Toxicity Specialists

TOXICITY TEST REPORT

For:
GIY Hydroponics

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Survey:
2022-05

Report reference:
GIY-SOP-A-22_TOX

Revision:
0

Project:
Southern Proteins

Samples:
Boiler ash

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Technical Signatory

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1. ANALYSES REQUESTED AND SAMPLE INFORMATION

Enclosed please find Test Certificate of analysis number GIY-SOP-A-22_TOX. The results relate only to the sample(s) tested. BioToxLab does not accept responsibility for any matters arising from the further use of the results. Tests marked "Not SANAS accredited" (NA or OS) in this Certificate of Analyses are not included in the SANAS Schedule of Accreditation for this Laboratory.

No part of this Certificate of Analyses may be quoted in isolation of the rest of the text without the written permission of BioToxLab. Opinions and Interpretations expressed herein are outside the scope of SANAS accreditation.

Please contact the Laboratory if further information is required.

Table 1: Analyses requested and description for the different samples, including sampling and delivery dates.

Sample name	Sampling date	Sample type (water, sediment, product etc)	Sampled by	Delivery date	Delivered by	Additional comments (sample description or deviations)	Screening	Definitive	*Daphnia magna*	*Poecilia reticulata*	*Allivibrio fischeri*	*Selenastrum capricornutum*	Phyto seeds	Ostracod
										Water			Sediment	
Boiler ash	2022/05/13	Leachate	GIY Hydroponics	2022/05/20	Client	Delivered >3 days after sampling		X	X	X		X		

Key:
Screening = 100% (undiluted) sample tested only
Definitive = Series of sample dilutions tested to enhance classification accuracy and to determine safe dilution

2. METHODOLOGY

Sampling and sample handling

Samples were analysed as received from the Client, following the standard leachate procedure as requested (1:10). The sample received from GIY Hydroponics was exposed as a definitive on 4 trophic levels (*Selenastrum capricornutum*, *Spirodela polyrhiza, Daphnia magna* and *Poecilia reticulata*).

Test Conditions

All toxicity tests were conducted in environmentally controlled rooms using standard techniques.

Quality Assurance

The BioToxLab Aquatic Toxicology Laboratory's Policy and Quality Manual, intended to support and maintain all aspects of the Quality System, is based on the application of ISO/IEC 17025. The following Quality Assurance information can be made available on request (1) inhouse reference toxicant test data and control charts (2) Proficiency Testing Scheme (PTS) test data (3) lot and batch numbers (4) raw toxicity test data.

Assessments

Given the limitations of substance-specific assessments, and the risk of allowing ecological toxicity hazards to go unchecked/undetected, water resource managers and scientists have for some time called for methodologies that will allow more complete assessment s of ecological toxicity hazards to be used in addition to the substance-specific approach. The National Water Act (Act no. 36 of 1998), providing for water in sufficient quantity and in sufficient quality for basic human needs and for maintenance of aquatic ecosystem function, implemented an approach known as the Direct Estimation of Ecological Effect Potential (DEEEP) protocol as a means of circumventing the shortcomings of direct toxicant monitoring. This protocol consists of a battery of tests to directly assess lethal (acute) and sub-lethal (chronic) toxicity, using test organisms from a range of trophic levels. These toxicity tests can demonstrate whether contaminants are bioavailable, it can evaluate the aggregate toxic effects of all contaminants in the medium and it can evaluate the toxicity of substances whose biological effects may not have been well characterized.

Lethal or sub-lethal toxicity testing (as applied for this assessment) is applied by exposing biota to water sources in order to determine the potential risk of such a water to the biota/biological integrity of the receiving water bodies and the

environment. A risk category is determined based on the percentage of mortalities (lethal) or inhibition (sub-lethal) the exposed biota. It is important to note that the hazard classification is based on the standardised battery of selected test biota and therefore represents the risk/hazard towards similar biota in the receiving aquatic environment. The toxicity hazard is therefore in terms of the aquatic biotic integrity and does in no way represent toxicology towards humans or other mammals.

Physical and chemical properties as required to be performed by the standard toxicity methods are also presented in this report as supplementary data to the toxicity testing data.

Standard, internationally accepted methods and materials were applied in order to conduct lethal and sub-lethal toxicity testing.

Selenastrum capricornutum sub-lethal growth inhibition test (A)

Synonym:	*Raphidocelis subcapitata; Pseudokirchneriella subcapitata*
BioToxLab method number:	QM7.2/TMH-02
Standard method:	SANS 8692:2015
Deviation from the method:	None
Test endpoint:	EC_{20}/EC_{50}
Exposure period:	72-hours
Test chamber type:	10cm path length long cells
Test sample volume:	25 mL
Number of replicates per sample:	3
Test temperature (21-25°C):	22.7 – 24.7°C
Test organism species name and source:	*Selenastrum capricornutum*, Printz algae beads (CCAP 278/4 Cambridge, UK)
Optical density measurement:	Jenway 6300 Spectrophotometer
Algal beads batch number(s):	SC 260422
Matrix dissolving batch number(s):	MD 190721
Nutrient batch number(s):	A: SC080222 B: SC240821 C: SC240821 D: 240821
Statistical methods used:	Microsoft Excel® spreadsheet formulated by supplier (MicroBioTests Inc., Belgium) – RegTox and Regression analysis
Date(s) of performance of the test(s):	2022.05.31
Uncertainty of measurement:	Available on request
Validity (from Regtox sheet: cell density factor ≥67):	Yes

Spirodela polyrhiza sub-lethal *toxicity test* (A)

BioToxLab method number:	QM7.2/TMH-15
Standard method:	ISO 20227: 2017
Deviation from the method:	None
Test endpoint:	EC_{20}/EC_{50}
Exposure period:	72-hours
Test chamber type:	Polystyrene plates (9x13 cm) with 48 wells (1 mL)
Test sample volume:	1 mL
Number of replicates per sample:	8
Test temperature (24-26°C):	25°C
Test organism species name and source:	*Spirodela polyrhiza* – Turions obtained from MicrobioTests test kit
Area measurement:	Image J from photograph taken of test plate
Spirodela batch number(s):	SPP 210122
Steinberg medium batch number(s):	SM 170322
Statistical methods used:	Microsoft Excel® spreadsheet formulated by supplier (MicroBioTests Inc., Belgium) – RegTox and Regression analysis
Date(s) of performance of the test(s):	2022.06.03
Uncertainty of measurement:	Available on request
Validity (mean growth of first fronds in cups of control column after 3 days incubation at 25°C and under 6000lux illumination ≥10mm²):	$18.23mm^{2e}$

Daphnia magna lethality toxicity test (A)

BioToxLab method number:	QM7.2/TMH-03
Standard method:	SANS 6341: 2015
Deviation from the method:	None
Test endpoint:	LC_{10}/LC_{50}
Exposure period:	24- and 48-hours
Test chamber type:	Polycarbonate test plates (6 rinsing wells and 24 testing wells)
Test sample volume:	25 mL
Number of replicates per sample:	4
Number of test organisms per chamber:	5
Test temperature (20-22°C):	21°C
Test organism species name, age & source:	*Daphnia magna* – ephippia obtained from MicroBiotests, <24h old
Feeding frequency during testing:	None
Ephippia batch number(s):	DM260122
ISO media batch number(s):	ISO191121
Statistical methods used:	Microsoft Excel®
Date(s) of performance of the test(s):	2022.05.30
Uncertainty of measurement:	Available on request
Validity criteria (control mortality≤10%):	0%

Poecilia reticulata lethality toxicity test (A)

BioToxLab method number:	QM7.2/TMH-04
Standard method:	SANS 7346-1: 2013
Deviation from the method:	None
Test endpoint:	LC_{10}/LC_{50}
Exposure period:	96-hours
Test chamber type:	250 mL disposable polystyrene cups
Test sample volume:	200 mL
Number of replicates per sample:	2
Number of test organisms per chamber:	6
Test temperature (20-22°C):	22°C
Test organism species name, age & source:	*Poecilia reticulata* – 7-21 days old. Obtained from external stock
Feeding frequency during testing:	None
ISO media batch number(s):	ISO191121
Statistical methods used:	Microsoft Excel®
Date(s) of performance of the test(s):	2022.06.02
Uncertainty of measurement:	Available on request
Validity criteria (control mortality≤10%):	0%

Physical and chemical properties

Parameter	BioToxLab Method number	Test temperature (25°C±3°C)	Instrument	Batch number(s)	Date(s) of test(s)
pH (A)	QM7.2/TMC-05	24.2°C	HQ440d	pH4: A0147 pH7: A0225 pH10: A1239	2022.05.25
EC (A)	QM7.2/TMC-06	24.2°C	HQ440d	1413µS/m: A1306	2022.05.25
Dissolved oxygen	QM7.2/TMC-07	24.2°C	HQ440d	N/A	2022.05.25

Uncertainty of measurement for accredited (A) methods available on request

3. HAZARD CLASSIFICATION METHODOLOGY

The toxicity unit (TU) for each test performed is calculated as 100% (full strength effluent expressed as percentage) divided by the effective concentration or LC_{50} expressed as percentage sample dilution (e.g. *Daphnia magna* and *Poecilia reticulata* lethal toxicity tests) and EC_{50} (e.g. *Selenastrum capricornutum* and *Spirodela polyrhiza* growth inhibition tests) (Tonkes & Baltus, 1997) (Table 2). If there is insufficient toxicity in a sample to allow for the determination of an EC_{50}/LC_{50} value, then a toxicity unit of <1 will be assigned to the sample.

Table 2: Toxicity Units (Tonkes and Baltus, 1997)

Toxicity Unit	Conclusion/Description
<1	Limited to no toxicity
1 – 2	Negligibly toxic
2 – 10	Mildly toxic
10 – 100	Acutely toxic
> 100	Highly toxic

A risk/hazard category is determined by using a hazard classification system developed by Persoone *et al.* (2003) whereby one can classify sites using the toxicity data of the non-diluted samples. The percentage effect (PE) of toxicity (mortalities, growth inhibition, luminescence inhibition) is used to rank the sample into one of five classes (Table 3 – effluent/waste samples) based on the highest toxic response obtained in at least one of the tests applied.

Table 3: Hazard classification system for definitive samples (effluents/wastes)

Class	Symbol	Hazard rating	PE	Percentage effect
I	☺	No lethal/sub-lethal hazard	≤10/20%	None of the tests show a toxic effect (i.e. an effect value that is significantly higher than that noted in the controls)
II	☹	Slight lethal/sub-lethal hazard	10/20%≤PE<50%	A statistically significant (P<0.05) PE is reached in at least one test, but the effect level is below 50% (TU<1)
III	☠	Lethal/sub-lethal hazard	50%≤PE<100%	The 50% effect level is reached or exceeded in at least one test but the effect level is below 100% (1≤TU<10)
IV	☠☠	High lethal/sub-lethal hazard	PE 100% in at least one test	The 100% effect is reached exceeded in at least one test (10≤TU<100)
V	☠☠☠	Very high lethal/sub-lethal hazard	PE 100% in all tests	The 100% effect is reached or exceeded in all the tests applied (TU≥100)

Each sample is furthermore weighted (Table 4) according to its relative toxicity level (out of 100%). Higher values indicate that more of the individual tests indicated toxicity within a specific class.

Table 4: Weight score allocation for each test type (Persoone *et al.* (2003))

Score	Category
0	No significant toxicity effect
1	Significant toxicity effect < PE50
2	Toxicity effect >PE50 but <PE100
3	The PE100 is reached

Class weight score calculated as follows:
Class weight score = ($\sum$ all test scores)/n)   where n is the number of tests performed
Class weight score % = (class score) / (maximum class weight score) x 100

EP (Percentage effect) = an effect measured either as mortality or inhibition (depending on the type of test). A >10% effect is regarded as slight lethal toxicity for *Daphnia* and *Poecilia* while a >20% effect is regarded as slight sub-lethal toxicity for *Selenastrum* and *Spirodela*. A 50% effect is regarded as a lethal/sub-lethal toxicity for all of the tests (*Daphnia*, *Poecilia*, *Selenastrum* and *Spirodela*)

The toxicity hazard for each <u>dilution level</u> used to assess the hazard and perform calculations and classifications are presented in Table 5 in order to asses/review data trends and are done according to the following scale:

Table 5: Hazard class per dilution level scale

Scale	Description
0-≤10% (*Daphnia, Poecilia*) 0-≤20% (*Aliivibrio, Selenastrum, Spirodela*)	Not toxic
10-<50 (*Daphnia, Poecilia*) 20-<50 (*Aliivibrio, Selenastrum, Spirodela*)	Slightly toxic
50-<100 (*Daphnia, Poecilia, Aliivibrio, Selenastrum, Spirodela*)	Toxic
≥100 (*Daphnia, Poecilia, Aliivibrio, Selenastrum, Spirodela*)	Highly toxic

4. RESULTS AND HAZARD CLASSIFICATION DATA

Table 6: Hazard classification of leachate <u>sample per test</u>

Site/ sample	Ducweed (A) *Spirodela polyrhiza*			Crustacea (A) *Daphnia magna*			Vertebrates (A) *Poecilia reticulata*			Weight %
	% effect	TU	Test score	% effect	TU	Test score	% effect	TU	Test score	
Boiler ash	-68.34	1.4	2	-100	1.4	2	-100	1.4	2	24

Table 7: Hazard classification of leachate <u>sample dilutions</u> (as per Table 5 methodology)

Sample name	Dilution level	Toxicity hazard	Sample name	Toxicity hazard	Sample name	Toxicity hazard
D. magna Boiler ash	100%	Highly toxic	*P. reticulata* Boiler ash	Highly toxic	*S. polyrhiza* Boiler ash	Toxic
	50%	Not toxic		Not toxic		Slightly toxic
	25%	Not toxic		Not toxic		Slightly toxic
	10%	Not toxic		Not toxic		Not toxic
	1%	Not toxic		Not toxic		Not toxic

Table 8: Site hazard classification of the leachate <u>sample</u>

Results		Boiler ash
Water quality	Test date yy/mm/dd	2022.05.25
	pH @ 25°C (A)	11.2
	EC (Electrical conductivity) (mS/m) @ 25°C (A)	48.9
	Dissolved oxygen (mg/l) (NA)	6.6
***S. polyrhiza* (duckweed) (A)**	Test started on yy/mm/dd	2022.06.06
	%72hour inhibition (-) / stimulation (+) (%)	**-68**
	EC/LC20 (72hours)	**24**
	EC/LC50 (72hours)	**71**
	Toxicity unit (TU) / Description	1.4
***S. capricornutum* (micro-algae) (A)**	Test started on yy/mm/dd	2022.05.31
	%72hour inhibition (-) / stimulation (+) (%)	**
	EC/LC20 (72hours)	**
	EC/LC50 (72hours)	**
	Toxicity unit (TU) / Description	**
***D. magna* (waterflea) (A)**	Test started on yy/mm/dd	2022.05.30
	%48hour mortality rate (-%)	**-100**
	EC/LC10 (48hours)	**53**
	EC/LC50 (48hours)	**74**
	Toxicity unit (TU) / Description	1.4
***P. reticulata* (guppy) (A)**	Test started on yy/mm/dd	2022.06.02
	%96hour mortality rate (-%)	**-100**
	EC/LC10 (96hours)	**51**
	EC/LC50 (96hours)	**73**
	Toxicity unit (TU) / Description	1.4
Estimated safe dilution factor (%) [for definitive testing only]		**24**
Overall classification - Hazard class*		Class III - Lethal/sub-lethal hazard
Weight (%)		100

Key:

% = for definitive testing, only the 100% concentration (undiluted) sample mortality/inhibition/stimulation is reflected by this summary table. The dilution series results are considered for EC/LC values and Toxicity unit determinations

**** = Algal test result inconclusive due to interference (caused by a coloured precipitate forming during testing). As the degree of inhibition/stimulation is unknown, individual test result was not used for overall hazard classification.

***** = The overall hazard classification takes into account the full battery of tests and is not based on a single test result. Note that the overall hazard classification is expressed as both lethal and sub-lethal levels of toxicity hazards.

Weight (%) = relative toxicity levels (out of 100%), higher values indicate that more of the individual tests indicated toxicity within a specific class

site/sample name shaded in orange = definitive test

Site	Hazard classification			Percentage Effect
Boiler ash	III	☠	Lethal/sub-lethal hazard	The 50% effect level is reached or exceeded in at least one test but the effect level is below 100% (1≤TU<10)

Based on the lethal (*D. magna* & *P. reticulata*) and sub-lethal (*S. polyrhiza*) results obtained on the 100% sample, the leachate of sample Boiler ash could be classified as Hazard Class III (i.e.the 50% effect level is reached or exceeded in at least one test but the effect level is below 100%).

5. COMMENTS

One sediment sample was delivered to the BioToxLab office on 2022.05.20. The pH level of the leachate of the sample was 11.15, which is above the acceptable range (pH 6-9) in which pH cannot be excluded as a driving factor for toxicity (USEPA, 1996). The conductivity (ECs) of the leachate of the sample was 48.9 mS/m. A dissolved oxygen (DO) concentration above 4 mg/L is required for aquatic organisms (USEPA, 1996) to survive. The DO level for the leachate of the sample was 6.62 mg/L.

Any queries regarding the results can be lodged with Lizet Swart within 14 days from the date of receiving this report after which the samples will be discarded. It is not advised to use these samples for any retesting other than range confirmation of chemical parameters – re-sampling must be done in the case of any queries relating to the results associated with the samples.

6. REFERENCES

DEPARTMENT OF WATER AFFAIRS AND FORESTRY, 2003. The Management of Complex Industrial Waste Water Discharges. Introducing the Direct Estimation of Ecological Effect Potential (DEEEP) approach, a discussion document. Institute of Water Quality Studies, Pretoria.

EVS-EN 12457-2:2003. Characterisation of waste – leaching – compliance test for leaching of granular waste materials and sludges – part 2: One stage batch test at a liquid to solid ration of 10l/kg for materials with particle size below 4mm (without or with size reduction).

INTERNATIONAL STANDARD ISO 20227:2017. Determination of the growth inhibition effects of waste waters, natural waters and chemicals on the duckweed *Spirodela polyrhiza* – Method using a stock culture independent microbiotest

PERSOONE G, MARSALEK B, BLINOVA I, TöRöKNE A, ZARINA T, MANUSADZIANAS L, NALECZ-JAWECKI G, TOFAN L, STEPANOVA L, TOTHOVA L, KOLAR B (2003). A practical and user-friendly toxicity classification system with Microbiotests for natural waters and wastewaters (personal communication).

SOUTH AFRICAN NATIONAL STANDARD, (SANS), ISO/IEC 17025:2017. General requirements for the competence of testing and calibration laboratories. 3rd Edition. South African Bureau of Standards, Pretoria.

SOUTH AFRICAN NATIONAL STANDARD, SANS 6341:2015. "Water quality – Determination of the inhibition of the mobility of *Daphnia magna* Straus (*Cladocera, Crustacea*) – Acute toxicity test.

SOUTH AFRICAN NATIONAL STANDARD, SANS 8692: 2015. "Water quality – Fresh water algal growth inhibition test with unicellular green algae

SOUTH AFRICAN NATIONAL STANDARD, SANS 7346-1:2013. "Water quality – Determination of the acute lethal toxicity of substances to a freshwater fish [*Brachydanio rerio* Hamilton-Buchanan (Teleostei, Cyrinidae) Part 1: Static method – also applicable to *Poecilia reticulata* (Teleostei, Poeciliidae)

TONKES M. and BALTUS C.A.M. 1997. Praktijkonderzoek aan complexe efflenetenmet de Totaal Effluent Milieubezwaarlikheid (TEM) – metodiek. RIZA – rapportnummer 97.033. RIZA, Lelystad, The Netherlands.

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY (USEPA), 1996. Ecological effects test guidelines. Fish acute toxicity test, Freshwater and marine. OPPTS 850.1075.

END OF REPORT