The Identification of a Suitable Culture Organism to Establish a Bio-Assay for Evaluating Sediment Toxicity

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Water Research Commission

by

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EXECUTIVE SUMMARY

BACKGROUND

Sediments act as a source and sink for a variety of organic and inorganic contaminants. These contaminants accumulate, resulting in extremely high concentrations even once the overlying water concentrations are at or below acceptable water quality guidelines (Burton *et al*., 2001). Any changes in the physical parameters of the overlying water can cause these pollutants to be released back into solution. These accumulated contaminants can be released at even higher concentrations than previously detected. In recent years sediment contamination has highlighted the need to monitor these previously overlooked pollutant sources in aquatic ecosystems. When the contaminants bound to sediments become toxic they pose a risk both to the aquatic organisms as well as Human health (MacDonald & Ingersoll, 2003).

RATIONALE

South Africa does not currently have standardized methods to assess sediment toxicity. Although international methods exist, they are largely untested in South Africa and the organisms needed to conduct these tests are not readily available.

OBJECTIVES AND AIMS

Aim 1

To identify ecologically relevant Benthic/Epibenthic organisms that can be successfully cultured under Laboratory conditions in South Africa.

Aim 2

To develop a stable culture of applicable Benthic/Epibenthic organisms that can be used for sediment toxicity testing.

Aim 3

To assist in the training of Resource Quality Services (RQS) Staff with culture maintenance, Good Laboratory Practices and application of sediment tests.

METHODOLOGY

Following on extensive literature review a few ecologically relevant organisms were identified that could be cultured under laboratory conditions in South Africa for the use in toxicity testing. Nineteen organisms were identified in this study that had previously been

used internationally to evaluate sediment toxicity. These organisms and methods were used as preliminary guidelines to select and culture laboratory organisms in order to identify a suitable culture organism to establish a bio-assay for evaluating sediment toxicity in South Africa.

The culture organisms used in this study were selected based on ease of handling, availability, culture maintenance, how often they are in contact with the substrate and to what extent, as well as their reproductive cycle. The initial culture methods were adapted from the organism culture methods that were identified in the literature review. The chosen organisms were *Heterocypris incongruens, Hydra* sp., Chironomid sp. (*C. transvaalensis* and *C. caffrarius*) and the snail *Melanoides tuberculata.* These organisms were cultured under controlled laboratory conditions. Some aspects of the identified methods were modified over time to optimise the culture methods. These aspects were the type and amount of food, the culture medium, substrate composition and general husbandry methods.

RESULTS AND DISCUSSION

Aim 1

Three ecologically relevant organisms were identified in the literature review that could be used for sediment toxicity testing in South Africa. Additionally a fourth organism was incorporated. These organisms are; a *Hydra* sp., the Ostracod *Heterocypris incongruens,* a Chironomid sp., and the snail *Melanoides tuberculata*.

Aim 2

The culture methods for the *Hydra* sp., Chironomid sp. (*C. transvaalensis* and *C. caffrarius*) and the snail *Melanoides tuberculata* were developed and are detailed in this report. During the culturing process it was however decided that the Ostracods were too small to culture and thus a test kit would be easier to use for toxicity testing purposes. The *Hydra,* Chironomid and snail cultures were stable and the populations grew at a sufficient rate.

Aim 3

Due to insufficient capacity and laboratory space at the Department of Water Affairs (DWA), Directorate: RQS, it was not possible to transfer a culture and train staff about the culture maintenance methods. For this reason multiple cultures were maintained at the Golder Associates Research Laboratory, in order to have a culture ready to transfer in the future should sufficient capacity, space and equipment be available at the DWA

GENERAL

Overall results showed that Hydra, Chironomid and *Melanoides tuberculata* cultures were stable.

CONCLUSIONS

Toxicity testing is vital in the remediation of environments that have been compromised by anthropogenic activities. Sediment toxicity tests can indicate effects that would not be evident in other toxicity tests.

Suitable ecologically relevant organisms were identified that could be used for sediment toxicity testing in South Africa. The culture methods for the *Hydra* sp., Chironomid sp. (*C. transvaalensis* and *C. caffrarius*) and the snail *Melanoides tuberculata* were developed. During the culturing process, attempts to culture Ostracods (*Heterocypris incongruens*) were made, however it was later decided that they were too small to culture successfully and thus a test kit would be easier to use for toxicity testing purposes. The *Hydra,* Chironomid and snail cultures were stable and the populations grew at a sufficient rate.

RECOMMENDATIONS FOR FUTURE RESEARCH

The following recommendations can be made, to:

- Conduct preliminary sediment toxicity test with the cultured organisms in order to evaluate their use for sediment toxicity testing. These results can then be used to prioritise tests for validation purposes.
- Transfer cultures to other testing laboratories and train personnel on the culture procedures. This will aid in increasing the skilled capacity of the country and contribute to the National Toxicity Monitoring Programme of DWA, in the future.
- Identify the Hydra sp. used in this culture method.

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LIST OF ABBREVIATIONS

ASTM	American Society for Testing and Materials
DO	Dissolved Oxygen
DWA	Department of Water Affairs
EC	Electrical conductivity
Env.C	Environmental Canada
GARL	Golder Associates Research Laboratory
ISO	International Organization for Standardization
OECD	Organization for Economic Cooperation & Development
RQS	Resource Quality Services
SSHW	Standard Synthetic Hard Water
USEPA	United States Environmental Protection Agency
WQ	Water quality
WRC	Water Research Commission

1 INTRODUCTION

Organic and inorganic compounds accumulate in the sediment of the aquatic environment and can be released into the water column, to the detriment of the environment, when the physical parameters of the system change (Ho *et al.,* 2002). The effects of contaminants and toxicants on the terrestrial and aquatic environment have internationally been studied for more than 30 years, and over this period numerous laboratory tests have been developed to assess the potential impact that contaminants and toxicants may have on the environment (USEPA, 2004).

Organizations such as the United States Environmental Protection Agency (USEPA), Environmental Canada, the International Organization for Standardization, the Organization for Economic Cooperation and Development (OECD), and the American Association for Testing Materials have establish guidelines and test methods for assessing the toxicity of sediment through using invertebrates as test organisms (Env.C, 1997).

Giesy & Hoke (1989) listed the criteria for selecting assays for sediment toxicity test as follows:

- The assay should be rapid
- Simple to perform
- Reproducible
- Cost effective
- Standardized
- Ecologically relevant and sensitive

The advantages of using invertebrates as test organisms is that they are easy to handle and culture, they have short life spans, reproduce frequently and in great numbers, they are inexpensive to maintain and they are an important component of the aquatic environment (Lagadic & Caquet, 1998).

Even though sediment toxicity tests have been used for many years internationally, it is only now being incorporated into testing methods in South Africa. For this reason it is important to develop assays that will be ecologically relevant to the indigenous aquatic environment. Oberholster *et al.* (2008) conducted a study on water pollution in the Rietvlei nature reserve wetland in South Africa using a battery of tests (including amongst others the *Hydra attenuate* and *Daphnia magna* test) to assess pollution effects on the wetland.

In order to develop these assays it is vital to study the tests used internationally in the past. A few test organisms, the culture and test methods used globally have been summarized in the literature review.

The aim of this project was to successfully culture where possible, a South African species for sediment toxicity testing. If this was not possible, the use of international accepted test kits for sediment toxicity would have been considered and tested under South African conditions.

In the literature review for this project (WRC Project No K8/946, GARL 2010) the following sediment toxicity indicator organisms were identified (from studies conducted globally) as candidates for laboratory cultures and toxicity testing: *Vibrio fischeri* (Bacteria), *Selenastrum capricornutum* (Algae), *Spirostomum ambiguum* (Ciliated protozoa), *Daphnia magna*, *D. pulex* (Crustacea), *Thamnocephalus platyurus* (Crustacea), *Hydra vulgaris*, *H. varidissima* (Hydrozoa), *Heterocypris incongruens* (Ostracoda), *Hydropsyche angustipennis*, *Cyrnus trimaculatus* (Thrichoptera), *Chironomus riparius*, *C. tentans* (Diptera), *Lumbriculus variegates*, *Lumbricus terrestris*, *Eisenia andrei*, *E. fetida* (Oligochaeta), *Caenorhabditis elegans* (Nematoda) and *Hyalella azteca* (Amphipoda) and *Melanoides tuberculata* (Mollusc).

After careful consideration of both the positive and negative points associated with culturing the above named organisms under laboratory conditions, as well as their use for routine sediment toxicity tests, the following three organisms were chosen as preliminary culture candidates (*Hydra* sp., Ostracod sp., & Chironomid sp.) with the addition of a fourth organism (*Melanoides tuberculata*), which was not part of the original literature review.

2 EXPERIMENTS

2.1 Potential Indicator Organisms

The following is a literature review of the potential indicator organisms:

2.1.1 Vibrio fischeri (Bacteria)

Vibrio fischeri (Table 1) is a luminescent bacteria found globally in all marine environments. This bacteria has been cultured in laboratories and used for toxicity testing since the early 1970s. A micro-biotest, namely Microtox, was developed as a rapid test for evaluating the toxicity of sediment samples (Doe *et al.*, 2005).

2.1.2 *Selenastrum capricornutum* (Algae)

Selenastrum capricornutum (Table 2) have in the past been cultured in laboratories with difficulty. The Algaltoxkit F was developed as a rapid growth inhibition laboratory test for water toxicity testing (Mankiewics-Boczek *et al.*, 2008) but has also been applied to sediment toxicity tests and have shown to be a sensitive test species for contaminants (Burton, 1991).

2.1.3 Spirostomum ambiguum (Ciliated protozoan)

Spirostomum ambiguum (Table 3) is a 2-3 mm long benthic (Day & Moor, 2002) ciliate that lives in small ponds in Europe. They are primary consumers that contribute to water purification. Protozoa graze on organic matter and bacteria that grow on substrates (e.g. sediment), thus bringing them in contact with numerous toxicants in the environment. They are highly sensitive to chemicals and can be used to test sediment pore water for toxicity. This protozoan can easily be cultured in a laboratory. For more than an hundred years, *Spirostomum ambiguum* have been used as a test organism (Nlecz-Jawecki, 2005).

2.1.4 Daphnia magna & *Daphnia pulex* (Crustacea)

Daphnia magna and *D. pulex* (Table 4) are small (3.5-6 mm) filter feeding crustaceans that have globally been used as a toxicity test organism for many years. *Daphnia* sp. is commonly found in freshwater ponds or section of streams. They are easily cultured in laboratories and have a short life cycle (Env.C, 1990). *Daphnia* sp. is sensitive to chemicals and has been observed feeding on the sediment surface during sediment assays (Burton, 1991).

Table 1: Culture and test methods for *Vibrio fischeri* (Bacteria)

Test organism	***Vibrio fischeri* (Bacteria)**		**Reference**
South African sp.	*Vibrio fischeri*		
Source (Ref. Site)	N/A		
Test type	Acute, static		Mankiewicz *et al.*, 2008
Test format	Solid phases, cuvettes		Doe *et al.*, 2005
Nr. of organisms	As per Vibrio test method		
Test time	30 min for water and 20 min for soils		Mankiewicz *et al.*, 2008
Medium to test	Water, pore water of sediment/soil, solid phase tests		Burton *et al.*, 2001
Test configuration	12 test concentrations and 3 controls. Disposable glass cuvettes		
Endpoints	Bioluminescence inhibition		Jennings *et al.*, 2001
Advantages	Rapid results, small sample volume, requires little bench space		
Disadvantages	Sample turbidity, salinity interferences, high and low pH, volatile samples		ISO, 2007
	Culture Requirements	**Test Requirements**	
Food	*	*	Doe *et al.*, 2005
Temperature	*	15 ± 0.5°C	
Light	*	Ambient	
pH	*	As per sample	
Hardness	*	As per sample	
Salinity	*	As per sample	
Dissolved O_2%	*	As per sample	
WQ Parameters	*	Temperature	
Culture procedure	Laboratory culture: Sterile cultures are streaked on plates containing culture medium every second week. After visual observation the brightest colonies are transferred to screw cap tubes. Tubes are incubated at 15°C until good growth is obtained. Tubes are closed and stored at 4°C Microtox Purchase freeze dried bacteria		Eley, 1972; Doe *et al.*, 2005; ISO, 2007
Test protocol	1.5 mℓ test solution, 20 µl reconstitute bacteria. Read initial illumination. Add sample – read at 5 min intervals		Eley, 1972
Equipment	Incubator, Luminometer, glass cuvettes , freezer, pipettes, test tubes, pH meter.		Doe *et al.*, 2005; ISO, 2007
Ref. toxicants	Certified reference sediment, $ZnSO_4$ & $K_2Cr_2O_7$		Doe *et al.*, 2005; ISO, 2007
Used by	Environmental Canada		Env.C, 1992

*No information available at time of Literature search

Table 2: Culture and test methods for *Selenastrum capricornutum* (Algae)

Test organism	***Selenastrum capricornutum* (Algae)**		**Reference**
South African sp.	N/A		
Source (Ref. Site)	N/A		
Test type	Acute ,chronic, static		
Test format	96 well micro plate		Blaise & Vasseur, 2005
Nr. of organisms	4-8 day old cells, 10000 cells/mℓ		Blaise & Vasseur, 2005
Test time	72/96 h		Mankiewicz *et al.*, 2008
Medium to test	Groundwater, sediment, wastewater,		Blaise & Vasseur, 2005
Test configuration	10 serial dilutions, 5 replicates, 10 controls		Blaise & Vasseur, 2005
Endpoints	Growth inhibition		Mankiewicz *et al.*, 2008
Advantages	Small sample volume, requires little bench space		
Disadvantages	Colored samples, High salt concentrations, volatile substances		Slabbert, 2004
	Culture Requirements	**Test Requirements**	
Food	Culture medium	Algal stock culture medium	Weber, 1993; Hall & Golding,1998; Slabbert, 2004; EC, 2007
Temperature	24 ± 2°C	24 ± 2°C	
Light	200 µmol m^{-2} s^{-1} PAR	24 h fluorescent illumination	
pH	7.5°±1	As obtained	
Hardness	*	*	
Salinity	*	*	
Dissolved O_2%	*	As obtained	
WQ Parameters			
Culture procedure	Algaltoxkit F , Prepare algae according to method		Algaltoxkit F, 1996;
Test protocol	10,000 ±1000 cells/mℓ, 200 µl test solution, 10 µl nutrient spike, 10 µl algae inoculums/well. 24 h fluorescent illumination For sediment: 2 g sediment, 20 mℓ algae, Inoculate solution, seal, vertical rotator 24 h, rest 5 min, 2 mℓ supernatant for reading		Blaise & Vasseur, 2005
Equipment	Incubator, cuvettes, pipettes, rrefrigerator, spectrophotometer. pH meter, beakers, centrifuge ,volumetric flasks		Blaise & Menard, 1998
Ref. toxicants	Zn^{2+} ($ZnSO_4$), Cu^{2+} ($CuCl_2$), $K_2Cr_2O_7$ or NaCl		Blaise & Vasseur, 2005
Used by	USEPA		Env.C, 2007

* No information available at time of Literature search

Table 3: Culture and test methods for *Spirostomum ambiguum* (Ciliated protozoa)

Test organism	***Spirostomum ambiguum, S. teres* (Ciliated protozoa)**		**Reference**
South African sp.	N/A		
Source (Ref. Site)	N/A		
Test type	Acute, static		Nalecz JaWecki, 2005
Test format	24 well micro plate (6x4 wells)		Nalecz JaWecki, 2005
Nr. of organisms	10 per well		Nalecz JaWecki, 2005
Test time	2, 24, 48 and 96 h		Nalecz JaWecki, 2005
Medium to test	Organic compounds, effluent, leachates, pore water, volatiles, pesticides		Nalecz JaWecki, 2005
Test configuration	Screenings. Definitive: 5 dilutions (3 replicates)		Nalecz JaWecki, 2005
Endpoints	Sub lethal effects (deformities, shortening, immobilization) and lethal		Nalecz JaWecki, 2005
Advantages	Short life cycle. Easy to culture. Susceptible to toxicants. Standard.		Nalecz JaWecki, 2005
Disadvantages	Culture medium is expensive, difficult to culture a large population under sterile medium conditions.		Twagilimana *et al*., 1998.
	Culture Requirements	**Test Requirements**	
Food	Flaked oats and alder leaves (50:1)	No feeding	Nalecz JaWecki, 2005; Le Du-Delepierre *et al.,* 1996
Temperature	15-25°C	25 ± 2°C	
Light	Darkness	*	
pH	5-8	*	
Hardness	0.3-250 mg/ℓ	*	
Salinity	3-1100	*	
Dissolved O_2%	0-100	*	
WQ Parameters	*		
Culture procedure	250 mℓ beaker, 200 mℓ water, 200 mg food. Cover, leave for 2 days add protozoan. Add 100 mg food/week. After 4 weeks add 600 mℓ culture water, 400 mg food to a 1 ℓ beaker. Cover and leave for 2 days. Add protozoans from 250 mℓ beakers. Once a week add 100 mg food per week.		Nalecz JaWecki, 2005
Test protocol	Transfer protozoans to 25 mℓ graduated cylinder. Add 20 mℓ sample per well		Nalecz JaWecki, 2005
Equipment	Balance, magnetic stirrer. Thermometer, beakers, volumetric flasks, pipettes, dissection microscope, graduated cylinders refrigerator.		
Ref. toxicants	Cd^{2+} as $Cd(NO_3)_2$, Zn^{2+} as $ZnSO_4$, sodium dodecyl sulphate		Nalecz JaWecki, 2005
Used by	Municipal water works in Poland, Medical University of Warsaw		Nalecz JaWecki, 2005

* No information available at time of Literature search

Table 4: Culture and test methods for *Daphnia magna* and *D. pulex* (Crustacean)

Test organism	***Daphnia magna, D. pulex* (Crustacean)**		**Reference**
South African sp.	*D. magna, D. pulex*		
Source (Ref. Site)	*D. pulex* obtained from the University of Johannesburg		
Test type	Acute, static, chronic		Env.C, 1990
Test format	Beakers and test cups/multi-wells		
Nr. of organisms	10 neonate organisms per concentration		Env.C, 1990
Test time	48 h		Env.C, 1990
Medium to test	Effluent, elutriate, chemicals, receiving water		Env.C, 1990
Test configuration	Screening and definitive, 3 replicates		Env.C, 1990
Endpoints	Mortality, immobility		Jonczyk & Gilron, 2005
Advantages	Fast, little space needed		Env.C, 1990; Daphnotox F, 1996
Disadvantages	Dark colored samples may impede observations, oil in the sample may cause organisms to float		Slabbert, 2004
	Culture Requirements	**Test Requirements**	
Food	Algae daily/formulated food	No feeding	Env.C, 1990;
Temperature	20 ± 2°C	20 ± 2°C	
Light	16 h Light: 8 h Dark, 40-800 Lux	16 h Light: 8 h Dark, 40-800 Lux	
pH	6.5-8.5	6-8.5	
Hardness	80-250 mg/l	*	Jonczyk & Gilron; 2005 USEPA,
Salinity	*	≤10‰ (1150 mS/m)	2000
Dissolved O_2%	60-100	40-100	
WQ Parameters	Temperature, DO, pH, every day	Temperature, DO, pH,	Env.C, 1990
Culture procedure	Laboratory culture; Reconstituted water. Glass culture vessel. *Daphnia* food according USEPA method. Replace culture water weekly Daphnia Kit: Hatch ephippia according to the Daphnotox F procedures		Daphnotox F, 1996; Env.C, 2000 USEPA, 2000
Test protocol	No aeration.		Daphnotox F, 1996
Equipment	Glass aquariums, Graduated cylinders. Pasteur pipettes. Volumetric flasks Petri dishes. Beakers. Dissection microscope. Pipettes		Jonczyk & Gilron, 2005
Ref. toxicants	Zn^{2+} ($ZnSO_4$. $7H_20$), $K_2Cr_2O_7$, NaCl & $CdCl_2$		Slabbert 2004; Env.C, 2000
Used by	Env.C, USEPA, ASTM, ISO, OECD		Env.C, 2000

* No information available at time of Literature search

2.1.5 Thamnocephalus platyurus (Crustacea)

Thamnocephalus platyurus (Table 5) commonly known as fairy shrimp occurs in freshwater pans in the United States of America. They sexually reproduce and release dormant eggs (cysts) into their environment. If the conditions are favourable these cysts will hatch, but will stay dormant for many years if the conditions are unfavourable. The cysts can hatch within 4-6 hours after being rehydrated (Brauschand & Smith, 2009).

2.1.6 *Hydra vulgaris and H. viridissima* (Cnidaria)

Hydra sp. (Table 6) are freshwater micro-invertebrates that can reproduce sexually or asexually depending on environmental conditions (Hickman *et al*., 2001). Numerous Hydra species occur across the world and have been used for metal toxicity tests in water and effluent. Hydras can easily be cultured under laboratory conditions. Hydras attach to plants and substrates e.g. sediment and are exposed to contaminants in both the water and sediment (Holdway, 2005).

2.1.7 Heterocypris incongruens (Ostracoda)

Ostracods (Table 7) are benthic macro-invertebrates and occur in both fresh and marine environments. They are scavengers and detritus feeders (Day *et al.*, 2001). Ostracods can reproduce sexually as well as asexually (Day *et al*., 2001). Neonates can be hatched form dormant cysts supplied in the Ostracodtoxkit F (2001) and are used to determine the toxicity of sediment samples (Oleszczuk, 2008).

2.1.8 Hydropsyche angustipennis and *Cyrnus trimaculatus* (Trichoptera - Caddis flies)

Hydropsyche angustipennis and *Cyrnus trimaculatus* (Table 8) are case-less caddis flies and have a semi aquatic life cycle. The caddis flies are mainly considered to be carnivores, but in the past it has been noted that they will also eat plant material. Larvae spin nets in-between hard substrates (e.g. sediment) in order to collect detritus and other small invertebrates; they have also been known to scrape food off the substrate (Greve *et al.*, 1998) and aid in the decomposition of organic material in the river system (Van der Geest *et al.*, 1999).

Table 5: Culture and test methods for *Thamnocephalus platyurus* (Crustacean - Shrimp)

Test organism	***Thamnocephalus platyurus*** **(Crustacean - Shrimp)**		**Reference**
South African sp.	N/A		
Source (Ref. Site)	NA		
Test type	Acute, static		Mankiewicz *et al.*, 2008
Test format	24 x multi-well test plates		Mitchell *et al*., 2002
Nr. of organisms	10x 2^{nd} -3^{rd} instar larvae per well		Thamnotoxkit F, 2005
Test time	Rapidtoxkit F: Feeding inhibition (60 min), Thamnotoxkit: Mortality (24h)		Rapidtoxkit F; 2000; Thamnotoxkit F,1995
Medium to test	Pure Compounds, Effluents, Sediments, Surface and Ground Waters, Wastewaters, pore water		Burton *et al*, 2001
Test configuration	3 replicates, definitive		Thamnotoxkit F, 1995
Endpoints	Mortality , feeding inhibition		Mankiewicz *et al.*, 2008
Advantages	Short testing time, minimal equipment needed		
Disadvantages	Kits are expensive and have a short shelf life		Chorus & Bartram, 1999
	Culture Requirements	**Test Requirements**	
Food	Liquid soya, *Spirulina* powder, fish oil, yeast	No feeding	Maeda-Martinez *et al.*, 1995
Temperature	20-25°C	*	
Light	2x fluorescent lamps (30Watt)	*	
pH	7	*	
Hardness	*	*	
Salinity	*	*	
Dissolved O_2%	*	*	
WQ Parameters	*	*	
Culture procedure	Hatching medium 1:8 (fresh water: dH20). 10 mℓ hatching medium/Petri dish, add cysts. Cover the hatching Petri dish. Incubate at 25°C for 20-22 hours Continuous light (3000-4000 Lux), 100 ℓ tank with tap water, aquarium filter Flow through system. Heater. 20 Watt fluorescent lamp		Thamnotoxkit F, 1995; Maeda-Martinez *et al.*, 1995
Test protocol	Place organisms in wells with sample. Cover with Parafilm. Incubate for 24 h at 25°C. in darkness. Calculate the mortality		Thamnotoxkit F, 1995.
Equipment	Incubator, fluorescent lamp, water pump, incubator, pipettes, filters, heaters, microscope		Maeda-Martinez *et al.*, 1995
Ref. toxicants	K_2CrO_7		Thamnotoxkit F, 1995
Used by	Canada		CCME, 2006

* No information available at time of Literature search

Table 6: Culture and test methods for *Hydra vulgaris* and *H. viridissima* (Cnidaria)

Test organism	***Hydra vulgaris, H. attenuata* & *H. viridissima*(Cnidaria)**		**Reference**
South African sp.	*Hydra* sp.		
Source (Ref. Site)	Magalies river		
Test type	Acute/chronic, Semi- static		Rosenkrantz, 2008
Test format	Petri dish/micro-plate		Holdway, 2005
Nr. of organisms	5 budding Hydras per plate		Holdway, 2005
Test time	7 days , or 96 h static bioassay		Holdway, 2005
Medium to test	Water, elutriate		Holdway, 2005
Test configuration	Screening		Oberholster *et al.*, 2008
Endpoints	Sub lethal (change in structure) and mortality		Arkhipchuk *et al*., 2006
Advantages	Rapid, cost effective, ease of culture		Holdway, 2005
Disadvantages	*		
	Culture Requirements	**Test Requirements**	
Food	Daphnia or Artemia	No food	
Temperature	25 ± 1°C	25 ± 1°C	Holdway, 2005; Karntanut & Pascoe, 2002
Light	16 h Light: 8 h Dark	*	
pH	7.8	*	
Hardness	209 mg/ℓ	*	
Salinity	498 µS/cm	*	
Dissolved O_2	6.9 mg/ℓ	*	
WQ Parameters	*	pH, temperature, EC	
Culture procedure	Litoralis medium or aged tap water		Galen, 1969
Test protocol	Renew solution daily. 35 mℓ sample/container		Holdway, 2005
Equipment	1 ℓ glass bowls. Beakers. Reconstituted water. Dissection microscope Air pump		Holdway, 2005
Ref. toxicants	4-chlorophenol		Pollino & Holdway,1999
Used by	Environment Canada		

* No information available at time of Literature search

Table 7: Culture and test methods for *Heterocypris incongruens* (Ostracoda)

Test organism	***Heterocypris incongruens* (Ostracoda)**		**Reference**
South African sp.,	*Heterocypris* sp.		
Source (Ref. Site)	Magalies river		
Test type	Acute/chronic, static		Mitchell *et al.*, 2002
Test format	Multi-well test plates/cups		Belgis *et al.*, 2003
Nr. of organisms	10 freshly hatched neonates per well		Belgis *et al.*, 2003
Test time	6 days		Ostracod toxkit F, 2001
Medium to test	Sediment, pore water		Ostracod toxkit F, 2001
Test configuration	6 replicates		Chial & Persoone, 2003
Endpoints	Mortality and growth inhibition		Ostracod toxkit F, 2001
Advantages	Easy and quick, low cost		Belgis *et al.*, 2003
Disadvantages	*		
	Culture Requirements	**Test Requirements**	
Food	Spirulina	Pre-feeding with Spirulina	Oleszczuk, 2008; Belgis *et al.*, 2003;
Temperature	25°C	25°C	
Light	*	Dark	
pH	6- 9.3	*	Yu *et al.*, 2009
Hardness	*	*	
Salinity	*	*	
Dissolved O_2%	*	*	
WQ Parameters	*	*	
Culture procedure	Place cysts in Petri dish. Cover and incubate at. 25°C for 52h under continuous illumination		Chial & Persoone, 2003
Test protocol	10 Ostracods per test cup. 1000 µl sediment, 2 mℓ water. Cover with Parafilm and Incubate at 25°C in darkness		Ostracod toxkit F, 2001
Equipment	Volumetric flasks, Refrigerator. Parafilm, Microscope. Petri dishes. Incubator, Test cups		Ostracod toxkit F, 2001
Ref. toxicants	$K_2Cr_2O_7$		Ostracod toxkit F, 2001
Used by	*		

* No information available at time of Literature search

Table 8: Culture and test methods for *Hydropsyche angustipennis* and *Cyrnus trimaculatus* (Trichoptera)

Test organism	***Hydropsyche angustipennis and Cyrnus trimaculatus* (Trichoptera)**		**Reference**
South African sp.	Hydropsychidae species		
Source (Ref. Site)	Elands river		
Test type	Acute, static		Van der Geest *et al.*, 1999
Test format	Glass vials containing water and sample in duplicate		Van der Geest *et al.*, 2000
Nr. of organisms	20 x 12 day old instar larvae		Greve *et al.*, 1998
Test time	7 days/96 h		Van der Geest *et al.*, 2000
Medium to test	Water, sediment		Greve *et al.*, 1998
Test configuration	Definitive		Van der Geest *et al.*, 2000
Endpoints	Survival		Greve *et al.*, 1998
Advantages	*		
Disadvantages	*		
	Culture Requirements	**Test Requirements**	
Food	Urtica, algae, fish food & Daphnia	Urtica	Greve *et al.*, 1998.
Temperature	20°C	20°C	
Light	16h Light :7 h Dark, 2 x 30 min twilight	16 h Light: 8 h Dark	Van der Geest *et al.*, 2000
pH	*	*	
Hardness	*	*	
Salinity	*	*	
Dissolved O_2%	*	*	
WQ Parameters	*	*	
Culture procedure	Egg masses in rearing container, 30 ℓ water, 2 cm stones. Flow through system. Renew water every two weeks. Fed 1 mℓ suspended 150 mg Urtica, 75 mℓ. Trouvit,40 mg Tetraphyll and 2.5 mℓ fresh algae in 25 mℓ water		Greve *et al.*, 1998. Van der Geest *et al.*, 2000
Test protocol	180 mℓ beakers, 100 mℓ water, 1.25 g Urtica/25 mℓ water		
Equipment	Glass aquariums. Glass beakers. Stones & silica sand		Van der Geest *et al.*, 2000
Ref. toxicants	$CuCl_2$		Van der Geest *et al.*, 2000
Used by	*		

* No information available at time of Literature search

2.1.9 Chironomus riparius and *Chironomus tentans* (Diptera)

The non-biting freshwater midge, *Chironomus riparius* (Table 9) occurs commonly in the Northern hemisphere. They reproduce sexually (Env.C, 1997). The larvae feed on detritus and live in tubes that they construct and are thus in close contact with the sediment substrate. *Chironomus* species are sensitive to sediment contaminants. These characteristics make them a suitable test organism for assessing the toxicity of sediment (Pery *et al.*, 2005). Laboratory based sediment toxicity tests have been conducted by American, European and Canadian scientists for numerous years (Env.C, 1997).

2.1.10 Lumbriculus variegates, *Lumbricus terrestris*, *Eisenia andrei* and *E. fetida* (Oligochaete)

These Oligochaete (Table 10 & Table 11) worms occur widely throughout the America and Europe and are considered ecologically relevant in terms of toxicity tests, the reason for this being that they ingest sediment particles and burrow into the substrate. In this way they are exposed to contaminants in their environment (Vandegehuchte *et al.*, 2007).

2.1.11 Lumbriculus variegates (Oligochaete)

Eisenia andrei, *E. fetida* and *Lumbricus terrestris* are terrestrial hermaphroditic Oligochaetes (Hickman *et al*., 2001; Hurdzan *et al.*, 2011) whereas *Lumbriculus variegate* is benthic aquatic Oligochaete that reproduces asexually (USEPA, 2000; Vandegehuchte *et al.*, 2007).Oligochaetes form an important part of the food web feeding on detritus and micro-fauna, they are also food for higher organisms (Invertebrates, fish and birds). In this way, persistent chemicals are transferred up the food chain (Williams, 2005). They inhabit shallow aquatic habitats and are sensitive to mesotrophic conditions (Dermott & Munawar, 1992).

2.1.12 Caenorhabditis elegans (Nematoda)

A vast number of nematode species occur worldwide with *Caenorhabditis elegans* (Table 12) being one of the best studied species (Khanna *et al.*, 1997). *Caenorhabditis elegans* lives in soil and its lifespan is short (3 weeks). *Caenorhabditis elegans* is sensitive to chemicals and organic compounds and has been used in the past to test the toxicity of sediment and water samples (Comber *et al.*, 2006; Yunhui *et al.*, 2009).

Table 9: Culture and test methods for *Chironomus riparius* and *C. tentans* (Diptera)

Test organism	***Chironomus riparius*, *C. tentans* (Diptera)**		**Reference**
South African sp.	*Chironomus transvaalensis* & *C. caffrarius*		
Source (Ref. Site)	Aquaculture		
Test type	Acute, static or intermittent renewal		Env.C, 1997; Pery *et al.*, 2005
Test format	Solid-phase assay		
Nr. of organisms	10 x 2nd instar larvae per beaker 1st instar larvae		Pery *et al*, 2005; Env.C, 1997
Test time	7 days		Pery *et al.*, 2005
Medium to test	Sediment		
Test configuration	Definitive		OECD, 2004
Endpoints	Growth, survival and Immobilization		Pery *et al.*, 2005,; OECD,2011
Advantages	Easy to handle and culture. Short life cycle. Requires little bench space		USEPA, 2004.
Disadvantages	Different response between the two species with the same toxicant		USEPA, 2000
	Culture Requirements	**Test Requirements**	
Food	1.5 mg Tetra-Min/larvae d	3.75 g Tetra-Min/day chamber	Env.C, 1997;
Temperature	21 ± 1°C, 23 ± 1°C	22 ± 1°C	
Light	16 h Light: 8 h Dark, 500-1000 Lux	16 h Light: 8 h Dark	Pery *et al.*, 2005
pH	7.8-8.2	*	
Hardness	90-100 mg/ℓ	*	
Salinity	330-360 µS/cm	*	
Dissolved O_2%	≥ 80%	*	
WQ Parameters	pH, hardness & ammonia	EC, temperature, pH, nitrates, DO, ammonia	Pery *et al.*, 2005
Culture procedure	20 ℓ closed aquarium, 2 cm silica sand. Aeration. Water renewal system, ± 200 larvae/20 ℓ culture tank, 7.5 ℓ water. 300 mg Tetra-Min/20 ℓ.d. Intermittent-renewal or continuous-flow, 25% water change per week.		Env.C, 1997 Pery *et al.*, 2005
Test protocol	Prior to test beakers are placed in water bath at 21 ± 1°C. 0.6 ℓ beakers 10 organisms/beaker. Sediment: water (1:4), 1.5-3 cm 300 mℓ glass beakers 100 mℓ sediment 175 mℓ water 5 replicates		Env.C, 1997
Equipment	3 x 20 ℓ aquariums, Water renewal system. Water bath. Silica sand or paper towel, Aeration. Beakers		Pery *et al.*, 2005
Ref. toxicants	Cu acute test		Pery *et al*., 2005
Used by	Environment Canada and USEPA		

* No information available at time of Literature search

Table 10: Culture and test methods for *Eisenia andrei*, *E. fetida* and *Lumbricus terrestris* (Oligochaete)

Test organism	***Eisenia andrei, E. fetida and Lumbricus terrestris* (Oligochaete)**		**Reference**
South African sp.	*Eisenia fetida*		
Source (Ref. Site)			
Test type	Static		Env.C, 2004
Test format	Whole sediment toxicity test,		Env.C, 2004
Nr. of organisms	5 worms per chamber (wet weight 3-10g) for *Eisenia* spp.3 for *L. terrestris*		Env.C, 2004
Test time	14 days		Env.C, 2004
Medium to test	Sediment/pore water		Env.C, 2004
Test configuration	Definitive: 5 concentrations (5 replicates)		OECD, 2006
Endpoints	Survival on day 7 and 14		Env.C, 2004
Advantages	Breeds readily in culture relatively large and easy to handle.		Addison, 2002
Disadvantages	Requires a pH of -7.6 to reproduce		Addison, 2002
	Culture Requirements	**Test Requirements**	
Food	Cooked oatmeal/alfalfa ones a week	No feeding	Env.C, 2004
Temperature	20 ± 2°C	20 ± 2°C	
Light	16 h Light: 8 h Dark , 400-800 Lux	16 h Light: 8 h Dark	
pH	6.0-7.5	*	
Hardness	*	*	
Salinity	*	*	
Dissolved O_2%	*	*	
WQ Parameters	*	pH, DO, ammonia, temperature	OECD, 2006
Culture procedure	Breeding box:25 ℓ capacity, covered with transparent perforated lid Substrate: 3 ℓ potting soil, 4 ℓ peat moss, 2 ℓ dH_2O,1.5 ℓ artificial soil. Monitor substrate temperature, pH and moisture content every week. Renew substrate every 2 months.		Env.C, 2004 Egeler *et al.*, 2006
Test protocol	500 mℓ glass jar. Perforated parafilm cover. 350 mℓ soil		
Equipment	Breeding box. Glass beakers. Centrifuge for pore water		Env.C, 2004; OECD, 2006
Ref. toxicants	KCl , CuSO4		OECD, 2008
Used by	Environment Canada, USEPA, ASTM, OECD, ISO		Env.C, 2004

* No information available at time of Literature search

Table 11: Culture and test methods for *Lumbriculus variegates* (Oligochaete)

Test organism	***Lumbriculus variegates* (Oligochaete)**		**Reference**
South African sp.	*		
Source (Ref. Site)	Elands river		
Test type	Static, acute		Sheedy *et al.*, 1998
Test format	2 mg live weight) per chamber/beaker		Dermott & Munawar, 1992
Nr. of organisms	15 per beaker (15 mm length worms)		Dawson *et al.*, 2003
Test time	28 days		Sheedy *et al.*, 1998
Medium to test	Sediment & water		Vandegehuchte *et al.*, 2007
Test configuration	5 replicates		Sheedy *et al*, 1998
Endpoints	Growth, survival & avoidance		USEPA, 2004
Advantages	Easy handling and culturing, tolerance to various experimental conditions, The organism ingest particles and has a high ecological relevance		Sheedy *et al.*, 1998
Disadvantages	Long testing time		USEPA,2004
	Culture Requirements	**Test Requirements**	
Food	200 µg Tetra-Min/organism day	No feeding	Sheedy *et al.*, 1998; Vandegehuchte *et al.*, 2007; Leppanes & Kukonen, 1998; Ingersoll *et al.*, 2003;
Temperature	23 ± 2°C	20 °C	
Light	16 h Light: 8 h Dark	16 h Light: 8h Dark	
pH	7.8-8.4	6.5	
Hardness	43-44 mg/ℓ	*	
Salinity	*	*	
Dissolved O_2%	7.1- 8.1 mg/ℓ	*	
WQ Parameters	*	DO, pH and temperature	Dawson *et al.*, 2003
Culture procedure	Same method as described for *Eisenia spp.above*		
Test protocol	1 ℓ glass beakers, 60 organisms/beaker, 500 mℓ test solution, renewed daily, 400 g sediment		
Equipment	Paper towel, aerator, silicon sand, Glass beakers. Glass pipettes		Dermott & Munawar, 1992
Ref. toxicants	KC or $CuSO_4$		OECD, 2006
Used by	USEPA, Canada centre for inland waters		Dermott & Munawar, 1992

* No information available at time of Literature search

Table 12: Culture and test methods for *Caenorhabditis elegans* (Nematode)

Test organism	***Caenorhabditis elegans* (Nematode)**		**Reference**
South African sp.	*Caenorhabditis briggsae*		
Source (Ref. Site)			
Test type	Acute/Chronic, Static		Khanna *et al*., 1997
Test format	Petri dishes (35 x 10 mm)		Peredney, 2004
Nr. of organisms	10 x 3-4 days old per chamber		Peredney, 2004
Test time	24/48/96 h		Peredney, 2004
Medium to test	Sediment		
Test configuration	3 replicates		Peredney, 2004
Endpoints	Mortality		Peredney, 2004
Advantages	Short life span, ease of culturing		Ura *et al*., 2002
Disadvantages	*		
	Culture Requirements	**Test Requirements**	
Food	Nematode growth medium & *E. coli*	*	Yunhui *et al.*, 2009; Peredney, 2004; Khanna *et al*., 1997;
Temperature	*	20± 2°C	
Light	*	none	
pH	7 ± 0.5	3.1-11.9	
Hardness	0.236 to 0.246 g/ℓ of $NaHCO_3$	*	
Salinity	Up to 15.46 g/ℓ NaCl and 11.51 g/ℓ KCl	*	
Dissolved O_2%	*	*	
WQ Parameters	*	*	
Culture procedure	Artificial soil: peat moss (10%), Silica sand 70 mesh grade (70%), $CaCO_3$ 40 um Kaolin clay (20%). After mixing add $CaCO_3$ equal to 0.40% of total weight. Add 56 mℓ water/125 g of soil to hydrate		Peredney, 2004
Test protocol	125 g dry sediment, (total amount), 2.33 g per replicate		
Equipment	100 mm Petri dishes. Containers. Volumetric flask. Scale.1-100 mℓ pipettes. Graduated cylinders. Stereo microscope		Peredney, 2004
Ref. toxicants	$CuCl_2$		Peredney, 2004
Used by	Washington State Department of Ecology		Peredney, 2004

* No information available at time of Literature search

2.1.13 Hyalella azteca (Amphipoda)

Hyalella azteca (Table 13) is a 6-8 mm amphipod which commonly occurs in North and South America. It has been used as a laboratory test organism in not only America but in numerous countries in Europe and Asia. They reproduce sexually (USEPA, 2000). This amphipod is a detritus feeder and lives in close contact with the sediment (Borgman *et al.*, 2005) in its environment (springs, rivers and marshes), for this reason *Hyalella* is considered a useful test organism for toxicity tests. In comparison with other amphipods, the *Hyalella* species is very sensitive to toxicants (Borgmann *et al.*, 2005).

2.1.14 Melanoides tuberculata

The snail *Melanoides tuberculata* is a freshwater mollusc (Bolaji *et al*., 2011) considered to be one of the most common and wide spread snails occurring in warm freshwater environments. They reproduce Parthenogenic.This species of snail has a distribution range covering Africa, southern Asia, Australia, central America and the southern parts of Europe (Ben-Ami & Hodgson, 2005). It is regarded as a native species of Africa. This snail can be found in brackish and marine environments with salinity levels as high as 33‰ (Bolaji *et al*., 2011). *Melanoides tuberculata* have been used in Biomarker, Bioaccumulation (Wepener *et al.,* 2005, Moolman *et al.,* 2007), pesticide analysis (Giovanelli *et al*., 2002) and toxicity test with blue green algae toxins in the past (Kinnear *et al*., 2007).

Table 13: Culture and test methods for *Hyalella azteca* (Amphipod)

Test organism	***Hyalella azteca* (Amphipod)**		**Reference**
South African sp.	Paramelidae		Day *et al.*, 2001
Source (Ref. Site)	*		
Test type	Acute, static		Env.C, 1997
Test format	Imhoff settling cones/beakers		Borgman *et al.*, 2005
Nr. of organisms	15 x 2-9 day old juveniles per container		Borgman *et al.*, 2005
Test time	Chronic test- 4 weeks,		Borgman *et al.*, 2005
Medium to test	Sediment and aqueous extracts (pore water & elutriates)		Env.C, 1997
Test configuration	4 replicates		USEPA, 2001
Endpoints	Survival and growth		Borgman *et al.*, 2005
Advantages	Ease of culturing and handling. Rapid growth. Sensitive to contaminates		Env.C, 1997
Disadvantages	*		
	Culture	**Test**	
Food	5 mg Tetra-Min per week/container	5 mg Tetra-Min 2x/week	Borgman *et al.*, 2005; USEPA, 2001
Temperature	23-25°C	23°C	
Light	16 h Light: 8 h Dark, 500 Lux	16 h Light: 8 h Dark 200 Lux	
pH	8-8.4	*	
Hardness	126 mg/ℓ	*	
Salinity	≤10‰	*	
Dissolved O_2%	90-100%	*	
WQ Parameters	*	Salinity, EC, DO, pH, and ammonia	USEPA, 2001
Culture procedure	30 adults in 2 ℓ container, 1 ℓ water, 5 x10 cm sterile cotton gauze strips. Once a week- shake animals off, filter, through 650 and 275 µm mesh to separate adults and young. Clean culture jars and return adults.		USEPA, 2001; Borgman *et al.*, 2005
Test protocol	2.3 cm sediment, 1 ℓ water/cone. Top up with dH_2O. Aerate gently. Equilibrate 1-2 weeks prior to test. 300 mℓ beakers, 100 mℓ sediment, 175 mℓ water per beaker. Automated renewal system		Borgman *et al.*, 2005
Equipment	Activated charcoal filter, silicone stopper, 2 ℓ containers, 250 µl pipette, tips, gauze, aerator, snap lids, cones, beakers		Borgman *et al.*, 2005
Ref. toxicants	96h sodium dodecyl sulphate (LC 50 – 0.4 mg/l)		USEPA, 2001
Used by	ASTM, USEPA, Environment Canada		Env.C, 1997

* No information available at time of Literature search

Table 14: Culture and test methods for *Melanoides tuberculata* (Snail) gastropod

Test organism	***Melanoides tuberculata***		**Reference**
South African sp.	*Melanoides tuberculata*		
Source (Ref. Site)	*		
Test type	Biomarker, Bioaccumulation, survival		
Test format	*		
Nr. of organisms	10 per cup		
Test time	*		
Medium to test	Sediment		
Test configuration	*		
Endpoints	Mortality & growth inhibition		
Advantages	*		
Disadvantages	*		
	Culture	**Test**	
Food	Shredded lettuce, algae	No food	Shuhaimi-Othman *et al.,* 2012;
Temperature	18-32ºC	18-32ºC	
Light	12 h Light: 12 h Dark	12 h Light: 12 h Dark 334-376 Lux	Ellis-Tabanor & Hyslop, 1997
pH	7.9-8.4		
Hardness			
Salinity			
Dissolved O_2%			
WQ Parameters		pH, EC, and DO	Shuhaimi-Othman *et al.,* 2012
Culture procedure	50 ℓ tank, aged tap water, aeration		Shuhaimi-Othman *et al.,* 2012
Test protocol	*		
Equipment	*		
Ref. toxicants	*		
Used by	*		

No information available at time of Literature search

2.2 Selected culture organisms

After careful consideration of both the positive and negative points associated with culturing the nineteen organisms identified in the literature review that can be cultured under laboratory conditions, and used for routine sediment toxicity tests, the following organisms were chosen as preliminary culture candidates:

- A *Hydra* sp.,
- An Ostracod sp.,
- A Chironomid sp.; and
- A Mollusc, *Melanoides tuberculata*

The selected culture organisms were selected for various reasons including ease of culturing, availability and usability.

2.2.1 Hydra (Hydrozoa)

Hydras are easy to culture as they only require a sufficient medium containing the most basic ions and an adequate supply of food. Little bench space is required for their culture containers which makes the culturing of Hydras very cost effective under laboratory conditions (Holdway, 2005). A stable culture proliferates rapidly if they are fed on a regular basis. *Hydra* spp. have been used internationally to test water, elutriate (Holdway, 2005) as well as sediment toxicity (Rosenkrantz *et al.*, 2008).

2.2.2 Ostracoda

Ostracods are easy to culture under laboratory conditions. They require a small amount of medium and feed on small food particles. An Ostracod culture does not take up a lot of bench space thus reducing the costs associated with regular culturing methods (Belgis *et al.*, 2003).

Ostracods lay numerous eggs at a time and thus the population in a culture can increase rapidly. In nature Ostracods are constantly in contact with the sediment (Day *et al.*, 2001) due to their feeding behaviour, and thus they have been used in the past for sediment toxicity testing. An Ostracod test kit is also available internationally (Ostracod toxkit F, 2001).

2.2.3 Chironomids

Chironomids have been cultured under laboratory conditions internationally for sediment toxicity tests (Pery *et al.*, 2005) with relative ease. They can be cultured in any type of container and require a substrate to live in. Due to their size, Chironomids are easy to

handle. They have a short life cycle and can produce egg sacs that can contain hundreds of eggs. Little bench space is needed for Chironomid culturing, thus reducing the costs (USEPA, 2004).

Chironomids are in direct contact with the substrate as they build tubes from small particles and will borrow into the substrate (Pery *et al.*, 2005). Their feeding habits also expose them to possible contaminants that may be present in the substrate, thus making them a suitable culture organism for sediment toxicity testing.

Another attribute of using Chironomids for sediment toxicity testing is that they can also be used for bioaccumulation (Roulier *et al.*, 2008) and biomarker studies (Domingues *et al.*, 2007) after they have been exposed to potentials contaminants. This can contribute to the knowledge of the specific sample and its potential effects on the environment.

2.2.4 Mollusca

Snails are easy to culture as they only require a substrate and a sufficient amount of food. Snails are easy to handle and can be kept in a medium sized container. Minimal costs are required to sustain a culture. Snails can produce a large population of individuals quit rapidly. They are in direct contact with the sediment as they feed from its surface and some snails will even borrow into the substrate. Besides their use for sediment toxicity tests they have been used in the past for biomarker (Moolman *et al.*, 2007) and bioaccumulation (White *et al.,* 2006) studies.

3 CULTURE METHODS

Over the years numerous culture methods have been develop globally for culturing organism to be used for water and sediment toxicity tests. In South Africa, the focus has mainly been on culturing organisms for water toxicity testing. Sediment toxicity testing with indigenous organism however was not developed. This report contains the training methods for culturing *Hydra* sp., (Figure 1A), Ostracod sp., (Figure 1B), Chironomid sp., (Figure 1C) and snails (*Melanoides tuberculata)* (Figure 1D) native to South Africa.

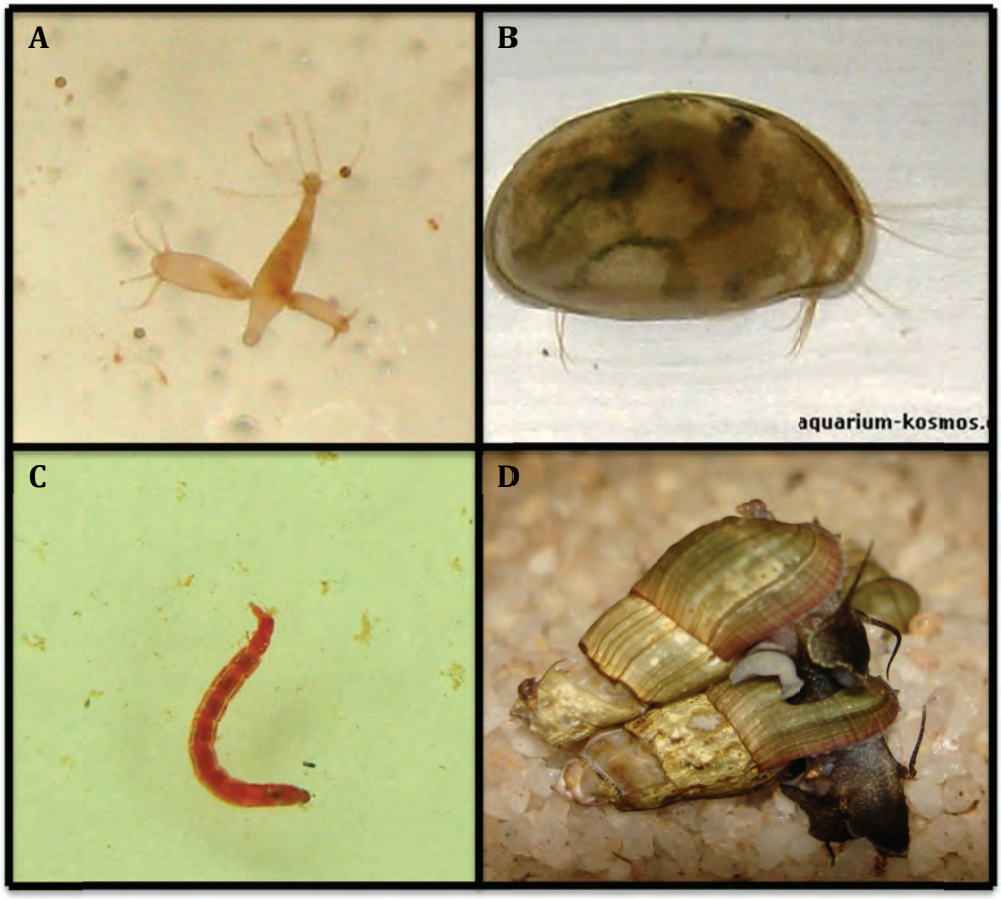

Figure 1: A Photo plate of the four culture organisms. A: *Hydra* sp., B. *Heterocypris incongruens* (Ostracod) (www.aquarium-kosmos.de). C: Chironomid sp., D. *Melanoides tuberculata* (Snail).

3.1 General culture

Culture methods for the selected culture organisms:

3.1.1 Hydra sp.

The *Hydra* species (Figure 1A) used for culturing was originally obtained from Happy Acres in Magaliesburg and were cultured at the University of Johannesburg. A subculture was

obtained and placed into 1.5 ℓ glass tanks (Figure 2A) with 500 mℓ of aged tap water. No aeration or substrate was added.

The culture medium was agitated slightly every second day in order to incorporate some oxygen. Originally a 200 mℓ water change was done every second day. The amount of medium in the beaker was then increase to 1 ℓ and the water changes increased to 500 mℓ, once a week. The room temperature was kept constant at a 22°C since the culture was started in August 2010. The culture was fed on live brine shrimp (*Artemia* sp.) or live *Daphnia pulex* every second day. The culture is stable and the *Hydra* population has increased steadily.

Hydra Culturing Method

Background

- Hydras are found in clean streams attached to rocks, sediments and vegetation and can reproduce sexual or asexually.
- *Hydra* species have been used in the past for toxicity testing in various countries.

Scope

- The method describes the culturing of *Hydra* under controlled laboratory conditions.

Summary of the method

- The Hydras were cultured in 1.5 ℓ glass tanks containing 1 ℓ aged tap water in a controlled environmental room.
- The Hydras were fed small to medium sized *Daphnia* juveniles three times a week.
- A 500 mℓ water change was done once a week for each culture beaker.
- Hydras reproduced by budding.

Equipment

- 1.5 ℓ glass tank.
- Calibrated pipettes with 1 mℓ and 5 mℓ tips.
- Polypropylene cups 250 mℓ.
- Analytic balance.
- Calibrated Thermometer.
- pH meter.
- Oxygen meter.

- Controlled environmental room 21 ± 2°C, equipped with glass shelves, a light timer (16 h: 8 h, Light: Dark)
- 20 ℓ washing tubs.
- Paper towel.
- Permanent marker.
- Petzl Headlamp.
- Container (25 ℓ) with tap.
- Fine brush
- Brine shrimp (*Artemia*) eggs

Reagent

- Sodium Hydrogen carbonate ($NaHCO_3$).
- Calcium sulphate ($CaSO_4$. $2H_2O$).
- Magnesium sulphate ($MgSO_4.7H_2O$).
- Potassium Chloride (KCI).
- Hydrochloric acid (HCI).
- Sodium hydroxide (NaOH).
- Marine salt

Quality Assurance

- Personnel culturing the *Hydra* should be well trained and should be competent in standard laboratory procedures.
- The *Hydra* should be disease free and should be obtained from a clean source.
- The temperature controlling equipment should be maintained properly in order to maintain the required culturing temperatures.
- The culturing rooms should be well ventilated and cleaned regularly.
- The water used for culturing purposes should be free of contaminants and metals.

Cleaning of glassware

- Glassware used for culturing purposes should be washed according to the laboratories standard operating procedures.
- Glassware used for culturing should be rinsed with Milli-Q or RO Water before use.

Culturing and Maintenance

Culturing medium

- Aged tap water

Food preparation and feeding

- Live *Daphnia* neonates are obtained from Laboratory cultures (See Appendix 3) or alternatively *Artemia* nauplii can be used as food (See Appendix 4).
- The amount of food should be enough for the *Hydra* to capture in 10 min.
- It is important to feed the Hydra at least every second day in order to keep them in an optimum condition and promote budding.
- The food can be added to the tanks by using a 5 mℓ pipette with a tip. Make sure to distribute the food evenly.
- Care should be taken so as to prevent the removal of *Hydra* on the pipette tip and too much disturbance of the water which could result in the *Hydra* being trapped on the sides of the glass beaker above the water line.

Culture temperature

- The best temperature for *Hydra* cultures is 22˚C ± 2˚C.
- Sudden changes in temperatures can stunt the growth of the *Hydra* and reduce budding.
- Room temperatures should be recorded every day in order to monitor the temperature equipment.
- If any parameters are outside the recommended parameters, adjust the equipment or determine the cause, report problem to supervisor.

Culture Container

- 1.5 ℓ glass tanks containing 1 ℓ of aged tap water.

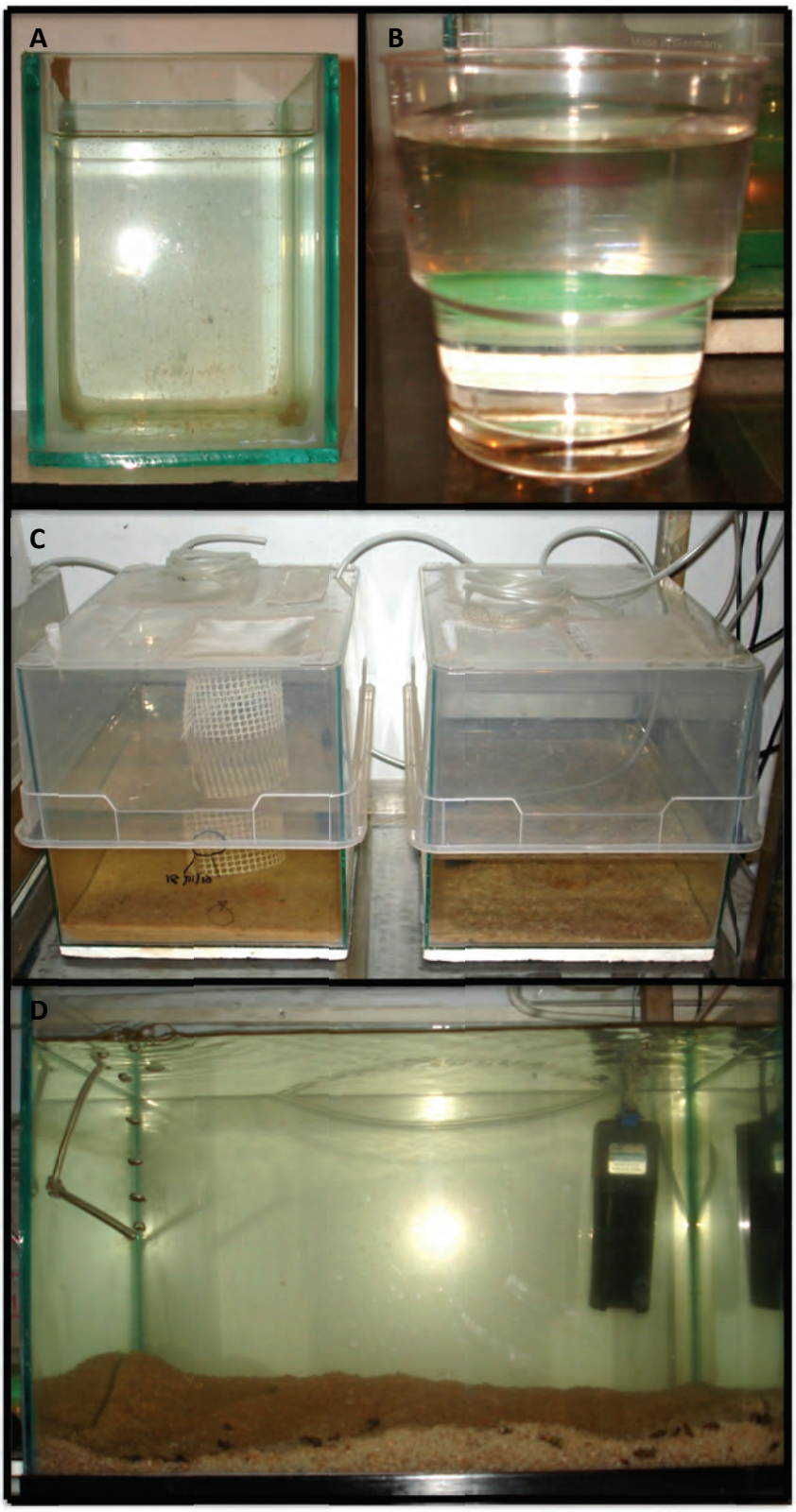

Figure 2: Culture Containers. A: 1.5 ℓ glass tank for the *Hydra* culture. B: 250 mℓ cup for the Ostracod culture. C. 12 ℓ Tanks for the Chironomid culture. D: 30 ℓ tank for the snail culture.

Aeration

- No aeration is necessary.

Illumination

- Ambient lighting with a light dark cycle of controlled by a timer (16 h: 8 h, Light: Dark).

Stock Culture

- Replace 500 mℓ of culture water per container each week with fresh aged water.
- Live *Daphnia*/*Artemia* can be added to the beaker with a 5 mℓ pipette.
- *Hydra* can be transferred to a new culture container with a fine brush.

Daily checks

- Ambient temperatures. If any parameters are outside the recommended parameters, adjust the equipment or room temperature of the culture room: minimum and maximum. Record all temperatures and determine the cause, report problem to supervisor.

3.1.2 Ostracod

The Ostracod *Heterocypris incongruens* (Figure 1B) used for culturing was originally obtained from the Ostracod toxkit F, 2001. The Ostracods were placed into a 250 mℓ cup (Figure 2B) with 200 mℓ of SSHW. No aeration or substrate was added. Half of the water in the cup was changed every second day, but this was reduced to only one water change per week.

The room temperature was kept constant at 22°C and the Ostracods were fed 200 µl *Daphnia* food prepared according to the USEPA (2002) standard method. The adults laid eggs at the bottom of the cup, and these hatched. It was however decided that the newly hatched Ostracods are too small to manage in a laboratory culture and that this would hamper its use in toxicity testing. Therefore the culturing was abandoned. The Ostracod toxkit F however is easy to use and would be the better method to use for the sediment toxicity test.

3.1.3 Chironomids

The Chironomid sp. (Figure 1C) was originally obtained from Aquaculture CC (Meyerton). A total of 150 individuals of different sizes were placed in 12 ℓ Glass tanks (Figure 2C) with 4 ℓ SSHW. A 1 cm mixture of acid washed fine and coarse silica sand was added to each tank. Constant aeration was supplied with an air stone. The tanks were covered with shade cloth

to prevent adults from escaping the tanks; this was later changed to plastic lids which were modified to allow for aeration.

A 1 ℓ water change was done every second day. This was later reduced to a 1 ℓ water change only once a week to reduce the amount of disturbances to the culture. The room temperature was kept constant at 22°C. The culture was fed a 15 mℓ food slurry made from a mixture of *Daphnia* food and 1 mℓ Sera Vipan flake every second day. Once a week the water quality (pH, O_2% & O_2 mg/ℓ) is measured and recorded using a HACH HQ 40d Multi-meter. Stable cultures will complete their entire life cycle in a laboratory and the following life stages will be visible; eggs (Figure 3A), larvae (Figure 3B), pupa (Figure 3C) and adults (Figure 3D).

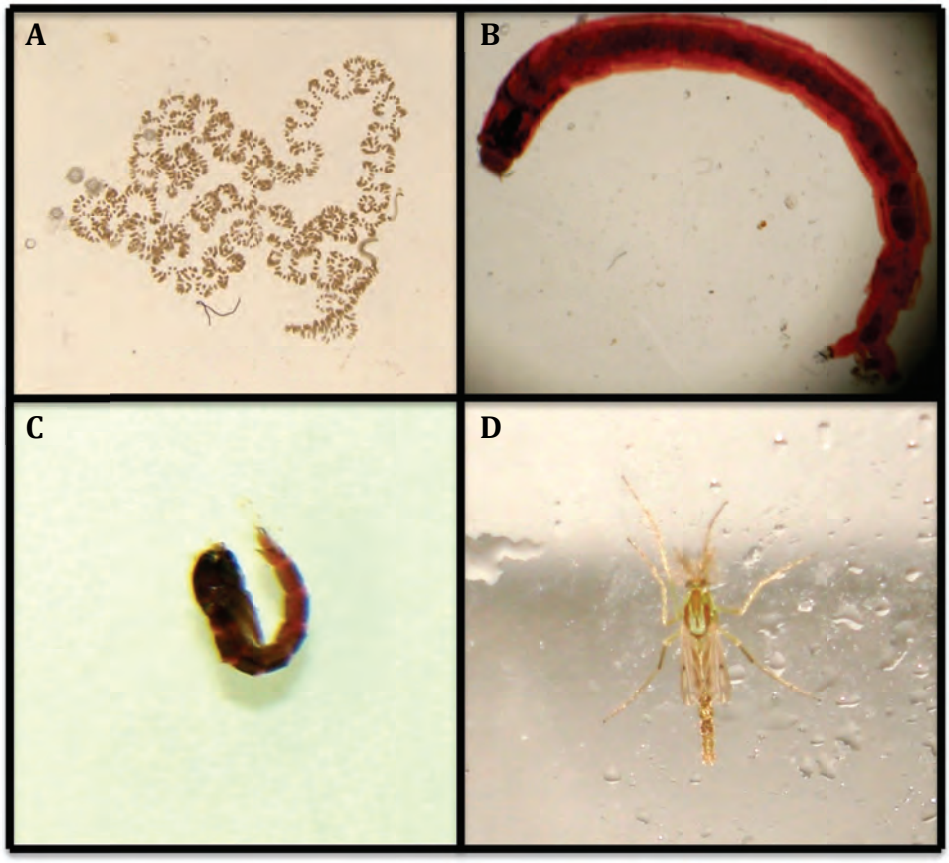

Figure 3: The Chironomid life stages. A – Egg mass, B – Chironomid larvae, C – A pupating chironomid, D – A Adult chironomid.

The eggs that are produced are carefully removed from the tanks and placed in a new 12 ℓ tank with aeration and SSHW in order to produce a monoculture. Specimens from the cultures were sent to The Institute for Water Research for identification purposes. Two species of Chironomids were identified from the GARL culture, primarily *Chironomus*

transvaalensis and to a lesser degree *C. caffrarius* (Personal communication Odumo, N.O., Rhodes University, 2011).

Chironomus transvaalensis

Chironomus transvaalensis can withstand low oxygen levels within the aquatic environment (McLachlan, 1970). Organic detritus is usually abundant where this species occur (Ogbeibu, 2001).They are considered to be a pioneer species and has a short life cycle. The short life cycle ensures that emergence time is quicker enabling the chironomids to escape harsh environmental conditions (McLachlan & Cantrell, 1980). It has been noted that *C. transvaalensis* populations will diminish when fine silt is deposited into their habitat (Ischinger *et al*., 1975).

Chironomus caffrarius

Very little information is available regarding *C. caffrarius* due to inconsistent use of its name and the use of its synonyms. The following are synonyms of *C. caffrarius: C. apricus, C. capensis, C. longicornis* (Bisby *et al*., 2011). *C. caffrarius* are distributed over the whole of South Africa, Namibia and the eastern parts of Africa and are usually found in standing or slow flowing water (Harrison, 2004)

There are two possible ways by which the larvae age can be determined, firstly is to note the specific date that they hatch from the egg sacs, and the second method is to determine the instar stage with the use of a microscope. The culture methods are summarized in Table 15.

Chironomid Culturing Method

Background

- Chironomid larvae are commonly found in fresh water and reside in the sediment or mud. They reproduce sexually and lay egg sacs in the water.
- The lifespan of a Chironomid varies depending species, food availability and water temperatures.
- Chironomid species have been used in the past for toxicity testing in various countries.

Scope

- The method describes the culturing of Chironomids under controlled laboratory conditions.

Summary of the method

- The Chironomids are cultured in 12 ℓ tanks containing 4 ℓ of SSHW in a controlled environmental room.
- A depth of 1 cm sand consisting of fine acid washed silicon sand (212-53 µm) and coarse silica sand (2000-500 µm) in a 20: 80 ratio was placed in each tank.
- The Chironomids are fed a slurry of *Daphnia* food mixed with Sera fish flakes three times a week.
- A 1 ℓ water change is done once a week for each culture tank.
- The pH, oxygen concentration and oxygen mg/ℓ are measured once a week.
- Egg sacs laid by the adults are transferred into new 12 ℓ glass tanks.

Equipment

- 12 ℓ glass aquarium tanks.
- Fine and course acid washed silicon sand (<2 mm).
- Plastic container with meshed openings as lids for the tanks.
- Air pump with small air stones and tubing.
- Calibrated pipettes with 1 mℓ and 5 mℓ tips.
- Glass beakers: 600 mℓ.
- Polypropylene cups 250 mℓ.
- Scissors.
- Analytic balance.
- Calibrated Thermometer.
- pH meter.
- Oxygen meter.
- Blender.
- Controlled environmental room 21 ± 2°C, equipped with glass shelves and a light timer (16 h: 8 h, Light: Dark).
- Measuring cylinder, 50 mℓ, 100 mℓ, 20 mℓ, 500 mℓ, and 1 ℓ.
- 20 ℓ washing tubs.
- Paper towel.
- Permanent marker.
- Petzl Headlamp.
- 200 µm Nylon Mesh.
- Silicon.
- Carpet cutter

- Container (25 ℓ) with tap.
- Magnetic tank cleaner.
- Magnetic stirrer.

Reagents and Materials

- Sodium Hydrogen carbonate ($NaHCO_3$).
- Calcium sulphate ($CaSO_4$. $2H_2O$).
- Magnesium sulphate ($MgSO_4.7H_2O$).
- Potassium Chloride (KCI).
- Hydrochloric acid (HCI).
- Sodium hydroxide (NaOH).
- Trout chow pellets.
- Dried yeast.
- Dried Alfalfa tablets.

Quality Assurance

- Personnel culturing the Chironomids should be well trained and should be competent in standard laboratory procedures.
- The chironomids should be disease free and should be obtained from a clean source.
- The temperature controlling equipment should be maintained properly in order to sustain the required culturing temperatures.
- The culturing rooms should be well ventilated and cleaned regularly.
- Water quality instruments should be clean and calibrated regularly.
- The water used for culturing purposes should be free of contaminants and metals.

Cleaning of glassware

- Glassware used for culturing purposes should be washed according to the laboratories standard operating procedures.
- Sediment should be washed in a 10% HCl solution overnight and should be rinsed properly in Milli-Q or RO water.
- Glassware used for culturing should be rinsed with Milli-Q or RO Water before use.

Culturing and Maintenance

Culturing medium

- Standard Synthetic Hard Water (SSHW) is used for culturing Chironomids

- The date of medium preparation should be noted on a record form with the physical parameters.
- Chronomids can survive over a large pH range but the preferred range should be between pH 6.5-8.5.
- The oxygen should be kept between 5-7 mg/ℓ.

Food preparation and feeding

- The food is prepared according to the method (Daphnia food) described proceeding the culture methods.
- The amount of food can be adjusted for the Chironomids according to their size and numbers. Adult worms need less food.
- It is important to feed the Chironomids at least every second day in order to keep them in an optimum condition.
- Fresh food should be prepared every week and all the preparation information (Date and time) should be recorded.
- Feed the Chironomids 15 mℓ of food slurry per 12 ℓ tank, once every second day (Monday, Wednesday, Friday). If the amount of food is adjusted it should be recorded.
- The food can be added to the tanks by using a 5 mℓ pipette with a tip. Care should be taken to distribute the food evenly.
- Food should be refrigerated for a maximum of 10 days.

Culture temperature

- The best temperature for Chironomid cultures are 22˚C with a range of 22-25˚C.
- Sudden changes in temperatures can stun the growth of the Chironomids.
- Room temperatures should be recorded every day in order to monitor the temperature equipment.
- If any parameters are outside the recommended parameters, adjust the equipment or determine the cause, report problem to supervisor.

Culture Container

- 12 ℓ rectangular glass tanks with (plastic lids) containing 1 cm (depth) mixed acid washed silicon sand and 4ℓ of culture medium.

Aeration

- An air pipe is inserted in to a hole in the lid and is fitted with an air stone. The airflow should be at a low strength, enough to circulate the water, but not to agitate the sediment.

Illumination

- Ambient lighting with a light dark cycle of controlled by a timer (16 h: 8 h, Light: Dark).

Stock Culture

- Adult Chironomids can be obtained from a clean source or a supplier for a new culture if no egg sacs area available to start a culture from.
- Approximately 150 Chironomids of similar sizes range are transferred to the 12 ℓ glass tanks. This will insure that all the organisms can moult at the same time and increase the amount of egg sacs that are produced.
- A new culture can be started from the egg sacs by transferring one or two egg sacs to a tank containing silicon sand. The tank should contain water from the original tank where the egg sacs came from mixed with fresh SSHW.
- The egg sacs can also be left in the original culture tank if the risk of damaging the egg sacs is too high.
- Alternatively adults can be removed from an established tank (without agitating the sediment too much) and can then be placed into a new tank to start a new culture. The new culture tank should follow the same acclimation method as is used for the existing tanks.
- Replace 1 ℓ of culture medium per tank each week with fresh medium.
- Prepared food can be added with a 5 mℓ pipette.
- Adults and egg sacs can be transferred with a 5 mℓ pipette and a 5 mℓ tip with 1/10 cut off from the front of the tip).

Daily checks

- Ambient room temperature of the culture room: minimum and maximum. Record all temperatures. If any parameters are outside the recommended parameters, adjust the equipment or determine the cause, report problem to supervisor.

3.1.3 Mollusca

The snail species *Melanoides tuberculata* (Figure 1D) used for culturing was originally obtained from the University of Johannesburg. A subculture was placed into a 30 ℓ glass tank (Figure 2D) with 25 ℓ SSHW. A substrate of 1.5 cm acid washed coarse silica sand was added into the tank. Constant aeration was supplied with an air stone and the water is filtered with an internal carbon filter. A heater was placed in the tank and the temperature was set at 27°C.

Initially a 50% water change was conducted in the tank every second day. But after the addition of the internal filter, this was reduced to a 3 ℓ water change once a week. The room temperature was kept constant at 22°C and the snails are fed 1 tablet of Sera Spirulina tabs per tank three times a week. The culture is stable. The culture methods are summarized in Table 15.

Melanoides tuberculata Culturing method

Background

- *Melanoides tuberculata* have been used in the past for toxicity testing, bioaccumulation and biomarker studies.

Scope

- The method describes the culturing of *Melanoides tuberculata* under controlled laboratory conditions.

Summary of the method

- The *M. tuberculata* are cultured in 30 ℓ glass tank filled with aged tap water in a controlled environmental room.
- The *M. tuberculata* snails are fed 1 tablet of Sera Spirulina Tabs three times a week.
- A 3 ℓ water change is done once a week.
- Snails reproduce by laying eggs.

Equipment

- 30 ℓ glass tank.
- Acid washed course silicon sand.
- Water heater set at 27˚C.
- A submerged water filter.
- Calibrated pipettes with and 5 mℓ tips.

- Calibrated Thermometer.
- Controlled environmental room 21 ± 2°C, equipped with glass shelves, a light timer (16 h: 8 h, Light: Dark).
- Paper towel.
- Petzl Headlamp.
- Reagents and Materials
- Sera Spirulina tabs.

Quality Assurance

- Personnel culturing the *M. tuberculata* should be well trained and should be competent in standard laboratory procedures.
- The *M. tuberculata* snails should be disease free and should be obtained from a clean source.
- The temperature controlling equipment should be maintained properly in order to maintain the required culturing temperatures.
- The culturing rooms should be well ventilated and cleaned regularly.
- The water used for culturing purposes should be free of contaminants and metals.

Cleaning of glassware

- Glassware used for culturing purposes should be washed according to the laboratories standard operating procedures.
- Sediment should be washed in a 10% HCl solution overnight and should be rinsed properly in Milli-Q or RO water.
- Glassware used for culturing should be rinsed with Milli-Q water or High Quality Reverse Osmosis (RO) Water before use.

Culturing and Maintenance

Culturing medium

- Aged Tap water was used for culturing *M. tuberculata.*

Food preparation and feeding

- *M. tuberculata* are fed Sera Spirulina tabs. The tablets were broken into pieces and were evenly distributed in the tank.
- It is important to feed the snails at least twice a week in order to keep them in an optimum condition and promote reproduction.
- Make sure to distribute the food evenly.

Culture temperature

- The best temperature for *M. tuberculata* cultures is 27˚C with a range of 25-30˚C.
- Room temperatures should be recorded every day in order to monitor the temperature equipment as well as the water temperature.
- If any parameters are outside the recommended parameters, adjust the equipment or determine the cause, report problem to supervisor.

Culture Container

- 30 ℓ Glass tank.

Aeration

- Aeration from a submerged filter.

Illumination

- Ambient lighting with a light dark cycle of controlled by a timer (16 h: 8 h, Light: Dark).

Stock Culture

- Replace 3 ℓ of water with fresh water once a week.
- Clean the water filter regularly.

Daily checks

- Ambient room temperature of the culture room: minimum and maximum. Record all temperatures. If any parameters are outside the recommended parameters, adjust the equipment or determine the cause, report problem to supervisor.

Safety

- *M. tuberculata* is a host for numerous parasites that can infect humans; however they require a first and second intermediate host like fish or crustaceans. Humans are infected by eating the raw or uncooked meat of the infected fish or crustaceans.
- *M. tuberculata* laboratory cultures aren't likely to infect personnel with parasites but as a safety precaution it is advised to work with gloves when handling the snails.

4 RESULTS AND DISCUSSION

A summary of the test conditions, for each of the organisms cultured for this project, is listed below in Table 15.

Table 15: A Summary of the organism culture conditions

Culture Organisms	***Hydra* sp.**	**Chironomid sp.**	***Melanoides tuberculata***
Temperature	22 ± 1°C	22 ± 1°C	27 ± 1°C
Light quality	Ambient laboratory illumination	Ambient laboratory illumination	Ambient laboratory illumination
Photoperiod	16 h Light: 8 h Dark	16 h Light: 8 h Dark	16 h Light: 8 h Dark
Culture container size	1.5 ℓ glass tank	12 ℓ glass tank	30 ℓ glass tank
Medium volume	1 ℓ	4 ℓ	25 ℓ
Renewal of culture medium	500 mℓ once a week	1 ℓ once a week	3 ℓ once a week
Number of organisms per container	±100	±150	±30
Cleaning of container	Once every 3 weeks	Sides of tank once every second week	Sides of tank once every second week
Feeding regime	*Daphnia pulex* or Brines shrimp	15 mℓ *Daphnia* food and 1 mℓ fish food slurry	1 tablet of Sera Spirulina tabs, three times a week
Aeration	None	Light aeration	Medium aeration

4.1 Hydra

The *Hydra* culture method was adapted from the one described by Holdway (2005). A few changes were however made. The GARL culture was kept at 22 ± 1ºC as opposed to 25ºC, whilst the food, photoperiod and light intensity were the same. When the culture was started in 2010, SSHW was used as the original culture medium. It was however noted that the *Hydra* did not proliferate and most of them had died within a week of starting this new culture. As a result it was decided to rather use aged tap water with the same temperature

as that of the other tanks in the culture room to culture the *Hydra*. With this change in medium, the culture has grown at a steady rate.

Hydra cultures will ingest both *Daphnia* sp. and *Artemia* sp. as food sources. Even though the Hydras can eat large adult *Daphnia,* young adults and juveniles were easier to catch. *Artemia* nauplii are easily hatched from eggs when needed and are therefore available in larger quantities. Whilst the nauplii are also easy to catch, they can cause the tank to become fouled faster than it would if the Hydras were fed *Daphnia*. For this reason extra care should be taken to keep the culture containers clean after feeding the *Hydra* with *Artemia*.

It was observed that the *Hydra* occasionally floated on the water surface rather than being attached to the culture container wall. It is possible that this behaviour is exhibited when the Hydras are hungry, likely in an attempt to disperse to an area where more food is available.

4.2 Chironomid

The following Chironomid culture methods were taken into account when the GARL culture was started: USEPA (2004), OECD (2004) and Env.C (1997). Environment Canada (1997) cultured *C. tentans* at a temperature of 23ºC±1ºC, 500-1000Lux light, oxygen percentage of higher than 80% and a pH value ranging from 7.8-8.2. The GARL culture had the same light period and aeration as that of the Environment Canada method.

In comparison to the USEPA culture method, the GARL culture was kept at a lower pH of (7-8), Oxygen saturation (60-90%), light intensity (ambient illumination) and temperature (22ºC±1ºC). The Tetramin flakes recommended by Environment Canada report as food for the chironomids were mixed with standards *Daphnia* food in order to make slurry, to feed the GARL Chironomid culture with. The GARL cultures were fed three times a week as feeding them every day as the Env.C (1997) report suggests might lead to a rapid organic content build-up.

It was noted that the chironomids in the culture has a tendency to move into the water column when the oxygen levels dropped to low or when insufficient food was available; they also tend to clump together in a section of substrate.

4.3 Molusca

Melanoides tuberculata snails were cultured at 27°C with a light day photoperiod of 16 hours light and 8 hours light. The Spirulina tablets used to feed the snails improved the condition

of the snails and the population is growing steadily. The SSHW are also improving the hardness of their shells. Minimal information regarding the culturing of *M. tuberculata* was found during the literature search.

The snails exhibited the general burrowing behaviour in the silica sand that was added to the tank. During periods where the water quality deteriorated the snails moved to the water surface.

5 CONCLUSION

The need for toxicity tests are increasing with the ever growing world population and the anthropogenic impacts that humans have on the aquatic environment. Toxicity testing is an important part of determining the health of an aquatic ecosystem and establishing remediation methods to limit the effect of the contaminants.

- The four species regarded as possible test organisms were cultured. After the cultures were initiated it was found that the Ostracod sp. was difficult to handle as the neonates are extremely small and may hinder culture and test procedures. It was decided that the use of the Ostracod toxkit F, 2001 would be easier for toxicity test purposes.

- It is important to develop effective, easy and rapid toxicity tests in order to assess impact. Thus research has to be continued on the culture of suitable species and refining and standardizing test methods globally. In South Africa aquatic invertebrate research has to continue in order to establish tests for indigenous invertebrate species that are applicable to the native circumstances and that are similar to species used across the world.

6 RECOMMENDATIONS

The current culturing methods still need to be refined and adapted continuously in order to optimize conditions for the organisms. This will allow for more control and better quality assurance of the test organisms.

The following recommendations are critical:

- To conduct preliminary sediment toxicity test to determine the application of each organism to the sediment toxicity tests and to develop sediment toxicity test methods.
- To transfer monocultures of Chironomids to other testing laboratories in order to sustain multiple cultures that can be used for sediment toxicity.
- To conduct training on the culture methods through a work shop and hands on experience.
- To Identify the *Hydra* sp. from the GARL culture

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APPENDIX 1: Standard Synthetic Hard Water

Standard Synthetic Hard Water reagents

• *Reagents*	• *Concentration (mg/l)*
• *$NaHCO_3$*	• *96.0*
• *$CaSO_4$. $2H_2O$*	• *60.0*
• *$MgSO_4$. $7H_2O$*	• *123.0*
• *KCl*	• *4.0*

Culture Medium Preparation:

- Place 19 ℓ Milli-Q water or High Quality Reverse Osmosis (RO) Water in a clean 25 ℓ container with a tap.
- Add 1.2 g $CaSO_4.2H_2O$ to Measuring cylinder containing 1 ℓ of Milli-Q/RO water and place on a magnetic stirrer until the $CaSO_4.2H_2O$ has completely dissolved.
- Add 2.46 g of $MgSO_4.7H_2O$, 1.92 g $NaHCO_3$ and 0.08 g KCl to the 25 ℓ container with the 19 ℓ Mili-Q/RO water.
- Add the dissolved $CaSO_4.2H_2O$ solution to the 25 ℓ container.
- Aerate the container overnight in order to dissolve all the chemicals.
- Record the preparation time and date on a data sheet.
- Freshly prepared culture medium is stable for 2 weeks when stored at room temperature (DWA, 2006).

APPENDIX 2: *Daphnia* food preparation

Daphnia Food preparation:

- Add 12.6 g trout chow pellets, 5.2 g dried yeast and 2 tablets (1 g) dried alfalfa to 1 ℓ Milli-Q water or RO Water in the blender, allow to soak for 5 min.
- After soaking, mix in the blender at a high speed for 10 min.
- Pour the blended food into a 1 ℓ plastic bottle and refrigerate for at least 6 hours.
- After most of the larger particles have settled out, carefully decant the top 800 mℓ of the food mixture into a container with a lid and discard the rest.
- Mark the food container with the date of preparation as well as the expiry date.

APPENDIX 3: *Daphnia* culture

- Stock cultures are maintained as follows:
  - The Number of Stock culture beakers maintained depends on the amount of Daphnia required.
  - Weekly, two new culture beakers are started to prevent overcrowding and ensure the maintenance of healthy organisms.
  - The Daphnia are cultured in 3 ℓ glass beakers filled with 2.5 ℓ of SSHW as culture medium.
  - A 5 mℓ pipette with a trimmed tip (approximately 1 cm removed to prevent turbulence and damage to the Daphnia) is used to transfer 50 adults and 50 Neonates to a new beaker containing fresh medium and food.
  - The date of transfer should be noted on each beaker.
  - Transfers are done on Mondays and Wednesdays, and the oldest cultures are discarded (4 week old cultures).
  - Care should be taken so to transfer as little as possible old medium with the Daphnia from the old stock culture to the new culture beaker.
  - Each culture beaker should be covered to prevent dust contamination as well as to limit evaporation from the culture.
  - The culture should be monitored on a daily basis and all dead Daphnia and Daphnia with Ephippia should be removed.
- Daphnia neonates can be obtained from a culture used in the laboratory or from various Universities.
- SSHW is used as culture medium
- Daphnia can tolerate a wide pH range.
- Daphnia are fed 4 mℓ of Daphnia food on a Monday, Wednesday and Friday (see section 1 for food preparation).
- The optimum temperature for the Daphnia is 20 ± 2˚C. The culture room temperature should be monitored and recorded every day.
- It is not necessary to aerate the culture medium in the beakers. The light dark cycle is 16H:8H, Light: Dark.

APPENDIX 4: *Artemia nauplii*

- *Artemia* eggs (Brine Shrimp) can be obtained from local pet stores.
- Fill a 2 ℓ upturned plastic bottle, which has had the base removed, with 1 ℓ.
- Following the manufacturer's directions, add approximately 3 teaspoons of marine salt to the 1 ℓ.
- Place an air stone in the bottle with a high airflow so as to prevent the eggs from settling on the bottom of the beaker.
- The eggs are then added to the water (dependent on the quantity of Artemia required for the Hydra).

Note: Never add more than 3 g of eggs per litre of water.

- The *Artemia* nauplii will hatch within 24-36 hours, depending on the room temperature.
- The *Artemia* nauplii are removed from the culture beaker with a 5 mℓ pipette.
- The nauplii are then rinsed through in a fine sieve with *Hydra* culture medium (to remove the excess salt) and then added to the *Hydra* culture beaker.

Note: Using *Artemia* as food does require more culture maintenance as the *Hydra* culture beaker becomes dirty very quickly as a result of the un-eaten nauplii which sink to the bottom of the beaker.