg counts in the preparation stages can sometimes take a bit longer than expected, etc. While speed is important, a 4 hour time frame might allow a little more carefulness sometimes that will ultimately improve the outcome of the tests. I also question the choice of the 3 hour period (is there a particular justification or data that suggest the need for this)?? From my experience with *Tripneustes,* the sperm remain viable for a very long time (at least 6-8 hours when kept cool), and the eggs are also pretty hardy. I really think 4 –6 hours would be fine. Therefore, you might state that the optimal time frame from start to finish is recommended as 3 hours, but longer time frames such as 4 – 6 hours may be allowed. The control fertilization criteria should then be used to eliminate problem assays. | Y | EC | Changed from 3 hrs to 4 hrs; consistent with the *S. purpuratus* time frame. |
| General | AR | (3) General comment. I did review the calculations for the assay and found them to all be correct. However I did not do a particularly detailed review of the statistical section – I will leave that to experts in that area. | N | OK--NO ACTION | |

KEY TO ACRONYMS

BP = Bryn Phillips

AR = Amy Ringwood

SB = Steve Bay

Y = Yes

N = No

CFD = CON Criterion for decision (CFD) = consistency with other test methods (CON)

EC = Editorial change was made

OK--NO ACTION = the comment is accepted; no action is needed

CFD = VAR The criterion for the decision (CFD) was that new methodologies suggested could add unknown variation (VAR)

REFERENCES

Ringwood, A.H. 1992. Comparative sensitivity of gametes and early developmental stages of a sea urchin species (Echinometra mathaei) and a bivalve species (Isognomon californicum) during metal exposures. Arch. Environ. Contam. Toxicol. 22: 288-295.

Spotte, S., G. Adams, and P.M. Bubucis. 1984. GP2 as an artificial seawater for culture or maintenance of marine organisms. Zool. Biol. 3:229-240.

USEPA. 1995. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to West Coast Marine and Estuarine Organisms. First Edition, EPA/600/R-95-136. August 1995.