Freshwater toxicity bioassays: BioTreat

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1. Introduction

This report summarises the methods and results of freshwater toxicity screening bioassays conducted by the CSIR, Natural Resources and the Environment (NRE) for Bluestream. The BioTreat sample that was tested is used in pit latrines and septic tanks. The purpose of the toxicity tests was to establish the acute toxicity potential of the test samples by performing a battery of acute bioassays.

2. Materials and methods

2.1 Sample preparation

The BioTreat sample was prepared according to directions provided on the sachet. Hundred grams (100 g) of sample was mixed into 2 L of tap water (de-chlorinated). The mixture was allowed to stand overnight at room temperature before it was filtered and diluted. A No. 1 Whatmann filter was used to remove debris from the sample. The strained water sample (regarded as the undiluted test sample) was diluted with de-chlorinated tap water and test organisms were exposed to two dilutions of the sample, namely a 0.05% and a 0.005% concentration.

2.2 Toxicity assays

Standard toxicity screening assays (ISO, 1998; USEPA, 2002; DWAF, 2003), using organisms (Table 1, 2, 3 and 4) from different trophic levels, were conducted under static conditions. The following test organisms were exposed to the test samples:

- 15-minute *Vibrio fischeri* (bacterium)
- 72-hour *Selenastrum capricornutum* (algae)
- 48-hour *Daphnia magna* (water flea)
- 96-hour *Poecilia reticulata* (fish)

The test conditions and guidelines used for each bioassay are summarised in the section below.

Dissolved oxygen, pH, conductivity and temperature of each sample were tested at the start and the end of the exposure period.

2.2.1 15-minute *Vibrio fischeri* screening assay

The inhibition of light emitted by the bioluminescent bacterium, *V. fischeri* (Figure 1) is the basis for this toxicity bioassay.

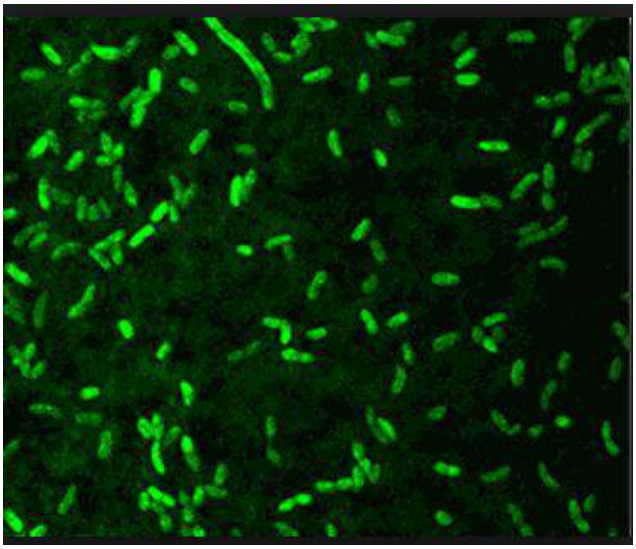

Figure 1: Micrograph of *Vibrio fisheri* (from https://microbewiki.kenyon.edu/index.php/Vibrio_fischeri)

In Table 1, test conditions for the *V. fischeri* test are summarised.

Table 1: Summary of test conditions and test acceptability criteria for the bacterium *V. fischeri* growth test (ISO, 1998).

Parameter	Condition maintained during test
Test type	Static non-renewal
Volume of test sample	0.5 mℓ
Exposure period	15 minutes
Number of replicate chambers	2
Measurement equipment	Titertek Berthold FB 14 Luminometer
Effects measured	Screening test - % growth inhibition or stimulation relative to control; Definitive test - EC20 and EC50 –values.
Interpretation	Inhibition / stimulation of >20% over control indicates toxicity.

2.2.2 72 / 96-hour *Selenastrum capricornutum* screening assay

Algae are especially suitable for bio-testing because of their sensitivity to environmental pollution and their abundance in aquatic systems. In addition, algae do not have roots, unlike higher plants, and only reflect the properties of the ambient water, rather than those of the soil, in which the higher plants are rooted. Algal bio-tests are simple and allow for the observation of multiple generations (Bae and Park, 2014). **Table 3** summarises the test conditions of the *S. capricornutum* algal assay. Growth inhibition of *S. capricornutum* (**Figure 3**) is the basis for this toxicity assay.

Figure 2: The unicellular alga *Selenastrum capricornutum (*from: http://www.suggestkeyword.com/c2VsZW5hc3RydW0/).

Table 2: Summary of test conditions and test acceptability criteria for green algae, *S. capricornutum*, growth toxicity tests with effluents and receiving waters (USEPA, 2002).

Parameter	**Condition maintained during test**
Test type	Static non-renewal
Temperature	25 ± 1° C
Light quality	"Cool white" fluorescent lighting
Light intensity	4306 lux
Photoperiod	24 hours light
Volume of test sample	10 mℓ
Age of test organisms	4 to 7 days
Initial cell density in test chambers	10 000 cells/ml
Number of replicate chambers	3
Shaking rate:	100 cpm continuous
Aeration	None
Dilution water	Algal stock culture media
Test duration	72 to 96 hours
Effects measured	Percentage inhibition or stimulation
Interpretation	Inhibition of ≥20% over controls indicates toxic activity, while growth of ≥20% over controls indicates stimulation (Oberholster *et al*., 2010)

2.2.3 48-hour *Daphnia magna* screening assay

The crustacean Daphnia are a major component of the freshwater zooplankton throughout the world and are sensitive to environmental toxicants, such as heavy metals, and an array of organic toxic chemicals (Bae and Park, 2014). Acute, 48 hour *D. magna* bioassays were conducted under static conditions to establish the short term toxicity potential of the test water samples. *D. magna* acute toxicity assays were performed in accordance with the U.S. Environmental Protection Agency's Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms (USEPA, 2002). The immobility / mortality of *D. magna* (**Figure 3**) is the basis for this toxicity bioassay.

Figure 3: The crustacean, *Daphnia magna (http://mblaquaculture.com/content/organisms/daphnids.php).*

A summary of the test and exposure conditions are summarised in **Table 3**.

Table 3: Summary of test conditions and test acceptability criteria for *Daphnia magna* acute toxicity tests with effluents and receiving waters (USEPA, 2002).

Summary of toxicity test	
Test system	*Daphnia* test
Test species	*Daphnia magna*
Age of test organisms	Less than 48h old
Trophic level	Grazer
Toxicity level	Acute toxicity
Test procedure	USEPA, 2002
Summary of test conditions for the Daphnia magna acute toxicity test	
Test type	Static-renewal
Water temperature	20 ºC ± 1 ºC
Light quality	Ambient laboratory illumination
Photoperiod	8 hours dark: 16 hours light
Feeding regime	Feed algae and commercial fish flakes while in holding prior to test
Aeration	None
Size of test chamber	50 mℓ
Volume of test sample	25 mℓ
Number of test organisms per chamber	5
Number of replicate chambers	4
Total number of test organisms per sample	20
Control and dilution water	Moderately hard, de-chlorinated water
Test duration	48 hours
Effect measured	Percentage lethality (no movement on gentle prodding), calculated in relation to control
Test acceptability	90% or greater survival in control
Interpretation	Lethality >10% indicates toxicity, provided that control lethality is ≤10%

2.2.4 96-hour *Poecilia reticulata* screening assay

The acute, 96 hour *P. reticulata* bioassay (Table 4) was conducted under static conditions. *P. reticulata* acute toxicity assays were performed in accordance with the OECD guidelines (OECD, 1992). The immobility / mortality of *P. reticulata* (Figure 4) is the basis for this toxicity bioassay.

Figure 4: *Poecilia reticulata* (guppy) (http://www.fishesofaustralia.net.au/home/species/3637)

Table 4: Summary of test conditions and test acceptability criteria for *Poecilia reticulata* acute toxicity tests with effluents and receiving waters (OECD, 1992).

Summary of toxicity test	
Test system	Fish test
Test species	*Poecilia reticulata*
Size of test organisms	2±1 cm
Toxicity level	Acute toxicity
Test procedure	OECD, 1992
Summary of test conditions for the Daphnia magna acute toxicity test	
Test type	Static
Water temperature	21 - 25 ºC
Light quality	Ambient laboratory illumination
Photoperiod	8 hours dark: 16 hours light
Feeding regime	None
Aeration	None
Test duration	96 hours
Size of test chamber	800 mℓ
Volume of test sample	500 mℓ
Number of test organisms per chamber	10
Number of replicate chambers	2
Total number of test organisms per sample	20
Control and dilution water	Moderately hard, de-chlorinated tap water
Test duration	96 hours
Effect measured	Percentage lethality (no visible movement, e.g. gill movement)
Test acceptability	90% or greater survival in control
Interpretation	Lethality >10% indicates toxicity, provided that control lethality is ≤10%

3. Results

Results are reported per bioassay. Physical parameters measured at the start and end of each test with a hand-held Hach HQ 40D multi parameter meter, are summarised in Appendix A.

3.1 15 / 30-minute *Vibrio fischeri*

The percentage *V. fischeri* growth inhibition measured after 15 minutes for the two diluted samples was as follows: 0.05% test sample: 4.29% and 0.005% test sample: 3.73%. No acute toxicity was observed.

3.2 72-hour *Selenastrum capricornutum*

According to the results, the algal growth rate was inhibited in both the 0.05% and the 0.005% test samples, relative to the control (Figure 5). In the 0.05% concentration test sample, the algal growth rate was 53.9% (46.10% inhibited) while in the 0.005% test sample, it was 53.99% (46.01% inhibited).

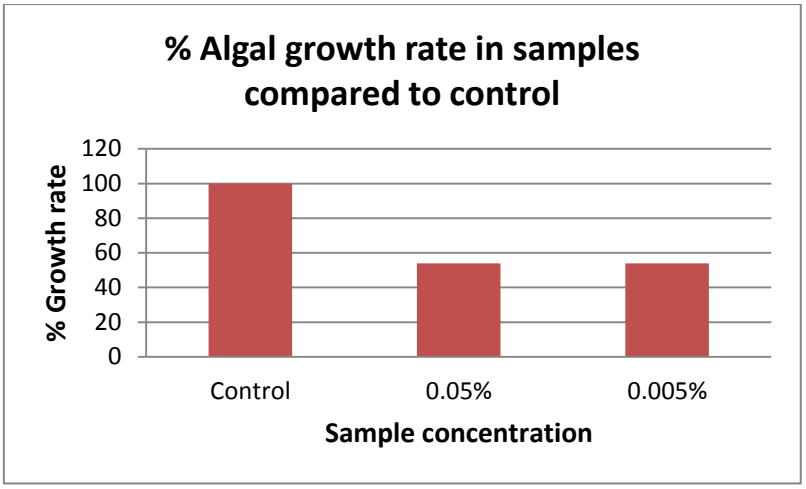

Figure 5: Percentage growth rate of *S. capricornutum* in test samples (0.05% and 0.005%), relative to the control.

3.3 48-hour *Daphnia magna*

No Daphnia mortality was observed in either of the two concentrations tested (i.e. 0.05% and 0.005%). Readings were taken after 24 and 48 hours.

3.4 96-hour *Poecilia reticulata*

No fish mortalities were observed in either the 0.05% or the 0.005% concentration test sample after 96 hours. Readings were taken at 24 hour intervals.

4. Discussion and conclusion

In Table 5, the results of the four toxicity tests, performed per test concentration, are summarised.

Table 5: Summary of toxicity tests and results for Bluestream test sample: Sani Treat

Sample dilution (%)	Toxicity test	Test duration	End point	Results	Toxicity hazard potential
0.05	*Vibrio fischeri*	15 min	% inhibition	4.29%	None
	Selenastrum capricornutum	72 hours	% inhibition	46.10	Slight acute hazard
	Daphnia magna	48 hours	% mortality	0%	None
	Poecilia reticulata	96 hours	% mortality	0%	None
0.005	*Vibrio fischeri*	15 min	% inhibition	3.73%	None
	Selenastrum capricornutum	72 hours	% inhibition	46.01	Slight acute hazard
	Daphnia magna	48 hours	% mortality	0%	None
	Poecilia reticulata	96 hours	% mortality	0%	None

For both test concentrations (0.05% and 0.005%), no acute toxicity was observed in any of the exposures, except the algal assay where both test concentrations posed a slight acute hazard to *S. capricornutum*.

5. References

Bae, M-J and Park, Y-S. 2014. Biological early warning system based on the responses of aquatic organisms to disturbance: A review. *Science of the Total Environment*: 635-649.

Department of Water Affairs and Forestry (DWAF). 2003. *The management of complex industrial wastewater discharges: Introducing the Direct Estimation of Ecological Effect Potential (DEEEP) approach.* A discussion document. 15pp.

ISO. 1998 (replaced by 2007). *Water quality -- Determination of the inhibitory effect of water samples on the light emission of Vibrio fischeri (Luminescent bacteria test) -- Part 1: Method using freshly prepared bacteria.* ISO 11348-1:2007.

OECD. 1992. *Fish acute toxicity test 203*. OECD Guidelines for testing of chemicals.

US Environmental Protection Agency (USEPA). 2002. *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms.* Fifth Edition. Report no.: EPA-821-R-02-012. USA. U.S. Environmental Protection Agency. USA.

APPENDIX A

(Physico-chemical readings)

Table A.1: Physical parameters measured at the start and end of the bioassays.

Test sample	Time (h)	Temperature	pH	Electrical conductivity	Dissolved oxygen	
		°C		µS/cm	mg/L	%
Control	Start	19.6	6.35	189.2	7.4	92.9
	End (A*)	22.0	8.20	199.1	8.05	98.7
	End (D*)	19.2	7.10	199.4	7.12	92.2
	End (F*)	19.8	7.20	201.3	6.94	89.6
0.05%	Start	19.4	6.85	206.4	7.48	95.2
	End (A)	22.3	7.90	208.1	7.60	96.7
	End (D)	19.3	6.95	210.6	4.06	50.7
	End (F)	19.6	6.99	238.0	6.26	81.1
0.005%	Start	19.7	7.49	188.3	7.07	90.8
	End (A)	23.0	8.40	194.2	7.60	96.7
	End (D)	19.9	7.73	193.8	6.94	89.6
	End (F)	19.8	7.83	225.0	6.26	81.1

*A: Algae; *D: Daphnia; *F: Fish

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29th JUNE 2015

ANALYTICAL REPORT

OUR REF: **W661Y15.REP**
COMPANY NAME: **BLUESTREAM**
COMPANY ADDRESS: **SUITE 57 PRIVATE BAG X 7**
CONTACT PERSON: **HAYDEN VON BLERK**
LAB. NO: **W661/15**
SAMPLE ID: **PRODUCT BIOTREAT SAMPLE**
DATE SUBMITTED: **26/05/2015**

One sample was submitted to the laboratory for various analyses. **These results are presented below.**

DETERMINAND	UNITS	W661/15 PRODUCT BIOTREAT SAMPLE	CLASS "A" LIMITS
Total Helminth's	Count per dry g	0	1 **viable** helminth ova
#pH @ 25°C	pH Units	7	
Total Solids at 105°C [Gravimetric]	% m/m	91	
Volatile Fatty Acids, VFA	mg/l CH_3COOH	3548	
Volatile Solids at 400°C [Gravimetric]	% m/m	56	
**Potassium as K	mg/kg	8238	
Phosphorous as P [ICP]	% m/m	2.76	
Nitrogen as N [Kjeldahl Digestion]	% m/m	4.03	

Comment: #pH was done on a 1:10 slurry
** The sample was prepared by means of an aqua-regia digestion and analysed for Potassium. The result was calculated back with mass and volume used in the digestion.

POLYAROMATIC HYDROCARBONS (PAH's)

DEERMINAND	UNITS	RESULTS W661/15 PRODUCT BIOTREAT SAMPLE	CLASS "A" LIMITS
Naphthalene	µg/kg	22	The sum of the concentrations is below 6,000 µg/kg
Acenaphthene	µg/kg	<20	
Acenaphthylene	µg/kg	<20	
Fluorene	µg/kg	<20	
Phenanthrene	µg/kg	<20	
Anthracene	µg/kg	<20	
Fluoranthene	µg/kg	<20	
Pyrene	µg/kg	<20	
Benzo[a]anthracene	µg/kg	<20	
Chrysene	µg/kg	<20	
Benzo[k +b]fluoranthene	µg/kg	<20	
Benzo[a]pyrene	µg/kg	<20	
Benzo[g,h,i] perylene	µg/kg	<200	
Dibenz[a,h]anthracene	µg/kg	<200	
Indeno[123-cd]pyrene	µg/kg	<200	

The samples were prepared by means of an aqua-regia digestion where the resultant digests were analysed by ICP-MS. These results were calculated back with mass and volume used in the digestions. **All results are presented below**.

DETERMINAND	UNITS	W661/15 PRODUCT BIOTREAT SAMPLE	CLASS "A" LIMITS
Arsenic, As	µg/kg	1299	<40,000
Cadmium, Cd	µg/kg	26	<40,000
Chromium, Cr	µg/kg	3697	<1,200,000
Copper, Cu	µg/kg	6494	<1,500,000
Lead, Pb	µg/kg	110	<300,000
Mercury, Hg	µg/kg	170	<15,000
Nickel, Ni	µg/kg	3697	<420,000
Zinc, Zn	µg/kg	29673	<2,800,000

Vanessa Talbot
LABORATORY DIRECTOR

TEST REPORT

SABS

MICROBIOLOGY DEPARTMENT 7218

Your ref:

Our ref:

Enquiries: 428-6172

Date: 2004-09-01

1907835/2419/X32902

BLUESTREAM

Attention: Mr. Mark Hinton

P.O Box 57

Kya Sand

2163

PRODUCT SAMPLES

1. DESCRIPTION OF SAMPLES

Two samples labeled as in paragraph 5, were received on 2004-07-29. One sample (BIO – TREAT POWDER) was re – tested on 2004-08-20.

2. TEST REQUESTED

Microbiological examination of above mentioned samples.

3. METHOD OF TEST

3.1 Sample preparation

25g of sample are aseptically weighed into 225mP sterile buffered peptone water. The mixture is homogenized to give a 1 in 10 dispersion.
*** (The sample was left, over – night, at room temperature for 72 hrs before being used for the test.) ***

3.2 Methods

1. ISO 4833 Microbiology – General guidance for the enumeration of micro-organisms – Colony count technique at 30°C.
2. The samples are plated out using the 1 in 10 dispersion. Tryptone bile X-glucuronide medium is used for the tests and incubated at 44°C for 18 hours. No resuscitation time at 37°C is given to the plates. The plates are then examined for the presence of blue/green colonies. Presumptive colonies are confirmed using the API 20E system.
3. ISO 6579 Microbiology – General guidance on methods for the detection of *Salmonella*
4. ISO 7937 Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of *Clostridium perfingens* – Colony count technique.
5. ISO 6888 Microbiology – General guidance for enumeration of *Staphylococcus aureus* – Colony count technique.

4. DURATION OF TESTS:

The tests commenced on the 2004-08-20 and were completed on 2004-08-27.

5. MICROBIOLOGICAL RESULTS:

Sample	Total bacterial count/g	*E. coli*/g	*Salmonella*	*S. aureus*	*Clostridium*
BIO – TREAT POWDER	1055 000 000	10	PRESENT	ND	ND

NOTE: ND = Not detected

R. ROOS
MICROBIOLOGY DEPARTMENT

A. OLIVIER
MICROBIOLOGIST (ANALYST)

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