Sinclair Inlet Toxicity Assessment :

Puget Sound Naval Shipyard & Intermediate Maintenance Facility Surface Water Copper Bioavailability and Toxicity Study

DRAFT REPORT
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For:
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INTRODUCTION

This document describes the results from a study designed to assess the bioavailability of copper in surface water from Sinclair Inlet, adjacent to the Puget Sound Naval Shipyard & Intermediate Maintenance Facility (PSNS & IMF). Physical and chemical characteristics of seawater influence the bioavailability and toxicity of metals to aquatic organisms (Stauber et al. 2000, Knezovich et al. 2001, Eriksen et al. 2001, Lorenzo et al. 2002; Rosen et al., 2004). Therefore, individual water bodies will differ in their potential to buffer against metal toxicity. One way of assessing relative bioavailability at a site is to spike site water samples with various concentrations of copper in the laboratory and compare resulting EC50 values in site water to those observed in concurrent exposures using laboratory water. This is the approach used in calculation of water effect ratios (WER), which are currently used for derivation of site-specific water quality criteria (WQC) for National Pollution Discharge Elimination System (NPDES) permits (USEPA 1994a).

Metal bioavailability can also be determined by measuring the complexation capacity of the water body. Copper complexation capacity (CuCC) is a chemical measurement determined with a copper ion selective electrode (CuISE), in response to systematic addition of copper in ambient water (Rivera-Duarte and Zirino, 2004). The response of the CuISE is indicative of the concentration of aqueous free copper ion ($Cu(II)_{aq}$), which has been shown to be a better predictor of toxicity than total or dissolved measurements (Sunda and Guillard, 1976; Sunda and Ferguson, 1983; Eriksen et al, 2001; Rivera-Duarte et al, 2004). Copper bioavailability in Sinclair Inlet was evaluated using both of the above-mentioned approaches. Toxicity tests employed the Mediterranean mussel (*Mytilus galloprovincialis*), which is the most sensitive test used for copper water quality criteria development in saltwater.

BACKGROUND

Sinclair and Dyes Inlets, Washington were listed on the 1998 303(d) list of impaired waters because of fecal coliform (FC) contamination in the marine waters and metals and organic contaminants in bottom sediments and fish tissues (WDOE 1998). The Puget Sound Naval Shipyard & Intermediate Maintenance Facility, Department of Ecology, U.S. Environmental Protection Agency (USEPA) and local stakeholders are working together on Project ENVVEST (an acronym for ENVironmental InVESTment) to address contamination issues and develop water cleanup plans for the watershed (Navy, EPA, and Ecology 2000, ENVVEST 2002). Significant progress has been made on the FC Total Maximum Daily Load (TMDL) study for Sinclair and Dyes Inlets (Ecology 2004; Figure 1), which has benefited from the collaboration and cooperation of many stakeholders within the watershed. Currently, the FC model verification sampling for the TMDL study is being planned (Johnston et al. 2004) and storm water flow monitoring is being initiated for representative storm water outfalls within the study area (TEC 2003a, b).

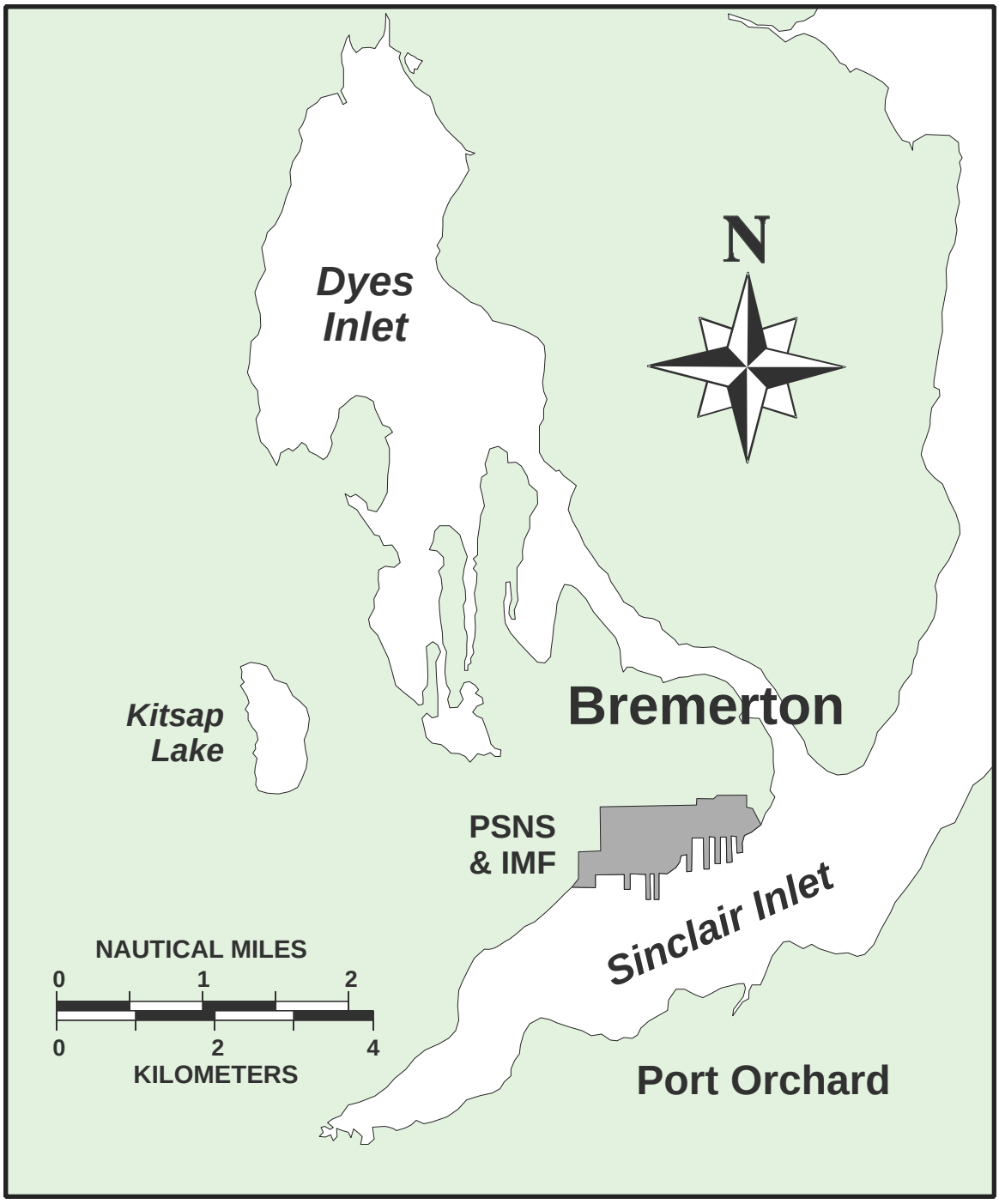


Figure 1. The location of PSNS & IMF in Sinclair and Dyes Inlets, WA.

Dry dock and storm water discharges from the Shipyard to Sinclair Inlet require NPDES permits issued by the USEPA. The current dry dock permit has average monthly and maximum daily total recoverable copper concentration limits. The average monthly concentration is 19 ppb and the maximum daily concentration is 33 ppb. The current NPDES permit contains the requirement to monitor storm water, but does not contain numerical limits (USEPA 1994b). In addition, the permit also contains loading limits for the dry dock discharges expressed in pounds per day. Although acute and chronic water quality criteria are not exceeded in Sinclair Inlet (Katz et al., 2004), the potential for adding to elevated copper levels in sediments adjacent to the Shipyard is a concern.

The objective of this study is to assess the assimilative capacity of surface water at sites adjacent to PSNS & IMF in Sinclair Inlet. In general, water bodies have a greater capacity to reduce a metal's bioavailability than the laboratory water used for derivation of national ambient water quality criteria (WQC), upon which discharge permits are generally based. This is because laboratory water is typically low in metal-binding particulate matter and dissolved organic matter compared to most ambient waters (USEPA 1994a, c). These differences in bioavailability are accounted for by USEPA's water effect ratio (WER) procedure in which metal-spiked site and laboratory waters are evaluated for toxicity in side-by-side exposures. The site water LC50/EC50 is then divided by the lab water LC50/EC50, resulting in a multiplier that can be used to adjust the national WQC. This work will follow the WER guidance to the extent that EC50 values for very sensitive marine invertebrates will be obtained in both site water and lab water to characterize the differences in bioavailability between the Sinclair Inlet sites and lab water, as well as among the selected Sinclair Inlet sites.

Copper complexation capacity (CuCC) is a chemical measurement that could be used as a surrogate for the types of toxicity tests described above. CuCC is defined as the capacity of ambient water to assimilate inputs of copper without associated adverse effects upon aquatic organisms. It is measured through a copper ion selective electrode (CuISE), in response to systematic addition of copper in ambient water. The response of the CuISE is indicative of the concentration of aqueous free copper ion ($Cu(II)_{aq}$) in solution, which according to the free-ion model (Buffle et al., 1990), and substantiated by experimental evidence (Sunda and Guillard, 1976; Sunda and Ferguson, 1983; Campbell, 1995, Ericksen et al, 2001; Rivera-Duarte et al., 2004), is the fraction of copper that is available to organisms, making it a better predictor of its potential toxicity than either the total or dissolved copper concentrations. Therefore, CuCC is a chemical measurement indicative of the toxicity of the water to organisms, and, as shown in Figure 2, is very much related to the results from toxicity tests (larval development EC50). As this figure indicates, larval development success of the Mediterranean mussel, *M. galloprovincialis,* decreases with the addition of copper. Similarly, the response of the CuISE indicates a change in slope that coincides with the EC50.

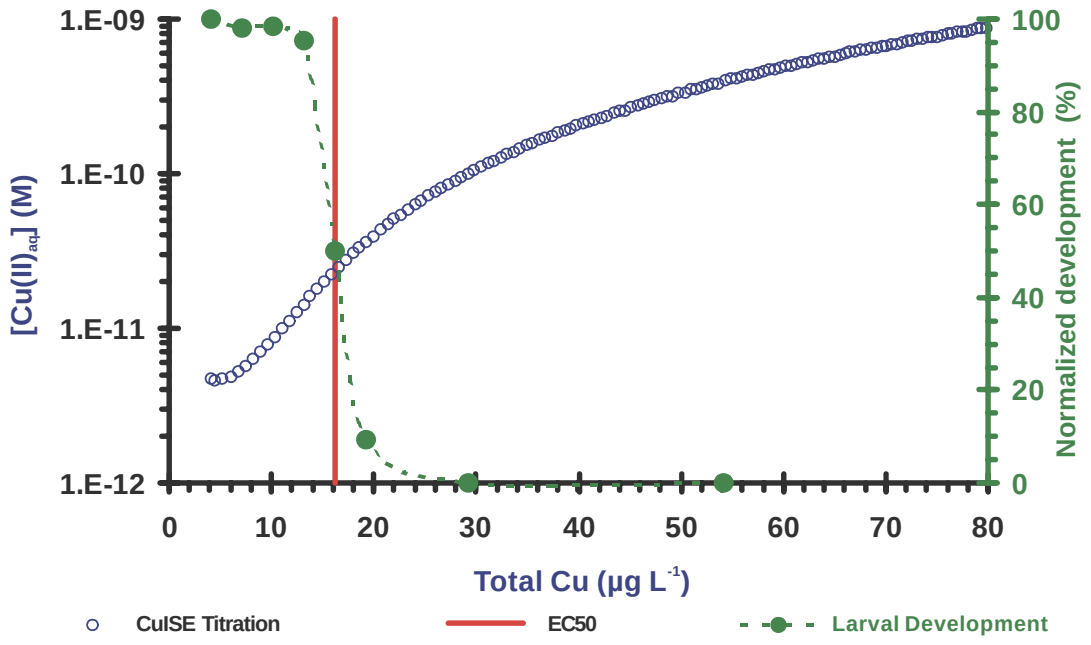


Figure 2. Relationship between toxicity and complexation capacity.

STUDY GOALS

The goals of this study were to measure the potential for surface water in Sinclair Inlet to buffer against copper associated toxicity. This was achieved through laboratory-based toxicity exposures following EPA's water effect ratio procedure for developing site-specific water quality criteria (USEPA 1994a), as well as via chemical measurements to determine the complexation capacity at the site.

STUDY SITE

Ambient Sampling Locations

Three ambient water samples from Sinclair Inlet were collected for the toxicity assessment (Figure 3). These stations were collocated with the sampling sites planned for the fecal coliform model verification study (Johnston et al. 2004). These stations were sampled for dissolved and particulate metals, total suspended solids, total organic carbon, dissolved organic carbon, salinity, alkalinity, and pH (Johnston et al. 2004).

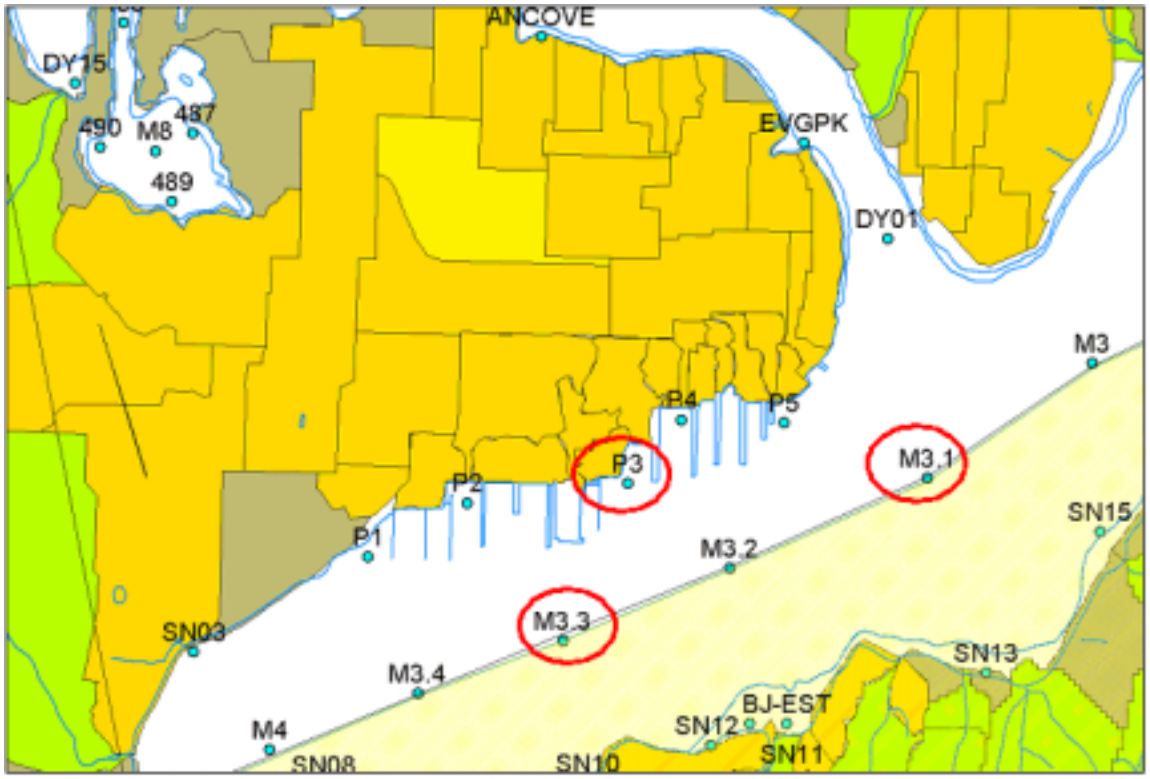


Figure 3. Marine stations near the Naval Station and Shipyard for the BREMERTON sampling event. The stations with red circles are the stations to be sampled to determine site-specific toxicity and assimilative capacity of copper.

METHODS

Toxicity Assessment

Sample Collection and Storage

Site water was collected from the water surface (depth of approximately 1 m) using clean techniques (US EPA 1995c) on March 31, 2004. Samples were stored in pre-cleaned 1-L HDPE containers, and shipped on ice overnight to SSC. Upon arrival, samples were 5 °C, and did not require additional storage, as testing was initiated immediately upon arrival. Testing began within approximately 24 h of receipt of samples, well within the 48 h period recommended (USEPA 1994a). Additional samples were collected for copper analysis (see Copper Measurements below), as well as total suspended solids (TSS) and dissolved organic carbon (DOC).

Site Water Preparation

Analysis of site water under the microscope indicated the presence of live zooplankton and phytoplankton, but predation on mussel embryos was not expected. Therefore, samples were tested without any treatment (e.g. coarse sieving) to remove ambient

organisms. Site water salinity was 29-30 ‰, within range of that tolerated by the test species. Therefore, no salinity adjustment was made to the samples. Laboratory water (filtered coastal seawater from Scripps research pier) was diluted to the site water salinity with 18-MΩ water

Test Species

Toxicity testing was conducted with embryos of the Mediterranean mussel, *Mytilus galloprovincialis*. This species is relevant because it is sensitive to copper at very low concentrations (e.g. < 10 ppb; USEPA 1995b), and is present as a commercially important species in the Puget Sound area (Taylor Shellfish Farms 2004). *M. galloprovincialis* has also been used as a test species for caged mussel deployments in Sinclair Inlet during the installation restoration investigations conducted for the Shipyard (URS 2001).

Toxicity Tests

Toxicity tests were conducted following ASTM and USEPA guidance for whole effluent toxicity (ASTM 1993, USEPA 1995a) and for determining WERs (USEPA 1994a). Site and laboratory water samples were spiked with as many as eight (depending on sample) nominal copper concentrations, including 0, 2.9, 4.1, 5.9, 8.4, 12, 17.2, 25, and 50 µg/L. Laboratory water was filtered (0.45 µm), open coastal seawater collected from the research pier at Scripps Institute of Oceanography (SIO). Copper stock solutions were made from copper sulfate and confirmed by stabilized temperature graphite furnace atomic absorption (STGFAA) spectroscopy prior to use. The same stock solution was used for laboratory and site waters, as well as a copper reference toxicant test. Test concentrations were prepared separately in acid-cleaned 125 mL Erlenmeyer flasks. From each flask, 10 mL was distributed to each of six new, seawater-conditioned, glass 20 mL scintillation vials for the bioassay. Five replicates were used for larval development assessment, while the sixth replicate was saved for later quantification of total recoverable and dissolved copper by STGFAA. An equilibration period of approximately 5 h was allowed following copper additions prior to addition of embryos.

M. galloprovincialis was obtained from Carlsbad Aquafarm, Carlsbad, CA on the same day tests were initiated. Mussels were induced to spawn by thermal shock (raising the temperature by about 10 °C). Approximately 200 embryos at or beyond the 2-cell stage (within four hours of fertilization) were added to each test vial. Vials were then incubated at 15 ± 1 °C for 48 h under a 16 h light: 8 h dark photoperiod. Water quality (pH, temperature, dissolved oxygen, salinity) was recorded at test initiation and test end. Two different endpoints were measured (normal survival and proportion normal), but normal survival was the one used for EC50 calculations, as this endpoint is more comprehensive, taking into account both survival and the number of normal D-shaped, straight hinged larvae relative to the number of embryos in a set of initial density vials. Larvae were evaluated with the aid of an inverted compound microscope.

Data Analysis

EC50s were calculated from normal survival calculations with ToxCalc™ version 5.0, using the Trimmed Spearman-Karber or Maximum Likelihood Probit methods. EC50 values were calculated from nominal, total recoverable, and dissolved copper

concentrations for each test. The potential for ambient toxicity was assessed by comparison of development success in the controls for each test (site water with no added copper) with test acceptability criteria. Control development in the site waters was also compared with that in the lab water using one-way ANOVA. Linear regression techniques were used to assess the relationship between EC50 and TSS, DOC, and copper complexation capacity (CuCC).

Copper Measurements

Concurrent with the toxicity samples, additional water samples were collected for measurement of total recoverable, dissolved, and free copper ion concentrations, as well as copper complexation capacity. Total and dissolved copper concentrations were used to support the toxicity assessment by allowing precise EC50 determination for each form of the metal. Free copper ion concentrations were used for copper complexation capacity determinations, to help demonstrate the method's usefulness as an alternative to biological testing.

Total recoverable and dissolved copper

Sampling protocols for the ambient waters were those of EPA Method 1669, EPA's Trace Metals Sampling Technique (USEPA, 1995c). These include the use of acid-cleaned apparatus and materials made of polyethylene, and "clean hands/dirty hands" techniques. Preservation, handling and analysis of the samples were conducted in class-100 trace metal clean working areas. Enough ULTREX grade nitric acid was added to the samples in order to decrease the pH to less than 2. Copper concentrations were measured by STGFAA spectroscopy either by direct injection (for effluent samples) or after liquid-liquid preconcentration with dithiocarbamates (for ambient samples) following Bruland et al (1985). The standard reference material (SRM) CASS4 (coastal seawater) from the National Research Council of Canada was used to quantify the recovery of the preconcentration, and SRM 1643d (trace metals in water) of the National Bureau of Standards was used to evaluate the precision and accuracy of the STGFAA analysis.

Free copper ion and copper complexation capacity

The concentration of the free aqueous copper ion ($[Cu(II)_{aq}]$) was measured with an Orion 94-29 Cu(II) ion selective electrode (Cu-ISE), following procedures used by Zirino *et al* (1998), and Cu-CC was measured as detailed in Rivera-Duarte and Zirino (2004); however, a brief description of the procedures is provided here. Both measurements were made in a dark, class-100 working station, with constant stirring at 25 ± 0.1°C, by the electrode potential (mV) between a Cu-ISE and an Orion Ag/AgCl double-junction reference electrode. The electrodes were calibrated with seawater Cu-activity buffers made with 2×10^{-4} M Cu in filtered (0.45 µm) seawater and either 1×10^{-3} M ethylenediamine or 1×10^{-3} M glycine (Belli and Zirino 1993, Zirino et al. 1998). Since $[Cu(II)_{aq}]$ in each buffer was calculated with a specific ion-interaction model for the measured pH and the concentrations of major ions (Belli and Zirino 1993), the calibrated response of the Cu-ISE is reported as the pCu (i.e., $-\log [Cu(II)_{aq}]$) of the solution.

The change in the response of the Cu-ISE during a titration with copper was used for the measurement of the Cu-CC (Rivera-Duarte and Zirino, 2004). The titrations were performed with a TTT 85 Titrator and an ABU 80 Autoburette, both from Radiometer Copenhagen, connected to a personal computer for continuous automatic recording of the data. First, the electrodes were calibrated and then allowed to equilibrate overnight in an aliquot of the seawater sample. The next day, an aliquot of 250-300 g of fresh seawater sample was weighed into a Teflon beaker, and the electrodes were allowed to equilibrate in it for several minutes before starting the titration. The titration proceeded automatically by additions of 10 µL each once the potential stabilized to within 0.1 mV sec^{-1} and was completed after 99 mL of the titrant was added. The titrant was made with 200 µL of 1000 ± 3 µg mL^{-1} High Purity Copper Standard added to 1L of 18-MΩ water containing 32 g NaCl. Cu-CC was estimated from the inflection point of the resulting titration curve using a MATLAB routine (Rivera-Duarte and Zirino, 2004).

Results and Discussion

Toxicity Tests and WER

Larval development in the ambient water samples was evaluated using two endpoints: proportion (percent) normal and normal survival, as defined above. Both methods resulted in very high survival in both lab and site waters, well above the 70% test acceptability criterion for controls (Figure 4). Percent normal averaged 98% among the lab and three site waters, while normal survival in the lab water was 93% and averaged 91% (range =87-93%) for the site waters. No statistically significant difference in larval development success was observed between site water and lab water (p=0.906 for percent normal; p=0.380 for normal survival). The absence of ambient toxicity is important because 1) it suggests that ambient conditions of the water body are not detrimental to very sensitive endpoints (e.g. mussel larval development), and 2) if ambient toxicity had been observed, it may have confounded the data interpretation from the spiked copper treatments, possibly preventing a WER estimate.

When copper was added to lab and site water, a dose response was observed in all cases. The proportion normal and normal survival data for each test concentration are provided in Appendix A. EC50 values, based on the normal survival endpoint, are shown in Table 1 and Figure 5. Nominal EC50s were within 10% of the total recoverable EC50s, indicating they closely approximated the total recoverable concentrations. Dissolved EC50s were 74.5 ± 3.7% of the total recoverable EC50 values. Regardless of the form of copper, the ranking of the EC50s from highest to lowest was M3.3 > M3.1 > P3 > LW (Table 1). The significance of this is that laboratory water (LW) has the expected lower capacity to buffer the effects of the added copper. Sample M3.3, on the other hand, had the greatest buffering capacity to neutralize the toxic effects of the added copper.

WERs are presented in Table 2. Like EC50s, WERs were highest for M3.3, followed by M3.1, and then P3. Because the WERs are within a factor of three, the three sites can be considered one site for the purposes of a final WER calculation, which is derived by determination of the geometric mean (USEPA 1994a). Based on the geometric mean of

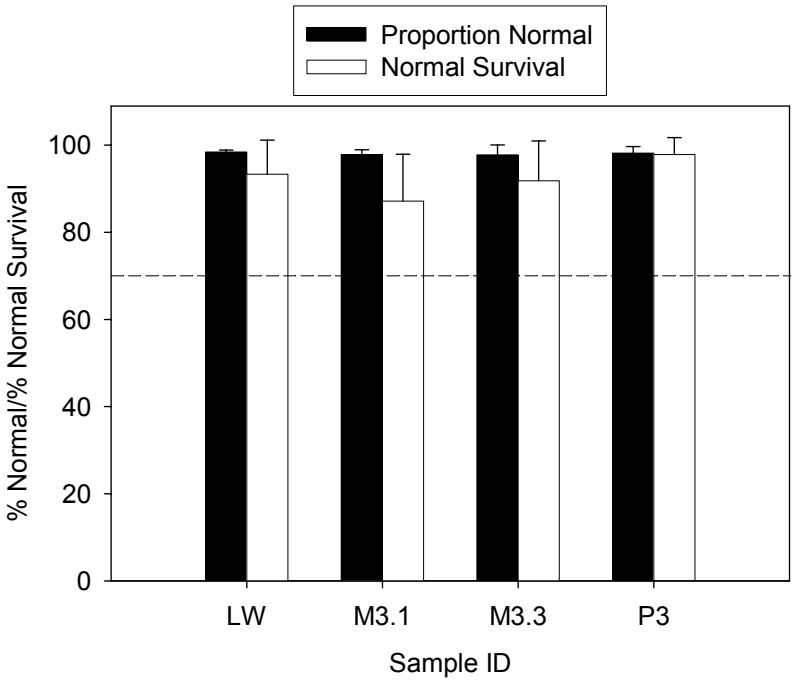
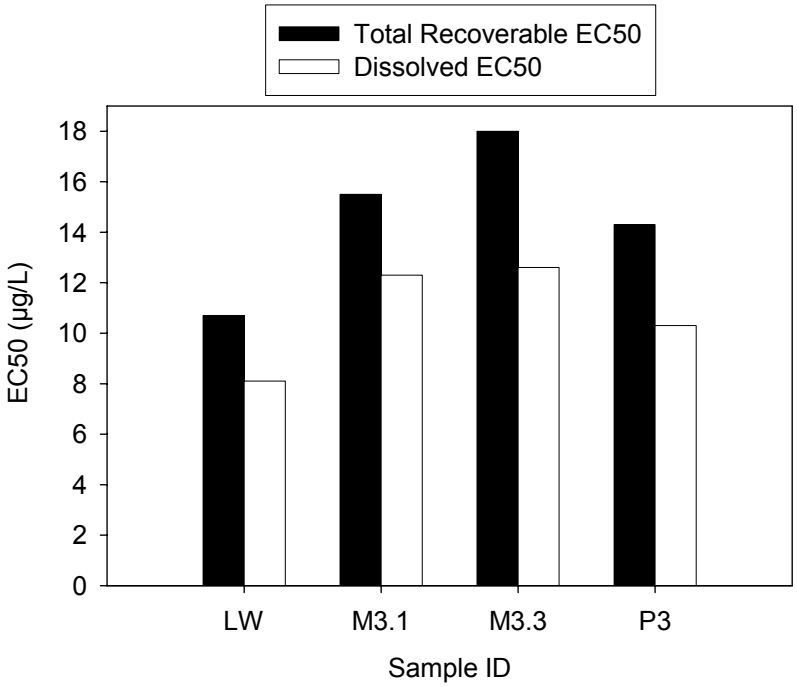


Figure 4. Control performance from toxicity tests performed with mussel (*Mytilus galloprovincialis*) embryos in laboratory water (LW) and three site waters adjacent to PSNS & IMF in Sinclair Inlet, WA. Proportion normal (percent normal) and normal survival are different endpoints commonly used in embryo-development tests**.** The dashed line represents the 70% minimum test acceptability requirement for controls.

Figure 5. Median effect concentrations (EC50) for copper exposures with mussel (*Mytilus*



galloprovincialis) embryos in laboratory water (LW) and three site waters samples collected adjacent to PSNS & IMF.

Table 1. Median effects concentrations (EC50) and associated 95% confidence limits (CL) expressed as nominal, total recoverable, and dissolved copper for laboratory water (LW), three surface water sites in Sinclair Inlet, WA, and a copper reference toxicant test.

	Nominal		Total Recoverable		Dissolved	
Sample ID	EC50 (µg/l)	95 % CL	EC50 (µg/l)	95 % CL	EC50 (µg/l)	95 % CL
M3.1	15.7	(15.5-15.9)	15.5	(15.3-15.8)	12.3	(9.1-13.1)
M3.3	16.4	(16.1-16.6)	18	(17.8-18.3)	12.6	(12.4-12.7)
P3	12.9	(11.9-13.9)	14.3	(13.3-15.3)	10.3	(10.1-10.5)
LW	10.1	(9.2-10.7)	10.7	(10.3-11.1)	8.1	(7.8-8.5)
Ref Tox	11.1	(11-11.3)	11.6	(11.5-11.8)	8.9	(8.8-9.0)

Table 2. Nominal, total recoverable, and dissolved water effect ratios (WER) for three Sinclair Inlet sites. Because of the similarity in the individual WERs, the area can be considered one site using the geometric mean of all three sites.

	Water Effect Ratio		
Sample ID	Nominal	Total Recov.	Dissolved
M3.1	1.56	1.45	1.52
M3.3	1.62	1.68	1.56
P3	1.28	1.34	1.27
Geo Mean	1.48	1.48	1.44

Table 3. Water quality characteristics for laboratory water (LW) and three surface water sites in Sinclair Inlet, WA. TSS=total suspended solids; TOC=total organic carbon; DOC=dissolved organic carbon; CuCC=copper complexation capacity; NM= not measured; ND= not detected.

Sample ID	TSS (mg/L)	TOC (mg/L)	DOC (mg/L)	Total Cu (µg/L)	Diss Cu (µg/L)	Free Cu (pCu)	CuCC (µg/L)
LW	ND	NM	0.9	1.0	0.6	NM	NM
M3.1	7	1.83	1.5	1.5	1.1	12.1	13.1
M3.3	6	1.29	0.9	1.5	1.0	12.0	13.3
P3	6	1.02	0.9	2.2	1.6	12.1	7.5

all three sites, total and dissolved WERs differed very little (<3%). This isn't surprising as the proportion of the total in the dissolved form differed very little between the mean of the three sites' (0.69 ± 0.08) and the lab water (0.67 ± 0.12). The standard deviations represent variability in the proportion among the different test concentrations.

Because WQC are expressed in terms of dissolved metal, the dissolved WER is the most appropriate for calculating a site-specific criterion. Using the dissolved WER, the acute and chronic site-specific criteria would be 6.9 and 4.5 µg/l, respectively, or 44% higher than the national criterion. These values are calculated as follows:

	National WQC		Diss. WER		Site-specific WQC
Acute	4.8 µg/l	X	1.44	=	6.9 µg/l
Chronic	3.1 µg/l	X	1.44	=	4.5 µg/l

The apparent reduction in copper bioavailability, as determined by less toxicity in surface water, in Sinclair Inlet compared to laboratory water suggests that adoption of site-specific criteria would still provide the same level of protection originally intended by the WQC derivation Guidelines (U.S. EPA 1985).

The copper reference toxicant test used to assess batch sensitivity resulted in an EC50 (Table 1) somewhat higher than typically observed in our laboratory, where the running mean is 6.1 ± 4.5 µg/l (mean ± 2 standard deviations). In general, the more sensitive the toxicity endpoint, the higher the outcome of the WER because of changes in metal speciation as the concentration of total metal increases in site water (Allen and Hansen 1996). This suggests that the WERs calculated in this study are probably on the conservative side (e.g. lower than they may have been with a more sensitive batch). This could be addressed in future sampling events, as WER studies should be conducted across different seasons and generally require a minimum of three sampling events (U.S. EPA 1994a).

A significant relationship between DOC and EC50 could not be established based on these data (r^2=0.223; n=3). Typically, DOC plays a key role in regulating the bioavailability of copper in aquatic environments. The generally low DOC concentrations measured in Sinclair Inlet and the limited data set produced by this study may explain why this relationship was not observed. A regression of TSS against EC50 resulted in a better relationship (r^2= 0.793; n=4). This calculation included the lab water, for which TSS were non-detectable.

Copper Measurements

Copper measurements and other water quality characteristics of unmodified (ambient) site water and laboratory water are summarized in Table 3. Dissolved Cu concentrations in ambient samples were all well below the national WQC (3.1 µg/l). Total recoverable and dissolved copper concentrations for all test solutions used in the toxicity tests are provided in Appendix A. Dissolved concentrations averaged 69 ± 8% of the total recoverable concentration across all test solutions for the three site waters, which is reflected in the total recoverable and dissolved EC50 values.

Free copper ion concentrations were similar in the three site water samples (Table 3). Previous work at SSC-SD suggests that free copper becomes toxic at a pCu < 11.0 (Blake et al. 2004). A pCu of ~12, observed in water samples from Sinclair Inlet, suggests that the free copper concentration would have had to be an order of magnitude higher before it became toxic to mussel embryos. This is emphasized by the pCu_{Tox}, which is the pCu in the ambient water with copper concentration identical to the EC50. The pCu_{Tox} were 11.18 for M3.3, 11.06 for M3.1 and 11.50 for P3; therefore, an increase in the concentration of free copper ion of at least an order of magnitude (i.e., from 1×10^{-12} M to 1×10^{-11} M) is needed in order to observe the toxic endpoint. The absence of toxicity in site water controls (ambient site water) is consistent with previous observations at similar pCu.

CuCC and EC50 values were quite similar for the three site water samples, resulting in a strong correlation between the two parameters (r^2=0.992; Figure 6). A good relationship has also been observed with larger data sets, such as those from several surveys in San Diego Bay, CA (Rivera-Duarte et al., submitted). Because measurement of CuCC is simpler and more cost effective than toxicity testing, demonstration of it usefulness as a predictor of toxicity is important. Although there are only three data points provided by this work, the relationship is apparent.

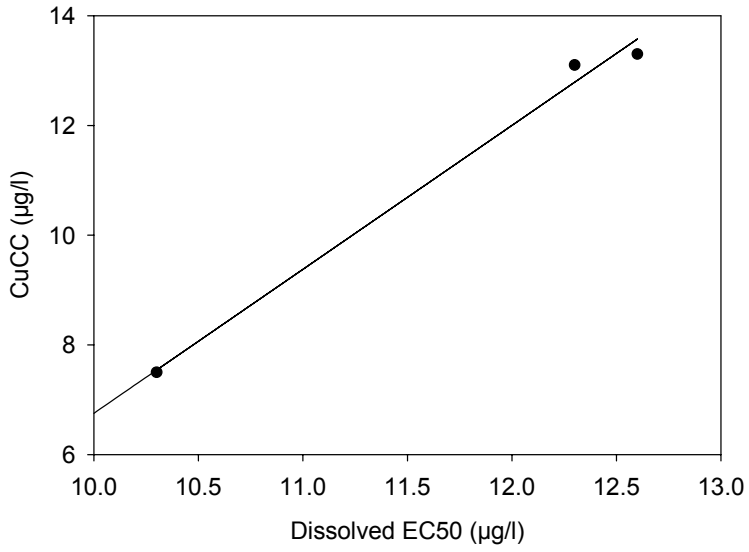


Figure 6. Relationship between copper complexation capacity (CuCC) and median effects concentration (EC50) from toxicity tests with mussel (*Mytilus galloprovincialis*) embryos for three site water samples collected in Sinclair Inlet adjacent to the PSNS & IMF.

Summary

A toxicity assessment was conducted to evaluate the bioavailability and toxicity of copper added to surface water samples collected from three sites in Sinclair Inlet, WA, adjacent to the PSNS and IMF. Unmodified site water samples were non-toxic to mussel embryos, and had dissolved copper concentrations well below ambient water quality criteria (3.1 [chronic] and 4.8 µg/l [acute]). Copper spiking to site water, however, resulted in EC50 values averaging 44% higher, on a dissolved basis, than spiked laboratory water. This equates to a WER of 1.44, which translates to acute and chronic site-specific criteria of approximately 6.9 and 4.5 µg/l, respectively, based on the one sampling event used for this study. Because the copper reference toxicant was somewhat higher than the mean observed in our laboratory, indicating somewhat reduced sensitivity, a higher WER may result from a more extensive study of the inlet. Copper complexation capacity (CuCC), a chemical measure of bioavailability based on free copper measurements, correlated very well with EC50 values. WER studies tend to very laborious and costly, while CuCC may be a valid alternative for derivation of site-specific criteria.

References

Allen HE, Hansen DJ. 1996. The importance of trace metal speciation to water quality criteria. *Water Environ Res* 68:42-54

American Society for Testing and Materials (ASTM). 1993. Standard guide for conducting static acute toxicity tests starting with embryos of four species of saltwater bivalve mollusk (E 724-89).

Belli, S.L.; Zirino, A. Behavior and calibration of the copper(II) ion-selective electrode in high chloride media and marine waters. *Anal. Chem*. **1993**, *65*, 2583-2589.

Blake AC, Chadwick DB, Zirino A, Rivera-Duarte I (2004). Spatial and temporal variations in copper speciation in San Diego Bay. *Estuaries* 27:437-447.

Bruland, K.W., Coale, K.H, and Mart, L. 1985. Analysis of seawater for dissolved cadmium, copper, and lead: An intercomparison of voltammetric and atomic absorption methods. Marine Chemistry 17: 285-300.

Buffle, J.; Altman, R.S.; Filella, M.; Tessier, A. Complexation by natural heterogeneous compounds: Site-occupation distribution functions, A normalized description of metal complexation. *Geochim. Cosmchim. Acta* **1990**, *54*, 1535-1553.

Ecology 2004. Sinclair/Dyes Inlets Water Cleanup Plan (TMDL). WA State Department of Ecology : Water Quality Home : TMDL Home : TMDLs by Watershed Home http://www.ecy.wa.gov/programs/wq/tmdl/watershed/sinclair-dyes_inlets/index.html

ENVVEST 2002a. PSNS Project ENVVEST Technical Work Masterplan, of May 2002, prepared by PSNS Project ENVVEST Technical Steering Committee. https://swdata.spawar.navy.mil/envvest/tech_master_plan_06_f2_web2b.pdf

Eriksen, R.S.; Mackey, D.J.; van Dam, R.; Nowak, B. *Copper speciation and toxicity in Macquarie Harbour, Tasmania: an investigation using a copper ion selective electrode. Mar. Chem.* 2001 *74*, 99-113.

Katz C.N, P.L. Noble, D.B. Chadwick, B. Davidson, and R.D. Gauthier. 2004. Sinclair Inlet Water Quality Assessment: Water Quality Surveys Conducted September 1997, March 1998, and July 1998. Prepared for Puget Sound Naval Shipyard Project ENVVEST. Space and Naval Warfare Systems Center, San Diego, CA. January 2004.

Johnston R.K., C.W. May, V.S. Whitney, J.M. Wright, and B. Beckwith, 2004. Fecal Coliform Model Verification Sampling Plan, Winter 2004: Addendum to the Fecal Coliform Total Maximum Daily Load Study Plan For Sinclair and Dyes Inlets. PSNS & IMF Project ENVVEST, Bremerton, WA, February 2004. http://www.ecy.wa.gov/programs/wq/tmdl/watershed/sinclair-dyes_inlets/w2004_fc_sap_final_ecy.pdf

Knezovich JP, Harrison FL, Tucker JS, 1981. The influence of organic chelators on the toxicity of copper to embryos of the pacific oyster, *Crassostrea gigas Archives of Environmental Contamination and Toxicology* 10:241-249.

Lorenzo JI, Nieto O, Beiras R. 2002. Effect of humic acids on speciation and toxicity of copper to *Paracentrotus lividus* larvae in seawater. *Aquatic Toxicology* 58: 27-41.

Rivera-Duarte, I.; Zirino, A. 2004. Response of the Cu(II) ion selective electrode to Cu titration of artificial and natural shore seawater and measurement of the Cu complexation capacity. *Environ. Sc. Technol*. 38: 3139-3147.

Rivera-Duarte, I.; Rosen, G., Lapota, D., Chadwick, D.B., Kear-Padilla, L, A. Zirino. 2004. Control of copper toxicity to larval stages of three marine invertebrates by copper complexation capacity in San Diego Bay, California. Environmental Science & Technology (submitted for publication).

Rosen G, Rivera-Duarte I, Kear-Padilla L, Chadwick DB, 2004. The use of …….

Stauber JL, Benning RJ, Hales LT, Eriksen R, Nowak B. 2000. Copper bioavailability and amelioration of toxicity in Macquarie Harbour, Tasmania, Australia. *Marine and Freshwater Research*, 51:1-10.

Sunda, W.G.; Ferguson, R.L. 1983 Sensitivity of natural bacterial communities to additions of copper and to cupric ion activity: A bioassay of copper complexation in seawater. In: Wong, C.S; Boyle, E.A.; Bruland, K.W.; Burton, J.D.; Goldberg, E.D. (Eds.), Trace Metals in Seawater, Plenum Press, New York, 1983, pp: 871-891.

Sunda, W.G.; Guillard, R.L.1976. The relationship between cupric ion activity and the toxicity of copper to phytoplankton. J. Mar. Res., 34: 511-529.

Taylor Shellfish Farms 2004. Mediterranean Mussels: *Mytilus gallaprovincialis*. http://taylorshellfish.com

(TEC) The Environmental Company 2003a. Draft Site Evaluation Report for Sampling and Analysis of In-Stream and Storm Water Chemical and Flow Characteristics PSNS Project ENVVEST Study Area Bremerton, Washington. TEC Inc. Bellevue, WA. Contract No.: N44255-98-D-4416, Task Order: 0068. 19 SEPT 2003. https://swdata.spawar.navy.mil/envvest/Watershed%20Monitoring/StreamStormSampling2002-2003/TEC_II_Site_Evaluation_Report_No_Appendices.pdf

TEC 2003b. Draft Sampling and Analysis Plan for Sampling and Analysis of In-Stream and Storm Water Chemical and Flow Characteristics PSNS Project ENVVEST Study Area Bremerton, Washington. TEC Inc. Bellevue, WA. Contract No.: N44255-98-D-4416 Contract Task Order: 0068. 19 SEPT 2003. https://swdata.spawar.navy.mil/envvest/Watershed%20Monitoring/StreamStormSampling2002-2003/TEC_II_Draft_QAPP.pdf

TEC 2003c. Annual Report 2002-2003 – In-Stream Storm Flow Sampling Puget Sound Naval Shipyard (PSNS) Project Environmental Investment (ENVVEST), September 2003. https://swdata.spawar.navy.mil/envvest/Reports/TEC_2003_Report/Final_2003_Annual_Report.pdf

(URS) URS Greiner Woodward Clyde Federal Services 2001. Draft final feasibility study report: Operable Unit B, Bremerton Naval Complex, Bremerton, WA, July 23, 2001. Contract No. N62474-89-D-9295 CTO 0131.

USEPA, 1985. Guidelines for deriving numerical national water quality criteria for the

protection of aquatic organisms and their uses. EPA/822/R-85/100. U.S. Environmental Protection Agency, Washington, DC, USA

U.S. EPA 1994a. Interim Guidance on Determination and Use of Water-Effect Ratios for Metals. EPA-823-B-94-001. Office of Water, Washington, D.C.

U.S. EPA 1994b. National Pollutant Discharge Elimination System Waste Discharge Permit for Puget Sound Naval Shipyard, Bremerton, WA.

U.S. EPA, 1994c. Development of Site-specific Copper Criteria for the NY/NJ Harbor Complex Using the Indicator Species Procedure. U.S. Environmental Protection Agency, Surface Water Quality Branch, Region II, June 3, 1994.

U.S. EPA, 1995a. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to west coast marine and estuarine organisms. EPA/600/R-95/136, Office of Research and Development, Washington, D.C

U.S. EPA, 1995b. Ambient Water Quality Criteria – Saltwater Copper Addendum (Draft), Aril 14, 1995. U.S. Environmental Protection Agency, Environmental Research Laboratory, Narragansett, RI.

U.S. EPA, 1995c. *Method 1669, Sampling Ambient Water for Determination of Trace Metals in Environmental Samples.* EPA/600-R-94-111.

U.S. Navy, US EPA, and Washington State Department of Ecology 2000. Project ENVVEST: Phase I Final Project Agreement for the Puget Sound Naval Shipyard, September 25, 2000 [Federal Register: October 23, 2000 (Volume 65, Number 205)]. http://www.epa.gov/ProjectXL/puget2/fpasigned.pdf

(WDOE) Washington State Department of Ecology 1998. Final 1998 Section 303(d) List -WRIA 15 . http://www.ecy.wa.gov/programs/wq/303d/1998/wrias/wria15.pdf

WDOE 2002. Water Quality Program Permit Writer's Manual. Department of Ecology, 92-109, Revised, 2002. http://www.ecy.wa.gov/biblio/92109.html

WDOE 2004. Guidance for Conducting Mixing Zone Analyses. http://www.ecy.wa.gov/programs/eap/mixzone/mixzone.html

(WDOH) Washington State Department of Health 2003a. Sanitary Survey of North Dyes Inlet. October 2003. Office of Food Safety and Shellfish Programs. 50pp.

WDOH 2003b. Shellfish harvesting opens in Northern Dyes Inlet, News Release Oct. 31, 2003. http://www.doh.wa.gov/Publicat/2003_News/03-176.htm

WDOH 2003c. Northern Dyes Inlet Proposed Classification Boundries. Office of Food Safety and Shellfish Programs. http://www.doh.wa.gov/ehp/sf/Pubs/DyesInlet.doc

Zirino, A.; VanderWeele, D.A.; Belli, S.L.; De Marco, R.; Mackey, D.J. 1998. Direct *measurement of Cu(II)aq in seawater at pH 8 with the jalpaite ion-selective electrode*. Mar. Chem., 61: 173-184.

Appendix A. Measured copper concentrations and larval development in test solutions

Sample ID	Nominal Cu (ppb)	Total Cu (ppb)	Diss Cu (ppb)	Normal Survival		Proportion Normal	
				Normalized to control (%)	Non-normalized (%)	Normalized to control (%)	Non-normalized (%)
M3.1	0.0	1.2	0.7	100	87	100	98
M3.1	4.1	4.6	3.2	100	87	100	98
M3.1	5.8	6.8	4.6	92	80	99	97
M3.1	8.4	9.0	5.6	112	98	99	97
M3.1	12.0	11.2	9.2	101	88	95	93
M3.1	17.2	17.2	13.3	29	25	26	26
M3.1	25.0	24.4	17.6	5	4	4	4
M3.1	50.0	53.2	37.7	0	0	0	0
M3.3	0.0	1.4	1.0	100	92	100	98
M3.3	4.1	5.5	4.1	101	93	99	97
M3.3	5.8	7.3	5.4	104	96	99	97
M3.3	8.4	9.1	6.6	95	88	101	99
M3.3	12.0	12.7	9.7	105	97	97	95
M3.3	17.2	19.5	12.1	43	40	45	44
M3.3	25.0	25.2	17.3	0	0	0	0
M3.3	50.0	56.6	29.1	0	0	0	0
P3	0.0	2.8	1.5	100	98	100	98
P3	4.1	6.1	3.9	98	96	100	98
P3	5.8	7.5	5.3	102	100	100	98
P3	8.4	10.1	7.5	96	94	100	98
P3	12.0	12.6	9.1	67	66	70	69
P3	17.2	19.3	13.1	11	10	11	10
P3	25.0	26.2	18.5	0	0	0	0
P3	50.0	53.9	39.8	0	0	0	0
LW1	0.0	1.0	0.6	100	93	100	98
LW1	4.1	5.7	3.0	102	96	100	98
LW1	5.8	7.6	5.9	96	89	99	97
LW1	8.4	9.4	6.9	91	85	93	91
LW1	12.0	12.0	9.4	13	12	12	12
LW1	17.2	17.4	13.2	0	0	0	0
Ref Tox	0.0	1.9	1.1	100	89	100	99
Ref Tox	4.1	6.1	3.6	98	87	100	98
Ref Tox	5.8	7.8	5.3	109	96	100	99
Ref Tox	8.4	9.7	6.7	103	91	97	95
Ref Tox	12.0	11.4	9.6	34	30	32	31
Ref Tox	17.2	17.9	12.8	0	0	0	0

Appendix B. Water quality parameters[1] during toxicity tests

Station ID	Nominal [Cu] µg/l	Temperature (°C)	pH	Salinity (‰)	D.O. (mg/L)
M3.1	0	15.8	7.93	30	7.6
	2.9	15.9	7.95	30	nd
	8.4	15.9	7.95	30	nd
	50	15.8	7.99	29	nd
M3.3	0	15.8	7.94	29	7.6
	2.9	16.1	7.95	29	nd
	8.4	15.8	7.95	29	nd
	50	16.1	7.95	29	nd
P3	0	15.9	7.89	30	7.5
	2.9	15.7	7.91	29	nd
	8.4	15.7	7.91	30	nd
	17.2	15.7	7.91	29	nd
LW1	0	16.0	8.01	29	nd
	4.1	16.2	8.10	29	nd
	8.4	16.2	8.01	29	nd
	17.2	16.1	8.01	29	nd
Ref. Tox.	0	15.8	8.00	34	6.0
	2.9	15.7	7.95	34	nd
	8.4	15.8	8.00	34	nd
	17.2	15.7	7.99	34	nd

nd=not determined

[1]Water quality measured upon initiation of tests