

Full Length Research Paper

Bioassay using juvenile mysids for rapid assessment of seawater: A case study from Reef HQ Aquarium (Townsville, Australia)

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Reef HQ Aquarium (Townsville, Australia) pumps its new exhibit seawater from a tidal inlet. This study presents the in-house calibration of a bioassay based on juvenile mysids to do a rapid assessment (presence or absence) of toxicity in the new seawater. Calibration tests were carried out for several substances: copper, sodium lauryl sulphate (SLS or SDS), ammonia, antifouling paint, bilge oil from a yacht, oil from a dive compressor, seawater cooling effluent from a commercial ferry vessel, and aquarium tank water. Results were compared with two other bioassays already in use at Reef HQ Aquarium, based on (a) artemia hatched from aquaculture cysts and (b) *Vibrio fischeri* bacteria (Microtox®). This study determined (a) that the juvenile mysids bioassay yielded meaningful results and was viable operationally, (b) its sensitivity with respect to likely local pollutants, and (c) how it compares in terms of sensitivity with the artemia and the Microtox® bioassays.

Key words: ARTOX, bioassay, mysid, Microtox®, Reef HQ Aquarium, seawater, toxicity.

INTRODUCTION

Reef HQ Aquarium (Townsville, Australia), the National Education Centre for the Great Barrier Reef, hosts the largest living coral reef tank in the world (2.5 ML), called the Coral Reef Exhibit (CRE aquarium). Since 2002, the aquarium has been pumping water from Ross Creek, a tidal inlet on its doorstep, as a supply of new seawater.

Water is pumped from the wharf alongside the aquarium building, about 1 km upstream from the creek mouth. This "creek water intake" takes place every two weeks when water is pumped continuously on either side

of the high spring tide for 6 to 7 h (taking ca. 630 m³). This timing aims at pumping water that is primarily coming from the ocean after flowing past the port at the creek mouth, as opposed to water that may have been standing in the upstream part of the inlet (Figure 1).

The new seawater is stored in a holding tank and recirculated in a closed loop for aeration, whilst it is screened for its potential toxic effect on corals and marine biological life in general (Figure 2). If the intake water passes all tests, it is mixed into the exhibits; if any

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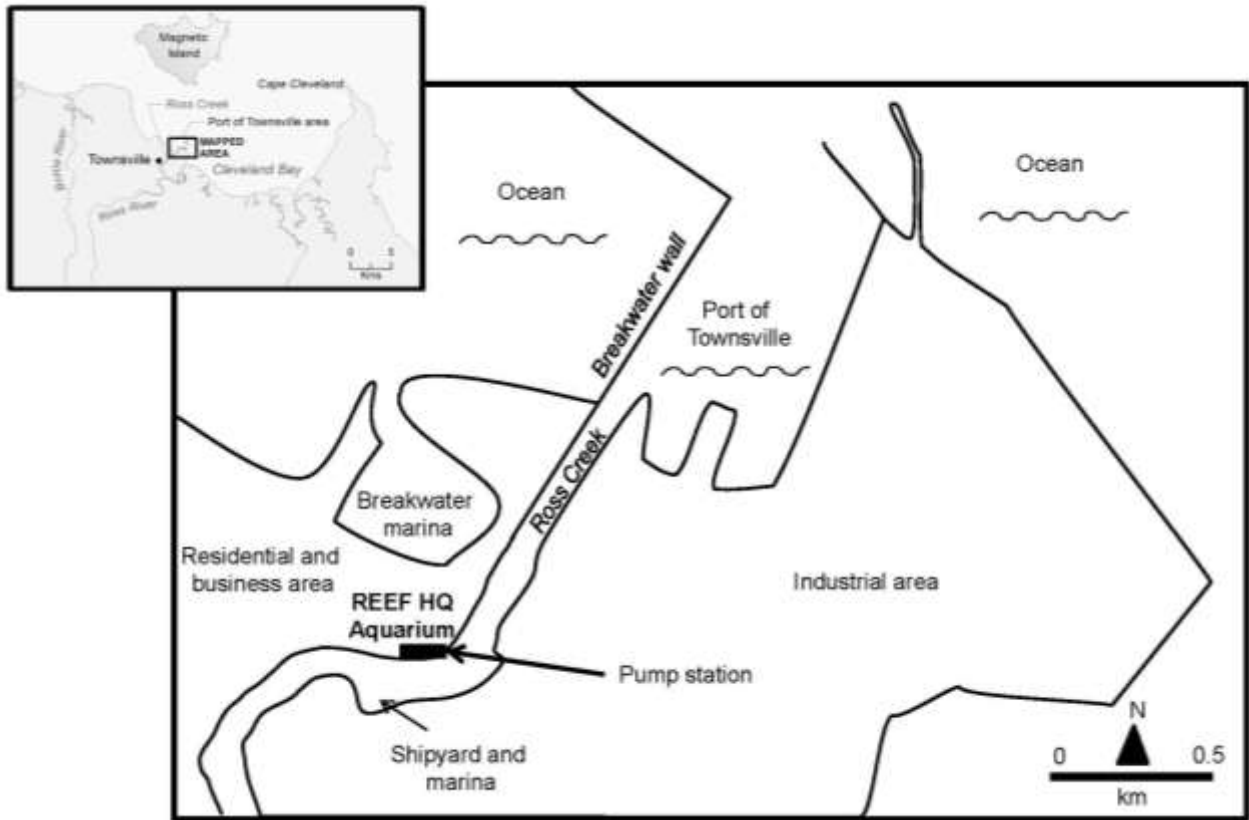


Figure 1. Aerial view of Townsville City centre, Aquarium and Pumping station, and Port area.

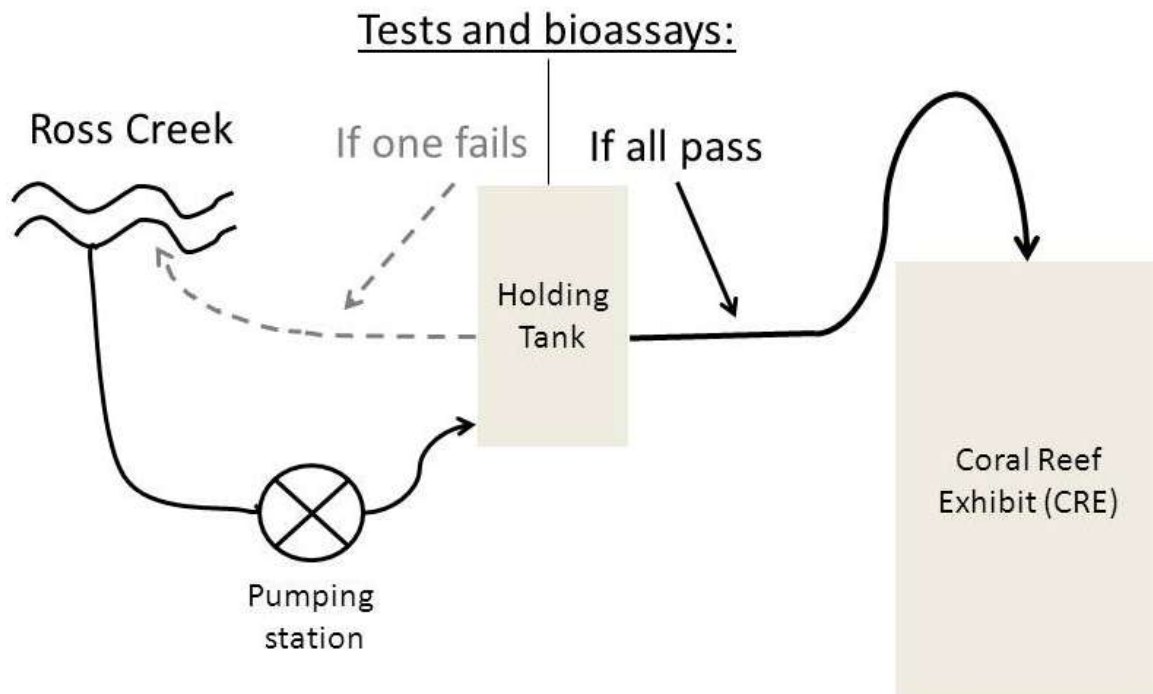


Figure 2. Diagram of creek water intake procedure and role of toxicity tests and bioassays.

one test fails, the water is rejected to the creek and a new intake is planned as soon as possible.

Tests to screen for potential toxicity of the new seawater have been developed or adapted from standard methods at Reef HQ Aquarium over the years with a focus on methods that are repeatable, affordable, have a sensitivity to toxic substances as close as possible to that of hard corals, require a reasonably low level of training, and have a response time of 24 h maximum. Such a short response time is necessary, because isolating the holding tank from the exhibits for longer would have too many other operational disadvantages, such as dropping water level in the exhibit and subsequent exposure of coral colonies due to the water being used to top up all other exhibit tanks; or loss of temperature control in summer as the cooling coil was circulating through the holding tank.

Whilst samples may be sent to external laboratories on an ad-hoc basis, the decision-making process of keeping or rejecting the new seawater cannot rely on external analyses due to the delays involved. In this context, the cause of a failed test is not of primary concern and is not always determined, nor are the selected tests aimed at identifying a specific toxic substance. Instead, a simple presence/absence estimate of the toxicity risk of the new seawater on marine life is required. Since no single test can provide this information reliably for all compounds, an array of toxicity tests has been used since 2002.

- A Juvenile fish test, where juvenile *Acanthochromis polyacanthus* (Bleeker, 1855) are exposed to the new seawater for 24 h. This test was ceased in June 2010 because of a lack of sensitivity and for ethics permit reasons.

- An Artemia nauplii test (ARTOXKIT M), where newly hatched brine shrimp nauplii *Artemia franciscana* (Kellogg 1906) are exposed to the intake water for 24 h. This test was discontinued in June 2012 due to restrictions on cysts imports into Australia.

- A Bacteria test (Microtox®), where the bioluminescent marine bacteria *Vibrio fischeri* (Beijerinck 1889) are exposed to the intake water. The reduction in intensity of light emitted by the bacteria is measured and the change in light output after 30 min exposure is an indicator of the health of the bacteria. This test is still in use in 2015 despite some doubts that it may not be sensitive enough for the purpose of the aquarium.

- A Coral microcosm tank, where hard coral colonies are exposed to the intake water for 24 h, and coral appearance (e.g. polyp extension, tissue colour, bleaching) is observed, looking for changes that may indicate stress or toxicity. This test is still in use in 2015 with a renewed coral population in late 2014 to host more sensitive coral species than has been done previously.

- Physico-chemical parameters, where pH, salinity, dissolved nitrate and phosphate concentrations are measured. These parameters are still measured in 2015 but an array of other parameters initially recorded has since been abandoned, namely: temperature, dissolved

oxygen, redox potential, alkalinity, calcium concentration, ammonia, nitrite, organic nitrogen and organic phosphate concentrations. These parameters either cannot be measured reliably in-house or are not useful indicators for the intake “keep or reject” decision.

Between July 2002 when the creek water intake procedure was instated and June 2015, 356 intakes have taken place and 9 batches have been rejected overall, based on the following tests giving a negative result: 1 ARTOX; 4 Microtox; 1 physico-chemical parameters; 3 nutrient levels.

At the onset of this project in 2013, two out of the four biological tests were thus no longer used (juvenile fish and artemia), whilst the remaining two (Microtox® and coral microcosm tank) gave no guarantee of being sensitive enough. Reef HQ Aquarium hence needed to increase its capacity to detect a water intake batch from Ross Creek that may be harmful to live marine organisms, in particular corals, and to further investigate the level of sensitivity covered by current operational protocols.

This study aimed at determining whether a mysids bioassay: (a) was meaningful and viable in terms of routine operation at Reef HQ Aquarium, (b) was sensitive enough to most likely local pollutants, and (c) how it compared in terms of sensitivity with the artemia and the Microtox® bioassays used at Reef HQ Aquarium.

MATERIALS AND METHODS

Study area

Ross Creek in Townsville (Queensland, Australia) is 3 km long and the pumping station is about 1 km from its mouth (GPS coordinates 19.258 S 146.824 E) (Figure 1). This short waterway harbours a commercial port at the mouth (with transit activity mainly from the mining and agricultural industries), a recreational yacht marina along its bank right across the aquarium, and it runs through the city centre of Townsville. Gunn and Barker (2009) classified the Ross Creek catchment area as 94% ‘urban’ and 6% ‘conservation, water and wetlands’ area. Potential diffuse sources of urban pollutants to Ross Creek have been identified as sediments, nutrients (principally nitrogen and phosphorus), biodegradable organic material, metals, garden and household chemicals, pathogenic micro-organisms, hydrocarbons and litter (Gunn and Barker, 2009). These substance classes have not all been assessed separately in Ross Creek, but Gunn and Barker (2009) reported that nutrient levels in Ross Creek were within water quality guidelines for organic and inorganic phosphate and nitrogen. Totals suspended solids (TSS) and chlorophyll a concentration, on the other hand, exceeded the guidelines levels (Wagner, 2008).

Despite a very small catchment area for Ross Creek, rain run-off in the wet season may be a concern with 2 artificial lakes forming the end of Ross Creek and acting as sediment and contaminant traps for the neighbourhood catchment. However, this particular concern remains low for Reef HQ Aquarium because (a) there is no developing urban area or agricultural land use in the catchment, two activities that can often contribute to an increased sediment load into waterways and (b) creek water intakes after a high rainfall event are restricted by low salinity in the tidal inlet anyway. By the time the salinity becomes high enough for taking new seawater, the suspended sediment concentration has usually dropped back to an

acceptable level. The water is also filtered upon pumping at a 20 µm nominal size, reducing the intake of suspended sediment.

Finally, port and marina activities are other potential pollutant sources considering the location of the pumping station at Reef HQ Aquarium (Figure 1). These may include anti-fouling paint, material being loaded at the port facility (including large tonnages of lateritic nickel ore and an array of metal concentrates) occasional bilge and sewage water coming from the boats, and oil spill.

A range of potential substances thus needed to be tested on the new mysids bioassay to cover the spectrum of scenarios that may happen, and to calibrate the response of the juvenile mysids to known substances and concentrations.

Selection and application of tested substances

A range of substances were investigated for their potential impact on marine life. These contaminants were selected based on their potential presence in Ross Creek or at Reef HQ Aquarium, or on their notoriety for being toxic to marine organisms. Tested substances, the rationale for choosing them and the concentrations at which they were tested are summarised in Table 1.

Known substances were applied by introducing a small amount of highly concentrated solution into the vial with the majority of the water being control water (CRE refugium) so that the dilution factor remained negligible. This applies for copper, SLS (also called SDS), and ammonia.

Bilge and compressor oil solutions were treated as highly concentrated solution, and a known volume was introduced into the testing vials filled with control water. Dilution was also considered negligible in this case.

Real life samples of unknown composition were treated as full exposure tests whereby the majority of the water in the test vial was the real life sample. This applies for high nutrient water, ferry coolant effluent, and antifouling paint soaked water.

For the antifouling paint test, 2 types of paint were used, one for aluminium (Al) and one for fiberglass (FG). Three coats of antifouling paints were applied to an aluminium and a fiberglass plate respectively (10 × 20 cm × 2 mm thick each). The plates were then soaked separately in 2.5 L plastic containers full of CRE water for several weeks. Results presented here are after 1 week of soaking. Results for a soaking duration of several weeks proved to be even more toxic than after 1 week. Calibrating the mysids bioassay on the least toxic scenario (1 week versus several weeks) was considered to provide the most sensitive results and was a conservative approach in terms of capacity to detect risks. Three reference treatments were also used for the antifouling test to rule out any toxic effect coming from the containers or from the aluminium/fiberglass plates themselves. The three reference treatments were carried out using a container full of culture water and (a) soaking an un-coated aluminium plate; (b) soaking an uncoated fiberglass plate; and (c) no plate at all in the container.

The containers were kept in an office at approximately 25°C during the tests to duplicate the conditions that the mysids bioassay would be run under in routine operations. Samples from the container were diluted with refugium culture water to obtain result with test concentrations from 1 to 100% of soaking water. These ratios were chosen to explore a range of concentrations and ascertain in the first place whether the mysids were sensitive to antifouling paint at all. The ultimate aim of this set of experiment was to determine whether the mysids would be a potential sensitive indicator of antifouling components leaching out from boats moored in the Ross Creek marina across the Aquarium. The aim, however, was not to quantify the concentration of chemicals contained in the paint. Instead, the 100% dilution was chosen to establish whether the bioassay would detect a worst case scenario, with maximum potential exposure where a freshly painted boat would be moored in

contact with the pumping station (which in real life is not physically possible). The 1% scenario (1% toxic water in 99% of clean water) was considered to be a more realistic case of some leaching occurring in the creek from boats nearby the pumping station but not in contact, with some dilution occurring before the water is pumped.

Validation of concentrations

Validation analyses of concentrations were carried out for most copper solutions by having the dissolved copper concentration present in the test water measured by an external laboratory, showing a good agreement between the nominal and the measured concentration (Figure 3). Other chemical analyses were done on ammonia and antifouling paint (copper concentration) to confirm concentrations of exposure during the tests. Concentrations measured by an external laboratory are summarised in Table 1 when available.

Control water was CRE refugium water where the mysids were bred and collected in the first place, or artificial seawater (for the *artemia* and *Microtox* bioassays).

Test protocols

Juvenile mysids bioassay

Prior to addressing the sensitivity of mysids to substances, a testing protocol was established. To this end, several test conditions recommended in various guidelines (USEPA, 1996; Garnacho et al., 2000; Den Besten and Munawar, 2005) were modified to suit Reef HQ Aquarium environment as summarised subsequently and detailed in the full protocol (available upon request to the corresponding author). The main steps are summarised here.

Juvenile mysids (*Mysidopsis* spp.) were selected as a candidate for an in-house bioassay because standard methods have been developed in the past with this species (Nimmo and Hamaker, 1982; USEPA, 1994, 1996, 2002). Mysids are small shrimp-like crustaceans, found in marine and estuarine environments. They are considered to be relatively easy to breed, with a short reproductive cycle (Mauchline, 1980) and a new generation every 30 days. A controlled mysids culture has not yet been established in-house. However several display tanks host a healthy mysids population at Reef HQ Aquarium, in particular in the refugium of the CRE. The refugium is a series of open air in-line tanks that are continuously fed with CRE water via a pump. The refugium tanks are about 50 cm deep and the water returns to the CRE by gravity. The species are present at Reef HQ Aquarium identified to belong to the family of Mysidae (Haworth 1825) and to the subfamily of Mysinae (Haworth 1825), but no detailed taxonomy could be defined.

It is possible that the mysids population that lives in the CRE at Reef HQ Aquarium has adapted to some form of chronic contamination present in the tank (should there be any), compared to the mysids population found in the wild along the coast. This may introduce a bias during the test and make them more resistant to some particular substances. However, this is not considered a problem in the light of the applications targeted at Reef HQ Aquarium, which is to detect a batch of new seawater that could be harmful to the CRE. Since the mysids grow and live in the same water as the corals (CRE water), it is expected that they would have the same acclimation conditions as the coral, and thus would still react to a change of acceptable conditions in the new seawater.

A collection technique of juvenile mysids from the CRE refugium was devised and standardised to retain only individuals that are smaller than 3 to 4 mm. This is done by placing live rocks into a collection tray and letting the mysids naturally present in the rocks swim spontaneously through a mesh that only lets juveniles

Table 1. List of substances used for the toxicity tests and their concentration in the test vials.

Contaminant	Reason for selection	Nominal concentration ($\mu\text{g/L Cu}$)	Measured concentration ($\mu\text{g/L Cu}$)
Copper	- Present in Townsville port loading facility. Known to be harmful to marine life. Typical metal used as model toxicant for bioassay calibration. - Test solution either made in-house with Copper II Sulphate Pentahydrate dissolved in deionised water or pre-made ACR elemental standard 1000 mg/L in 2% HNO_3 matrix).	25	51
		50	NA
		75	90
		100	132
		150	NA
		250	NA
		500	NA
		1000	913
		(mg/L SLS)	(mg/L SLS)
Sodium lauryl sulfate (SLS) also called sodium dodecyl sulphate (SDS)	- Detergent and pet pesticide. Often used as model toxicant for bioassay calibrations (Craig et al., 2003) - Test solution made in-house with Ajax Sodium Lauryl Sulphate dissolved in deionised water.	1	
		1.5	
		2	
		4	
		5	No analysis done
		10	
		15	
		20	
		(ppm NH_3)	(ppm NH_3)
Ammonia (total)	- Can be present in aquaria upon system start-up or crash, or animal death. - Test solution pre-made ACR analytical reagent 1000 mg/L NH_3 in water matrix.	0.01	NA
		0.1	NA
		1	4.3
		10	9.7
		(% Oil)	(% Oil)
Bilge oil	- Marina and port facilities present in Ross Creek. - A non-soluble oil, collected in the engine / bilge compartment of a yacht. Appearance was very thick, sticky and black. The sample was stored at ambient laboratory temperature until testing.	0.1	
		0.5	
		1	No analysis done
		4	
		(% Oil)	(% Oil)
Compressor oil	- Substance present on site, although no clear mechanism has been identified for this oil to enter the exhibits. - A soluble oil collected at Reef HQ Aquarium from the dive compressor waste. Appearance yellowish and fatty; sample stored at ambient laboratory temperature until testing.	1	
		2	
		4	No analysis done
		5	

Table 1. Contd.

		10	
		20	
Ferry cooling effluent	Collected from a commercial passenger ferry that regularly docks at the Reef HQ Aquarium wharf. The cooling water outflow can be as close as one meter from the aquarium pumping point. Water sample was collected directly from the ferry cooling outlet approx. 5 minutes after engine start up. The sample was stored in a fridge until testing.	84%	No analysis done
Antifouling paint	Marina, port and shipyard facilities present in Ross Creek. - Paint brand = International Ultra, designed for fiberglass hulled boats (with specifications of 417.1 g/L cuprous oxide and 40.5 g/L dichlofluamid as active ingredients). - Paint brand = International Trilux 33, designed for aluminium hulled boats (with specifications of 104-125 g/L cuprous thiocyanate and 40-50 g/L zinc pyrithione as active ingredients)	Description of soaking water (100%) Fiberglass plate not painted Fiberglass plate painted Aluminium plate not painted Aluminium plate painted	(µg/L Cu) 43 669 82 997
High nutrient	- No particular concern of nutrient toxicity as nutrient levels are measured directly during water intakes. - Collected from Reef HQ turtle and shark tanks before water change	84%	(Dissolved) NOx: 390 µM Phos: 25µM

through (mesh cylinder with hole size of 3 × 0.5 mm). Mysids under 2 mm long are considered to be approximately 24 h old (UESPA, 1994), which is the age targeted for this study as the most sensitive life stage. However, mysids up to 5 days old have also been used for toxicity tests (USEPA, 1994).

Juvenile mysids are sampled one by one in the refugium water using a manual pipette set on 2 ml. In total, 20 mysid juveniles are transferred to 20 plastic vials (each of 30 ml capacity). The vials are then immersed to about two thirds of their height in the refugium water, shaded from direct sunlight, and left to acclimatise for an hour. This setup aims at isolating the mysids for easy observation, yet modifying the test environmental conditions as little as possible compared to their breeding conditions, since

water quality, temperature and light levels are kept almost constant compared to refugium conditions.

Ten mysids per test condition were used throughout the study (that is 10 control individuals + 10 test individuals). This sample size was chosen (a) as guidelines on mysids bioassays recommend a minimum of 10 mysids per condition (USEPA, 1996) and (b) to calibrate the bioassay under conditions that would be as simple and as rapid as possible for routine implementation, should the bioassay prove useful for routine testing. Test vials were 30 ml polyethylene vials cleaned with lab-grade detergent and rinsed with deionised water between tests.

After acclimating for an hour, 10.5 ml of either test water (that is, with the tested substance) or control water (that is, breeding refugium water) are added to the vials so that the

concentration inside each test vial was as listed in Table 1. A maximum of 84% exposure was reached for the 100% ferry coolant water, high nutrient water, and water in which antifouling paint had been soaking in, due to the 2 ml of breeding water initially present in the testing vials for the acclimation period. 84% is also ultimately the percentage of creek water concentration in the routine protocol. The test duration was set at 24 h to suit the aquarium's rapid response requirement.

Environmental conditions: Salinity that mysids are exposed to at Reef HQ Aquarium is dictated by the CRE salinity, which ranges from 32 to 37 over a typical yearly cycle from wet to dry season. The salinity of the new seawater is often close to that of the CRE salinity (usually

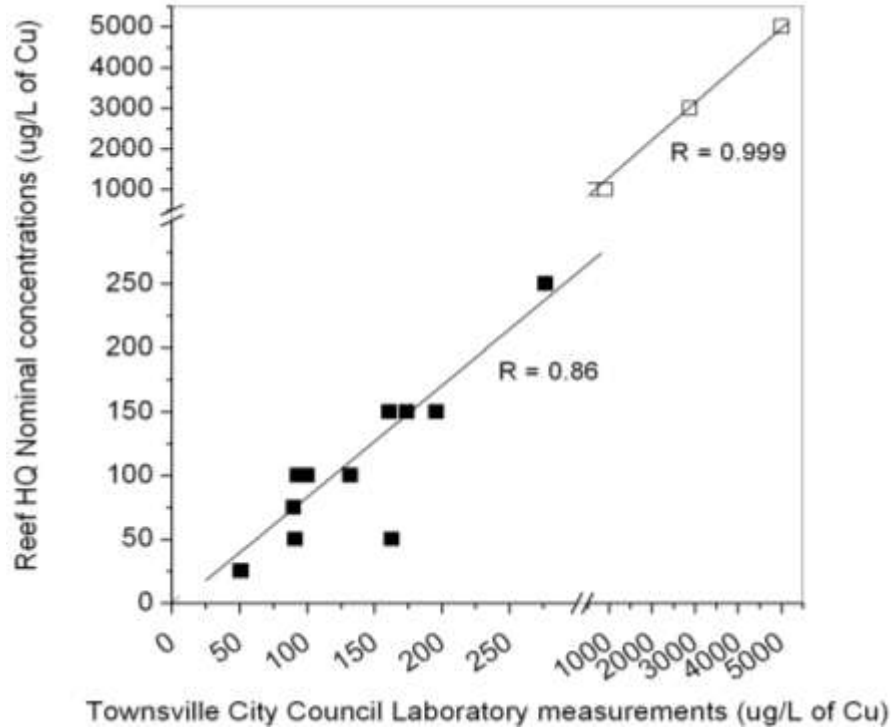


Figure 3. Validation graph for copper concentrations showing satisfactory agreement between nominal (y axis) and measured (x axis) dissolved copper concentrations, both at low and high concentrations.

within 0.5). In order to minimise environmental changes for the bioassay mysids, it was decided not to drop salinity despite recommendations in some guidelines on mysids bioassays to do it (USEPA, 1996). This was done in order to avoid introducing an unknown impact caused by a salinity change that could disguise a potential toxicity effect of the new seawater. This was also supported by the fact that mysids and corals live in the same environment inside the exhibit, and disturbing the salinity for the bioassay would only decrease the relevance of the test results in terms of potential toxicity detection.

Temperature in the CRE varies from 21 to 29°C with the seasonal cycle, in a similar way to Ross Creek temperature. The bioassay development and calibration were carried out in a controlled temperature room at ca. 25°C (quarantine room). The final protocol however was set to be run at the natural variable temperature to match the breeding conditions of the mysids stock, as for salinity. This was validated by several comparison trials showing no difference in toxicity results between the temperature controlled tests and the non-temperature controlled ones.

Mysids are exposed to natural light in the breeding tank, with daylight approximately from 6 am till 6 pm all year round (+/- 1 h). The light regime in the test room (quarantine room), where tests were carried out for this study, was lights exposure from 8 am till 4:30 pm, with no dimming effect to turn lights on or off. Here again the final protocol varied from the testing protocol in that mysids were kept in vials immersed for 2/3 of their height in the refugium during testing, with natural ambient light, but shaded from direct sunlight by a low-lying roof structure. Test vials were also loosely covered with a mesh to reduce evaporation and to minimize the entry of dust.

Dissolved oxygen concentration, temperature, salinity, and pH were measured at the beginning and end of the test during part of

this study. No variation was ever recorded and measurement of these parameters was ultimately not included in the final protocol.

After 24 h mysids are observed for signs of health, stress or death, and a standardised mortality value is defined as follows:

$$\frac{\text{Mysid behaviour index} \times \text{Number of mysids per condition}}{\text{Total mysids number}} \times 100 = \text{Standardised Mortality (\%)}$$

The mysid behaviour index is defined as 0 for a healthy mysid, 0.5 for weak mysid, and 1 for a dead mysid.

As an example, if at the end of a 24 h test 3 out of 10 mysids died, 1 displayed a weak behaviour, and 6 remained healthy, the standardised mortality would be:

$$\frac{(3 \times 1) + (1 \times 0.5) + (6 \times 0)}{10} \times 100 = 35\%$$

Standardised mortality is calculated for the control group and for the test group separately. A test is considered valid if the control group mortality is <10%. This is the natural variation allowed in the US EPA acute toxicity methods (US EPA, 2002) and corresponds to an acceptable noise level.

Artemia bioassay

The ARTOXKIT M test was interrupted at Reef HQ Aquarium in 2012 because the specific cysts strain required was no longer available. The Artemia test was nevertheless revived for this project using aquaculture Artemia cysts (Gulf Breeze Aquaculture) instead

of the recommended cysts, with the rest of the protocol being unchanged. The aim was to assess whether the artemia bioassay run with aquaculture cysts could still be used as a screen for potential toxic intake water. Besides the cysts strain, the protocol used in this study was the same as the one described in the ARTOXKIT M test available from MicroBio Tests Inc. The main steps of the protocol are summarised subsequently.

Artemia cysts are exposed to culture seawater 24 h before the test starts, because this is the time for artemia to reach the sensitive Instar II/III phase of their life. The number of artemia individuals present in a 100 µl sample of culture water is counted under a microscope (magnification ×40). This provides the number of artemia present in a given volume and the base to calculate mortality.

A 24 microwell plate (4 rows of 3 ml wells) is used for exposing the artemia to test water (12 wells) and control water (12 wells). Control water in this case was artificial sea water (ASW). Test and control wells were designated in alternate rows.

The final total volume added to each well is 2.5 ml as follows:

- For contaminant tests wells, 2.3 ml of ASW + 100 µl of artemia culture solution + 100 µl of the contaminant stock solution prepared such that the nominal concentration in the test wells was as shown in Table 1.

- For control wells, 2.3 ml of ASW + 100 µl of artemia culture solution + 100 µl of deionized water.

- For tests with oil as a contaminant, the concentration of oil was not known nominally, but as a percentage of the total volume (2.5 ml). So 100 µl of artemia from the culture solution were added to a variable volume of refugium water and oil solution to fit the desired percentage of oil (e.g. 0.1, 1, 10%...)

- For full exposure tests, test wells received 2.4 ml of creek water + 100 µl of artemia culture solution (96% exposure).

The well plate was then left undisturbed in the dark in a cardboard box at room temperature (ca. 25 °C) for 24 h. No food was provided to the artemia during the hatching or testing period.

After 24 h, dead artemia are counted in each well under a microscope and a standardised mortality is calculated as follows (separately for control and test wells):

$$\text{Standardised mortality (\%)} = \frac{\text{Average number of dead artemia per well}}{\text{Average total artemia number per well}} \times 100$$

As for mysids, a test is considered valid if the control group mortality is <10%.

Microtox bioassay

The Microtox® bioassay is carried out with a Microtox Model 500 laboratory-based photometer according to the manufacturer's standard protocol for "Comparison test for marine and estuarine samples" (AZUR Environmental, 1995), with a total exposure time of the *Vibrio* bacteria to the test water of 30 min. The control water is artificial seawater.

Response to toxicity is observed as a change in luminescence, which is a by-product of cellular respiration. This change can be used to calculate a percent inhibition of *Vibrio fischeri* that directly correlates to toxicity. Microtox® results are thus expressed in luminescence units, which are arbitrary and used as a relative comparison between test and control water.

Toxicity thresholds

A threshold was defined for each bioassay as the maximum acceptable mortality level for the new seawater to be mixed into the aquarium tanks with no significant risk of decline in marine

organisms' health. This threshold was called the "toxicity threshold" and was set at 20% standardised mortality for the mysids and artemia bioassays on the basis of a clearly detectable signal (that is, twice the 10% acceptable mortality as noise level), yet low enough to provide a warning tool and not a dramatic impact indicator.

A maximum difference in light emission of 2 in arbitrary units was set for the Microtox® between the control and the test group as the maximum acceptable difference for the new seawater to be declared non-toxic (that is, the threshold set when the Microtox® bioassay was first introduced at Reef HQ Aquarium in 2002).

RESULTS

Figure 4 shows mortality results of toxicity tests for mysids, artemia and Microtox® bioassays for each tested substance.

Wherever enough data points were available for the mysids and artemia bioassays, a linear extrapolation was done on Figure 4 to identify 4 levels of mortality (dotted lines on Figure 4 and summary of extrapolated values in Table 2) as follows:

- The No Observed Effect Concentration (NOEC) defined as the highest concentration of the toxicant tested that yields no statistically significant deviation from a control. Since the maximum standardised mortality in the control population for a test to be valid was set at 10%, this is also the mortality level set to define the NOEC.

- The Reef HQ toxicity threshold, set at 20% standardised mortality. This is the level defined as maximum acceptable for mysids and artemia bioassays at Reef HQ Aquarium.

- The Lethal Concentration 50 (LC50) defined as the level of substance that results in a standardised mortality of 50% after 24 h exposure.

- The Lethal Concentration 90 (LC90) defined as the level of substance that results in a standardised mortality of 90% after 24 hours exposure.

The three bioassays were compared to each other by dividing the toxic threshold concentration for each bioassay by the toxic threshold concentration for the mysid bioassay, for each tested substance. This yields a sensitivity of bioassays relative to mysids as displayed on Figure 5.

Figure 6 shows standardised results for all tested substances with each bioassay on a "wheel of mortality". This gives an overview of relative toxicity strength of substances for the mysids and artemia bioassays, and a toxic/non-toxic classification for the Microtox® bioassay results.

As an example, SLS at a concentration of 5 mg/L caused a standardised mortality well above the Reef HQ toxicity threshold to the mysids bioassay, whilst it caused a non-detectable standardised mortality to the artemia bioassay. Even though SLS was not applied at 5 mg/L to the Microtox bioassay, it can be assumed that it would have given a toxic signal since a concentration of 2 mg/L

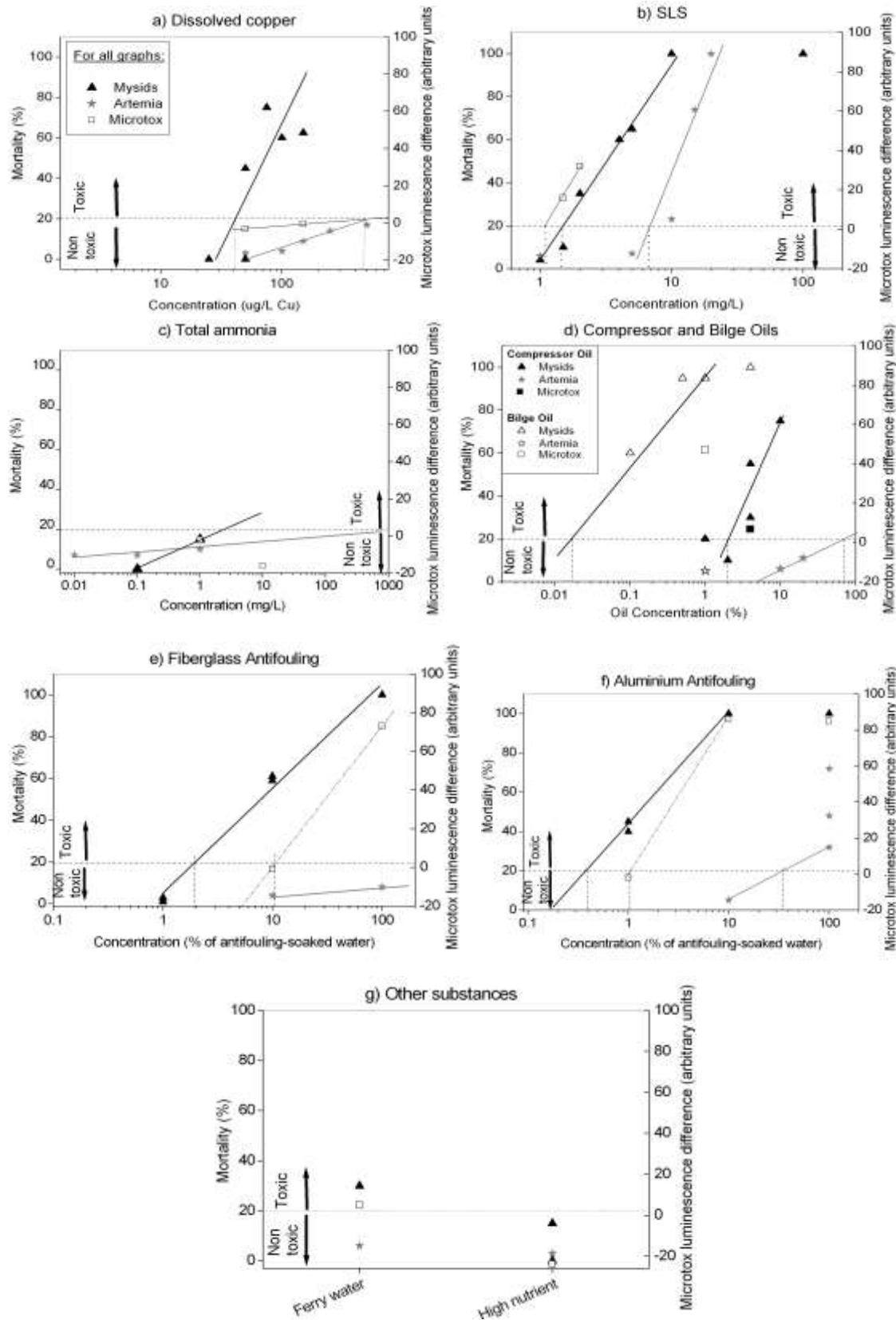


Figure 4. Results of toxicity tests for mysids, artemia and Microtox bioassays carried out with various contaminants: a) copper, b) SLS, c) ammonia, d) dive compressor and yacht bilge oils, e) antifouling paint applied to fiberglass and f) to aluminium, and g) effluents sampled in Reef HQ Aquarium's environment.

Table 2. Concentration of various substances required to cause a standardised mortality of 10, 20, 50 and 90 % for each bioassay and each tested substances. Values are extrapolated from results shown in Fig 3. When only one data point was available no value could be extrapolated (ferry coolant, high nutrient water, oils).

Parameter	NOEC	Reef HQ toxicity threshold	LC ₅₀	LC ₉₀
Mortality (%)	10	20	50	90
Mysids				
Copper (ug Cu/L)	44	52	85	160
SLS (mg/L)	1.2	1.5	3.5	10
Ammonia (mg/L)*	0.4	1.6	>10	Out of range
Aluminum antifouling (%)^	0.3	0.4	1.4	7
Fiberglass antifouling (%) ^	1.2	1.9	8	50
Bilge oil (%)	0.01	0.014	0.06	0.5
Compressor oil (%)	1	1.5	5	25
Artemia				
Copper (ug Cu/L)	130	335	Out of range	
SLS (mg/L)	6	7	11	20
Ammonia (mg/L)*	1	NA	NA	
Aluminum antifouling (%)^	16	35	Unclear (100%?)	
Fiberglass antifouling (%)^		Not detected		
Bilge oil (%)	1	Cannot extrapolate, only 1 point		
Comp. oil (%)	17	55	No such mortality	
Microtox ®				
	NA	Light difference <2	NA	NA
Copper (ug Cu/L)		400		
SLS (mg/L)		1.1		
Ammonia (mg/L)*				
Aluminum antifouling (%)^		1.1		
Fiberglass antifouling (%)^		11		
Bilge oil (%)		Cannot extrapolate, only 1 point		
Comp. oil (%)		Cannot extrapolate, only 1 point		

*Ammonia is the total ammonia N including NH₃ and NH₄⁺ forms. ^Values represent dilution strength of the initial soaking water.

was already showing a toxic signal.

DISCUSSION

The environmental guideline for dissolved copper is 3 µg Cu/L for a protection level of 90% of marine species according to water quality guidelines (ANZECC, 2000). The toxicity threshold for juvenile mysids was found in this study to be 52 µg Cu/L, which is a factor 10 higher than the environmental guideline as a first approximation. Whilst this is not a perfect toxicity screen, it is still a very sensitive tool and makes the protocol developed in-house at Reef HQ Aquarium meaningful in terms of copper toxicity detection for its live coral exhibit. Specifically, Schwartz et al. (2013) report that corals (*Montastraea franksi*) exposed to 30 µg/L copper for 48 h incurred genetic and DNA impact. This suggests that the juvenile mysids bioassay compares well with the coral response

to copper in terms of sensitivity.

SDS Material Safety Data Sheets report a NOEC concentration of 0.65 mg/L measured for the most sensitive species known, the clam *Corbicula fluminea* (30 day-NOEC = 0.65 mg/L) (OECD SIDS). The study presented here measured a NOEC of 1.2 mg/L for the juvenile mysids bioassay after 24 h, and a concentration of 1.1 mg/L for the Microtox ® toxicity threshold (20% mortality). These values converge and indicate that both the juvenile mysids and the Microtox ® bioassays (a) would be able to detect a SDS-related pollution likely to affect the live coral exhibit and (b) would perform equally in terms of sensitivity.

Recommendations for ammonia levels in aquaria is that it should remain non detectable. The toxicity threshold of 1.6 mg/L total ammonia found for juvenile mysids can therefore be considered too high for the bioassay to be able to detect ammonia in an aquaria application. This information is a valuable result to understand the

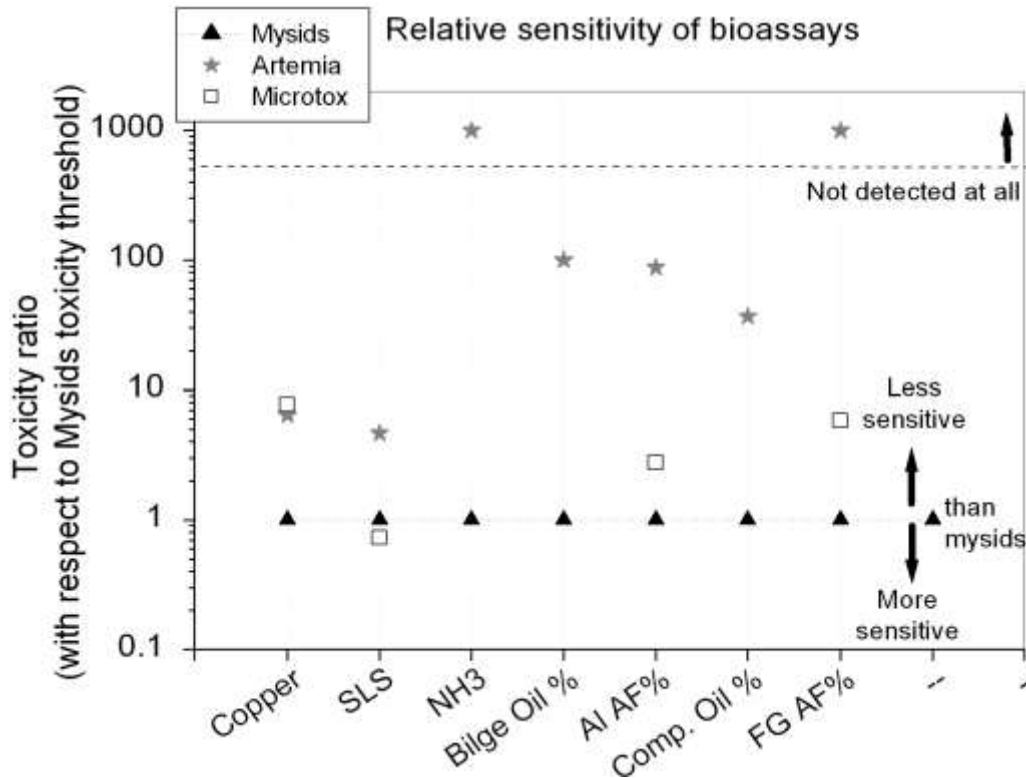


Figure 5. Bioassay sensitivity relative to the juvenile mysids bioassay calculated as the ratio of toxic threshold concentrations for each bioassay and each substance (when available). SLS = Sodium Lauryl Sulphate; NH3 = Ammonia; Comp. Oil = Compressor oil; Al = Aluminium; AF = Antifouling paint; FG = Fiberglass.

limits of the bioassay. However, in the specific context of Reef HQ Aquarium, ammonia is more likely to be occurring from within the system (e.g. from a shark dying inside an exhibit) than from the new source of Ross Creek water. It is therefore not considered critical that the bioassays applied to the new creek water be able to detect ammonia, and this limitation does not significantly drop the usefulness of the test on creek water.

As a summary, and given the substances tested and Reef HQ Aquarium in-house definition of the toxicity threshold, the juvenile mysid bioassay is consistently the most sensitive of the three bioassays tested because the toxicity threshold of 20% mortality is reached at the lowest concentrations for each tested substance. The only exception is SLS, for which the Microtox® bioassay registers approximately as sensitive as the mysids bioassay. For all other substances, the next most sensitive test is the Microtox®, with a sensitivity 2 to 10 times lower than mysids. The artemia bioassay tests carried out with aquaculture cysts consistently showed a sensitivity 10 to 100 times (or more) less sensitive than juvenile mysids.

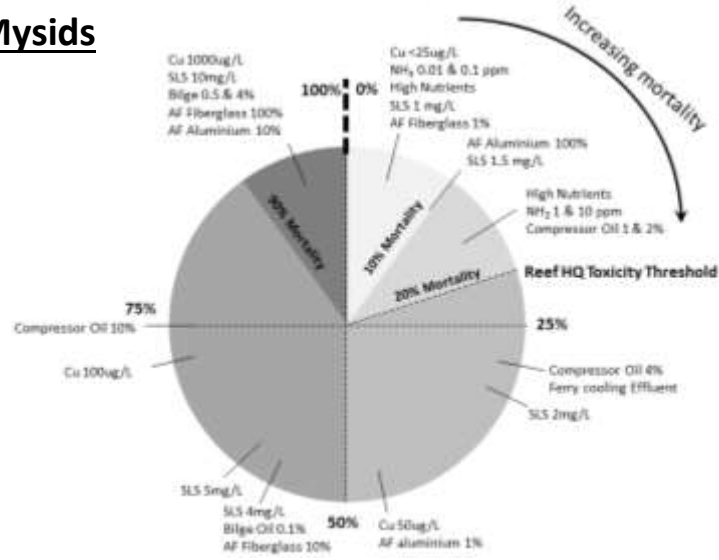
In terms of operations at Reef HQ Aquarium, these comparisons mean that the mysids bioassay is the best option available to date as an early warning indicator of a

potential toxicity effect of the new seawater on marine life, in particular if used in conjunction with other tests. The second best option is the Microtox® bioassay, whilst the artemia test with the aquaculture cysts appears to be uninformative due to its comparative lack of sensitivity.

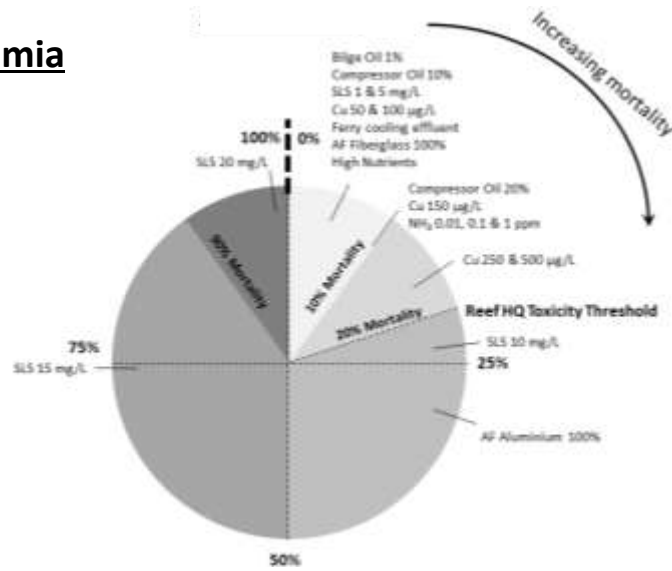
The Microtox® bioassay takes the shortest time of the three methods, with a completion time of 1.5 h. The artemia bioassay also requires ca. 1.5 h of work, but this time is spread over 48 h from the start of the hatching process to the results. The mysid bioassay requires approximately 2 h of work from the operator, and that time is spread out over ca. 26 h from collection time to results. This does not take the time required to breed and maintain a healthy mysid population into account since it occurs spontaneously at Reef HQ Aquarium.

All three bioassays can be run by non-specialists after adequate training, the easiest being the mysids bioassay and the most complex being the Microtox®. In terms of equipment, the mysids bioassay is also the simplest as only a manual pipette and plastic vials are required. The artemia is slightly more complex, requiring a small breeding chamber with at least an air pump and a microscope to count individuals. The Microtox® bioassay is the most technical, requiring the unit itself and a vial of the reagent bacteria for each test kept at -20°C, making

Mysids



Artemia



Microtox

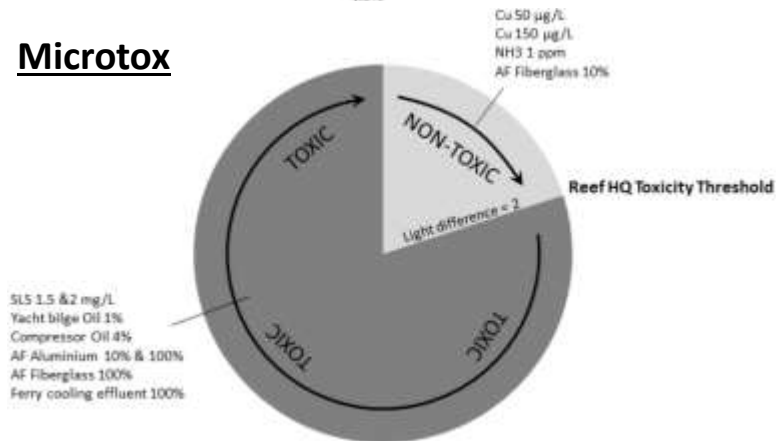


Figure 6. “Wheel of mortality” going clockwise from 0 to 100% standardised mortality for mysids and artemia bioassays for all substances and concentrations tested in this study. Microtox® bioassay results are displayed in 2 distinct toxic/non-toxic segments for the same substances and concentrations.

the cost very high compared to the other two bioassays.

In view of these parameters, and considering the relative sensitivity of the bioassays, the juvenile mysids bioassay developed in-house is the best option for rapid screening of toxicity: it takes approximately the same amount of time as the other bioassays, it is the simplest of all, and it yields results about 10 times more sensitive than the next best one. For the same amount of time on the other hand, the modified artemia bioassay (with aquaculture cysts) does not provide any useful information, confirming the decision to phase out this bioassay at Reef HQ Aquarium. The Microtox® is in between in terms of sensitivity and still provides useful information in parallel to the juvenile mysids bioassay, allowing redundancy for a moderate running cost (not including the upfront investment to purchase the instrument) and sufficient training of the operators.

Reef HQ Aquarium operation would benefit from an experiment comparing the sensitivity of the juvenile bioassay to that of live corals living inside the CRE. In such an experiment, the same substances tested in this study would be applied to live coral fragments and to juvenile mysids for 24 h, at the concentration found to be toxic for the juvenile mysids and summarised in Table 2 (column for 20% mortality).

Further calibration could also be carried out for nickel, zinc and lead toxicity for the juvenile mysids bioassay given that these substances are handled in large quantities at the Townsville Port loading facility. Finally, real samples from general rain runoff into Ross Creek could be applied to the calibration process to determine any potential harmful input into the estuarine system, in particular from the industrial port area.

Conclusion

Calibration of the mysids bioassay with various potential toxicants has enabled Reef HQ Aquarium to reinforce its capacity to detect toxic seawater during its procedure of new seawater intake. It has yielded concentration levels considered toxic for the aquarium exhibits for a range of substance groups: metal (copper), detergents (SLS), nutrient (inorganic phosphate and nitrate), and effluent from the aquarium and maritime industry (soluble and non-soluble oils, boat engine, cooling effluent, antifouling paint).

The toxicity threshold defined as 20% standardised mortality was established for the three bioassays for most tested substances and a 10, 50 and 90% standardised mortality concentration was also established whenever possible to help compare various tests sensitivity in the future.

For all substances, the mysids bioassay was the one showing an impact at the lowest concentration of all three bioassays, followed by the Microtox® and then by the artemia test carried out with an aquaculture strain of cysts (as opposed to a specific bioassay strain of cysts).

The average range of sensitivity in relative terms based on a value of 1 for the environmental guidelines was found to be 10 times less sensitive for the mysids bioassay, 100 times less sensitive for the Microtox® and 1000 less sensitive for the artemia bioassay as a first approximation.

This study has thus established that (a) the juvenile mysids bioassay is the most sensitive, (b) the Microtox® bioassay provides useful redundancy and (c) the artemia bioassay run with aquaculture cysts instead of certified ARTOXKIT M cysts is not useful for Reef HQ Aquarium screening procedure for toxic water.

The in-house protocol developed at Reef HQ Aquarium for a rapid screening of seawater toxicity using the juvenile mysids is available for other users to reproduce or adapt.

Conflict of Interests

The authors have not declared any conflict of interests.

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