

## TOXICITY TEST REPORT

**For:**  
GroundTruth

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## 1. Analyses requested and sample description

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

Sample name-B3:P13	Sampling date	Sample type (water, sediment, product etc)	Sampled by	Delivery date	Delivered by	Additional comments (sample description or deviations)	Tests requested - Marked with X							
							Screening	Definitive	Water				Sediment	
									<i>Daphnia magna</i>	<i>Poecilia reticulata</i>	<i>Allivibrio fischeri</i>	<i>Selenastrum capricornutum</i>	Phyto seeds	Ostracod
SW1	2021.12.14	Water	GroundTruth	2021.12.20	GroundTruth	Delivered >3 days after sampling Slightly discoloured		X	X	X	X	X		
SW2	2021.12.14	Water	GroundTruth	2021.12.20	GroundTruth	Delivered >3 days after sampling		X	X	X	X	X		
SW3	2021.12.14	Water	GroundTruth	2021.12.20	GroundTruth	Delivered >3 days after sampling		X	X	X	X	X		
CS3	2021.12.14	Water	GroundTruth	2021.12.20	GroundTruth	Delivered >3 days after sampling Slightly discoloured		X	X	X	X	X		
CS4	2021.12.14	Water	GroundTruth	2021.12.20	GroundTruth	Delivered >3 days after sampling		X	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

### 2.1 Sampling and sample handling

Samples were analysed as received from the Client. QM7.3/SOP-09 (guidance document for sampling) is available on request.

### 2.2 Bio-toxicity assessments

Acute (and short-chronic/chronic) toxicity testing (as applied for this assessment) is applied by exposing biota to water and sediment sources in order to determine the potential risk of such waters to the biota/biological integrity of the receiving water bodies and environment. A risk category is determined based on the percentage of mortalities (or inhibition-stimulation) of 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.

Standard, internationally accepted methods and materials were applied for the water samples in order to conduct acute and short-chronic toxicity testing and hazard classification based on 4 trophic levels (4 taxonomic groups, namely *Allivibrio fischeri* (bacteria), *Daphnia magna* (crustaceans), *Selenastrum capricornutum* (micro-algae) and *Poecilia reticulata* (fish)) at each of the selected sites/samples.

All tests were conducted in environmental controlled rooms using the following internationally standardized methods:

#### 2.2.1 *Allivibrio fischeri* bioluminescent test (A)

BioToxLab method: QM7.2/TMH-01; Standard method: SANS 11348-3:2013

Deviation from method: None

Exposure period: 15 and 30 minutes

Test sample volume: 500 µl

Number of replicates: 2

Test temperature: 14 - 16°C

Measurement equipment: Luminoscan TL, Hygiena Monitoring System

Test endpoint: Definitive test - EC<sub>20</sub> and EC<sub>50</sub> -values

Statistical method used: Manual plotting – Normalized regression of relevant data points

Batch numbers/expiry dates: VF 200505 / 2023-12; RD 200505 / 2023-12; SD 200505 / 2023-02

SD 200505 / 2023-09

Uncertainty of measurement available on request

Correction factor (validity of test): 1.00/1.20/1.10 (valid if between 0,6 & 1,8)

### 2.2.2 *Selenastrum capricornutum* growth inhibition test (A)

BioToxLab method: QM7.2/TMH-02; Standard method: SANS 8692:2015  
Deviation from method: None  
Test species: *Selenastrum capricornutum*, Printz (CCAP 278/4 Cambridge, UK)  
Exposure period: 72h  
Test sample volume: 25 ml  
Test chamber type: 10 cm long cell  
Algae batch number: SC181121  
Matrix dissolving batch number: MD110620  
Bead batch number: A-SC190321; B-SC190321; C-SC190321; D-SC190321  
Number of replicates: 3  
Test temperature: 21 - 25°C  
Measurement equipment: Jenway 6300 spectrophotometer  
Test endpoint: Definitive test - EC<sub>20</sub> and EC<sub>50</sub> values  
Statistical method used: EXCEL spread sheet formulated by supplier (MicroBioTests Inc., Belgium) - RegTox  
Uncertainty of measurement available on request  
Validity of test: Yes (valid if cell density factor  $\geq 67$ )

### 2.2.3 *Daphnia magna* acute toxicity test (A)

BioToxLab Method: QM7.2/TMH-03; Standard method: SANS 6341:2015  
Deviation from method: None  
Test species: *Daphnia magna*  
Test species age: Less than 24h old  
Exposure period: 24 and 48h  
Test sample volume: 25 ml  
Number of test organisms per well: 5  
Replicate number of wells per sample: 4  
Test temperature: 21  $\pm$  2°C  
Test endpoint: Definitive test – LC<sub>10</sub> and LC<sub>50</sub> values  
Statistical method used: Graphical interpolation by linear regression of relevant data points, EXCEL spread sheet  
Batch numbers: Ehippia – DM230921; ISO control medium – ISO070621  
Uncertainty of measurement available on request  
Control mortality/immobility rate (validity of test): 0% (valid if  $\leq 10\%$ )

### 2.2.4 *Poecilia reticulata* acute toxicity test (A)

BioToxLab Method: QM7.2/TMH-04; Standard method: SANS 7346-1:2013  
Deviation from method: None  
Test species: *Poecilia reticulata*  
Test species age: Less than 21 days  
Exposure period: 96h  
Test sample volume: 200 ml  
Number of test organisms per beaker: 6  
Replicate number beakers per sample: 2  
Test temperature: 21 $\pm$ 2°C  
Test endpoint: Definitive test – LC<sub>10</sub> and LC<sub>50</sub> values  
Statistical method used: Graphical interpolation by linear regression of relevant data points, EXCEL spread sheet  
Batch numbers: Control medium – ISO191121  
Uncertainty of measurement available on request  
Test validation: 0% control mortalities (valid if  $\leq 10\%$ )

### 2.2.5 pH (A)

BioToxLab Method QM7.2/TMC-05

Test temperature: 25°C±3°C

Instrument used: HQ440D multimeter

Uncertainty of measurement available on request

Batch numbers of buffers: pH4 – A0147                      pH7 – A0114                      pH10 – A0150

### 2.2.6 Electrical conductivity (A)

BioToxLab Method QM7.2/TMC-06

Test temperature: 25°C±3°C

Instrument used: HQ440D multimeter

Uncertainty of measurement available on request

Batch numbers of buffers: 1413µS/cm - A1181

#### Quality assurance

The following quality assurance information would be made available on request:

- In-house reference toxicant test data and control charts.
- Additional lot, batch numbers and raw test data.
- Participation in proficiency testing scheme and interlaboratory and intra-laboratory comparisons (SABS, NLA)

### 2.3 Toxicity test results classification system

A risk/hazard category was determined by application of the DEEEP<sup>1</sup> DWA recommended protocols and is broadly based on the hazard classification system of Persoone *et.al.* (2003). This risk category equates to the level of acute/chronic risk posed by the selected potential pollution source (water sample).

After the determination of the percentage effect<sup>1</sup> (EP), obtained with each of the battery of toxicity tests performed, the sample is ranked into one of the following five classes, based on the highest toxicity unit (TU) found in the battery of definitive testing protocols:

#### Hazard classification system for definitive tests

<b>Class I</b>	<b>No acute/short-chronic environmental toxicity hazard</b> - none of the tests shows a toxic effect (i.e. an effect value significantly higher than that in the control)
<b>Class II</b>	<b>Slight acute/short-chronic environmental toxicity hazard</b> - the percentage effect observed in at least one toxicity test is significantly higher than in the control, but the effect level is below 50% (TU is <1)
<b>Class III</b>	<b>Acute/short-chronic environmental toxicity hazard</b> - the L(E)C50 is reached or exceeded in at least one test, but in the 10 fold dilution of the sample the effect level is less than 50% (1≤TU≤9,99)
<b>Class IV</b>	<b>High acute/short-chronic environmental toxicity hazard</b> - the L(E)C50 is reached in the 10 fold dilution for at least one test, but not in the 100 fold dilution (10≤TU≤99,99)
<b>Class V</b>	<b>Very high acute/short-chronic environmental toxicity hazard</b> - the L(E)C50 is reached in the 100 fold dilution for at least one test (TU is ≥100)

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

<sup>1</sup> DEEEP = Direct Estimation of Ecological Effect Potential. This is a battery of tests that can measure toxicity of complex mixtures based on a set of parameters stemming from the results of effects, even if all constituents are not known. A hazard class is determined based on the resulting parameters of the battery of tests and is considered to be interpretation of the individual results

<sup>1</sup> EP (Percentage effect) = an effect measured either as a mortality rate or inhibition rate (depending on the type of test). A >10% effect is regarded as slight acute toxicity for *Daphnia* and *Poecilia*, while a >20% effect is regarded as slight short-chronic toxicity for *Selenastrum* and *Allivibrio*. A 50% effect is regarded as an acute/short-chronic toxicity for all the tests (*Daphnia*, *Poecilia*, *Selenastrum* and *Allivibrio*).

### 3. Results and discussion

#### 3.1 2021-12 survey - water

Refer to table 2 below for individual test results and overall hazard classification of the different water samples.

**Table 2:** Test results and risk classification for water samples (December 2021).

Results		SW1	SW2	SW3	CS3	CS4
weWater quality	Test date yy/mm/dd	2021.12.21	2021.12.21	2021.12.21	2021.12.21	2021.12.21
	pH @ 25°C (A)	8,7	8,3	7,5	7,9	7,3
	EC (Electrical conductivity) (mS/m) @ 25°C (A)	85,9	76,1	123,7	82,3	36,8
	Dissolved oxygen (mg/l) (NA)	3,74 Adjusted 7,5/7,92	7,5	5,5	2,38 Adjusted 6,58/7,84	3,63 Adjusted 7,31/4,90
A. fischeri (bacteria) (A)	Test started on yy/mm/dd	2022.01.05	2022.01.05	2022.01.04	2022.01.07	2022.01.07
	%30min inhibition (-) / stimulation (+) (%)	<b>38</b>	<b>14****</b>	<b>45****</b>	<b>38</b>	<b>26</b>
	EC/LC20 (30 mins)	n.r.	n.r.	n.r.	n.r.	n.r.
	EC/LC50 (30 mins)	n.r.	n.r.	n.r.	n.r.	n.r.
	Toxicity unit (TU) / Description	<1	<1	<1	<1	<1
S. capricornutum (micro-algae) (A)	Test started on yy/mm/dd	2021.12.21	2021.12.21	2021.12.21	2021.12.21	2021.12.21
	%72hour inhibition (-) / stimulation (+) (%)	<b>2****</b>	<b>-11****</b>	<b>11</b>	<b>7</b>	<b>14</b>
	EC/LC20 (72hours)	n.r.	n.r.	n.r.	n.r.	n.r.
	EC/LC50 (72hours)	n.r.	n.r.	n.r.	n.r.	n.r.
	Toxicity unit (TU) / Description	<1	<1	<1	<1	<1
D. magna (waterbug) (A)	Test started on yy/mm/dd	2021.12.20	2021.12.20	2021.12.20	2021.12.20	2021.12.20
	%48hour mortality rate (-%)	<b>-15</b>	<b>-5****</b>	<b>0</b>	<b>-5</b>	<b>-10</b>
	EC/LC10 (48hours)	<b>83</b>	n.r.	n.r.	n.r.	n.r.
	EC/LC50 (48hours)	n.r.	n.r.	n.r.	n.r.	n.r.
	Toxicity unit (TU) / Description	<1	<1	<1	<1	<1
P. reifusa (guppy) (A)	Test started on yy/mm/dd	2022.01.13	2022.01.13	2022.01.13	2022.01.13	2022.01.13
	%96hour mortality rate (-%)	<b>-42</b>	<b>-8****</b>	<b>-17****</b>	<b>-8****</b>	<b>0****</b>
	EC/LC10 (96hours)	n.c.	n.r.	n.c.	n.r.	n.r.
	EC/LC50 (96hours)	n.r.	n.r.	n.r.	n.r.	n.r.
	Toxicity unit (TU) / Description	<1	<1	<1	<1	<1
Estimated safe dilution factor (%) [for definitive testing only]		<1	<1	<1	<1	<1
Overall classification - Hazard class***		Class II - Slight acute/short-chronic hazard	Class I - No acute/short-chronic hazard	Class II - Slight acute/short-chronic hazard	Class I - No acute/short-chronic hazard	Class I - No acute/short-chronic hazard
Weight (%)		50	0	25	0	0

**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  
n.r. = not relevant, i.e. the 100% concentration caused less than 10/20/50% (effective concentration) mortalities or inhibition  
n.c. = not calculable, although the 100% concentration led to more than 10/50% mortalities, the 10% mortality rate was exceeded throughout the test  
\*\*\*\* = 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 acute/short-chronic level of toxicity, due to the fact that the *S. capricornutum* (micro-algae) and the *A. fischeri* tests are regarded as short-chronic levels of toxicity tests and the overall classification therefore contains a degree of short-chronic toxicity assessment.  
\*\*\*\* = Despite data used as per standard classification protocol to conduct the overall sample hazard classification as presented in this table, other data trends were noted throughout the dilution series - details are discussed below this table - based on these trends, no safe dilution factors could be established and a different hazard class should be considered based on these trends as discussed  
**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

Using the standard classification protocol where the 100% data effects are used (as presented in the table above), samples SW2, CS3 and CS4 would be classified as having “no acute/short-chronic environmental toxicity hazard” and samples SW1 and SW3 as having a “slight acute environmental toxicity hazard” (Class II). However, the following trends were noted for the different samples, which may result in a Class II classification consideration for samples SW2, CS3 and CS4:

- SW1: The 1.91% micro-algae growth *stimulation* noted for the 100% sample deteriorated to a 34.64% growth *inhibition* for the 0.1% dilution of the sample. A 15% macro-invertebrate mortality effect was noted for the 100% sample with a calculated 83% minimum effect percentage for this trophic level. The 38.21% bacterial light emission *stimulation* deteriorated to a 13.38% light emission *inhibition* on the 0.195% dilution of the sample (above the acceptable ≤20% minimum effect level). A 42% vertebrate mortality effect was noted on the 100% sample with a 25% mortality effect noted on the 0.1% dilution of the sample. Based on the trends noted as discussed, no safe dilution factor can be provided for this sample as a trend of increasing toxicity effects with increasing dilution levels on certain trophic levels were noted.

- SW2: Although a 10.98% micro algae growth *inhibition* (below the minimum acceptable effect level of  $\leq 20\%$ ) was noted, the growth *inhibition* effect increased to 26.82% on the 50% dilution of the sample, after which it reduced to an acceptable 4.93% *inhibition* level on the 1% dilution of the sample, but then increased significantly to 30.37% *inhibition* on the 0.1% dilution of the sample. A similar trend was noted on the macro-invertebrate level of testing, where an acceptable 5% mortality rate was noted on the 100% sample and 0% on the 50% sample, but then an increase up to 15% was noted on the 10% dilution of the sample, again reducing to 0% on the 0.1% dilution of the sample. The 14.48% light emission *stimulation* effect noted on the bacterial level of testing deteriorated to a 28.69% light emission *inhibition* effect on the 0.195% dilution of the sample. Similarly, the acceptable 8.33% mortality effects noted on the 100% and 50% dilutions, increased to 16.67% on the 25%, 10% and 1% dilutions of the sample, but improved to 0% on the 0.1% dilution. Based on the data trends noted above, no safe dilution factor can be estimated for this sample.
- SW3: No significant toxicity effects were noted on the micro-algae or the macro-invertebrate levels of testing. The 45.01% bacterial light emission *stimulation* effect noted on the 100% sample deteriorated to a 21.02% light emission *inhibition* on the 0.195% dilution of the sample. The 16.67% mortality rate noted on the 100% sample during the vertebrate level of testing improved to 0% as from the 50% dilution up to the 10% dilution of the sample, however a 25% mortality rate was noted for both the 1% and the 0.1% dilution of the sample. Based on the above data trends, no safe dilution factor can be provided for this sample.
- CS3: No significant toxicity effects were noted on the micro-algae, macro-invertebrate or bacterial levels of testing. However, the 8.3% mortality rate noted on the vertebrate level of testing, deteriorated to 33.33% up to the 0.1% dilution of the sample. No safe dilution factor can therefore be provided for this sample.
- CS4: No significant toxicity effects were noted on the micro-algae or the macro-invertebrate levels of testing. The 25.92% bacterial light emission *stimulation* deteriorated to a -19.55% light emission on the 0.195% dilution of the sample. Although no significant mortality effects were noted on the 100% to 1% dilutions of the sample, a slight 16.67% mortality effect was noted on the 0.1% dilution of the sample.

#### 4. Comments

Low oxygen levels were observed for samples SW1, CS3 and CS4 – the oxygen levels were adjusted during testing as per agreement with GroundTruth in order to comply with standard method testing requirements and to eliminate low oxygen biased toxicity effects from the results presented.

## 5. Literature 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.

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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.

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SOUTH AFRICAN NATIONAL STANDARD, SANS 11348-3: 2013. "Water quality – Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria test). Part 3: Method using freeze-dried bacteria

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, Cyprinidae) Part 1: Static method – also applicable to *Poecilia reticulata* (Teleostei, Poeciliidae)

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