

CITALOPRAM

Acute toxicity testing of Citalopram on the green algae
Pseudokirchneriella subcapitata



Toxicon Report E22-018
Härslöv October 2022



Acute toxicity testing of Citalopram on the green algae *Pseudokirchneriella subcapitata*

This report accounts for the results obtained from “Freshwater Algae and Cyanobacteria, Growth Inhibition Test” performed by Toxicon AB.

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Reference analysis: Ola Svahn (MoLab)

I, the undersigned, hereby declare that this report constitutes a complete, true and accurate representation of the study and its results.

Anders Walstad
.....
Anders Walstad, Study Director

2022-10-31
.....
Date

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Summary

In April 2022, an ecotoxicological test on the test item “Citalopram.HBr” was performed by Toxicon AB, Härslöv, Sweden. The study included one method for ecotoxicological testing. The test item was provided by H. Lundbeck A/S.

The method used was:

- OECD Guidelines for Testing of Chemicals No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test (2011).

The test was performed on freshly prepared dilutions of the test item. The stock solution (40 mg/L) for the algae test (OECD 201) was made using reverse osmosis water. All concentrations mentioned in this report, consider Citalopram without HBr.

Results from the algae test are presented in Table 1.

Table 1. NOEC, E_rC_{10} and E_rC_{50} of *Pseudokirchneriella subcapitata* determined after 72 hours of exposure to the test item Citalopram. NOEC is the highest test concentration that did not display a growth inhibition that was significantly different from the control ($p < 0.05$).

Test item	NOEC (µg/L)	E_rC_{10} (µg/L)	E_rC_{50} (µg/L)
Citalopram	977	1254	3846

Results from the reference analysis (UPLC-MS/MS) indicate that the test substance did not degrade during a 72 hours incubation time. Results from the reference analysis are presented in Table 2.

Table 2. Measured concentrations compared to nominal concentration after 0 hours and 72 hours incubation.

Test item	Incubation time (hours)	Nominal concentration (µg/L)	Measured concentration (µg/L)
Citalopram	0	29.8	25.7
Citalopram	72	29.8	24.2
Citalopram	0	10000	10217.6
Citalopram	72	10000	12703.8

Introduction

A sample of the product Citalopram.HBr was received at Toxicon AB on April 6th, 2022 for ecotoxicological testing. The test was performed according to OECDs Guidelines for testing of chemicals. All concentrations mentioned in this report, consider Citalopram without HBr.

Test item

Chemical name:	Citalopram.HBr
CAS number:	59729-23-7
Batch number:	2001976
Purity:	99.9 %
Appearance:	White to off-white, solid
Molecular formula:	$C_{20}H_{21}FN_2O \cdot HBr$
Relative molecular mass:	405.31 g/mole
Water solubility:	Approximately 30 mg/ml
Stability:	Stable at normal temperature and pressure
Expiry date:	2025-03-24
Toxicon identification code:	E22-017-1
Storage conditions:	Room temperature, in dark storage.

The test item was provided by H. Lundbeck A/S.

Test duration

Experimental starting date: April 25th, 2022.

Experimental completion date: April 28th, 2022.

Test methods

The method used was:

- OECD Guidelines for Testing of Chemicals No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test (2011). Toxicon SOP T 003-9.

Freshwater Alga and Cyanobacteria, Growth Inhibition Test – OECD 201

The purpose of the test is to determine the effects of a substance on the growth of a freshwater microalgae. Exponentially growing cultures of algae are exposed to a range of concentrations of the test item over a period of 72 hours. Despite of the relatively brief test duration, effects over several generations can be assessed. The response variable is the reduction of growth in a series of algal cultures (test units) exposed to various concentrations of a test substance. The response is evaluated as the function of the exposure concentration in comparison to the average growth of an unexposed control culture. Growth, and growth inhibition, are quantified from measurements of the algal biomass (dry weight per volume) as a function of time. In this study, cell counts (cells/ml) are used as a surrogate parameter to dry weight (OECD TG No. 201). The test endpoint is inhibition of growth, which is expressed as the logarithmic increase in biomass (average specific growth rate) during the period of exposure.

All test vessels contain 10 000 cells per mL at the test start.

Deviations from the standard OECD TG No. 201

There was no deviation from the standard OECD TG No. 201:

Test organism

The test organism is a laboratory culture of the green alga *Pseudokirchneriella subcapitata*. The inoculum is taken from Toxicon's culture of *P. subcapitata* (NIVA CHL 1), which originates from NIVA, Oslo, Norway. A qualification test using potassium dichromate was performed on December 3rd, 2021, to assure the quality of the test organism. E_rC_{50} was found to be between 0.9 and 1.5 mg of potassium dichromate per litre (1.4 mg/l), which confirmed that the culture was suitable as a test organism according to OECD TG No. 201.

Growth medium (dilution solution)

The composition of the growth medium was as specified in Annex 3 in OECD TG No. 201.

Recipe for the growth medium is presented in Table 3.

The growth medium had a pH of 8.1 ± 0.2 .

Table 3. Preparation of the growth medium. The substances were added to reversed osmosis water, $<10 \mu\text{S}/\text{cm}$.

Single substance	$\mu\text{g}/\text{L}$	Single substance	$\mu\text{g}/\text{L}$
NH_4Cl	15000	H_3BO_3	185
$\text{MgCl}_2 \times 6 \text{ H}_2\text{O}$	12000	$\text{MnCl}_2 \times 4 \text{ H}_2\text{O}$	415
$\text{CaCl}_2 \times 2 \text{ H}_2\text{O}$	18000	ZnCl_2	3
$\text{MgSO}_4 \times 7 \text{ H}_2\text{O}$	15000	$\text{CoCl}_2 \times 6 \text{ H}_2\text{O}$	1,5
KH_2PO_4	1600	$\text{CuCl}_2 \times 2 \text{ H}_2\text{O}$	0.01
$\text{FeCl}_3 \times 6 \text{ H}_2\text{O}$	64	$\text{Na}_2\text{MoO}_4 \times 2 \text{ H}_2\text{O}$	7
$\text{Na}_2\text{EDTA} \times 2 \text{ H}_2\text{O}$	100	NaHCO_3	50000

Preparation of test solutions

A fresh stock solution was prepared at room temperature ($20\text{--}22^\circ\text{C}$) in normal office lighting. The test item was weighed in on a weighing boat and then applied to a glass bottle containing reverse osmosis water. The stock solution (40 mg Citalopram per Litre) was stirred at a speed that created a small vortex on the surface (approximately 100 rpm) until the test item was dissolved. The stock solution was subsequently diluted into the different test concentrations. Part of the diluted test item was used for ecotoxicological testing, and part was stored in freezer for future chemical analysis.

The nominal test concentrations were 29.8; 95.4; 305; 977; 3125 and 10000 $\mu\text{g}/\text{L}$. All concentrations mentioned in this report, consider Citalopram without HBr.

Ecotoxicological testing

The nutrient concentration in the growth medium was the same in the control as in the test solution. Six replicates each were used for the control while the test concentrations were exposed in triplicates. Test bottles were 250 ml glass E-flasks with 100 ml solution, incubated on a shaking table with approximately $100 \mu\text{E m}^{-2} \text{ s}^{-1}$ continuous illumination at a temperature of $21\text{--}24 \pm 2^\circ\text{C}$. The pH (SS-EN ISO 10523) was measured in all solutions at the test start, and again in all replicates after 72 hours. The incubation period was 72 hours with sampling at 24, 48, and 72 hours. As a measurement of the biomass in the samples, the number of cells/ml was counted using an optical microscope.

Calculations/Statistics

For the test results to be acceptable in all the OECD member countries, both the response variables *Average growth rate* and *Yield* are calculated and presented.

Average growth rate

Cell count is one of the proposed surrogate parameters for biomass according to the OECD TG No. 201. The average specific growth rate (μ) for a specific period is calculated as the logarithmic increase in the biomass from the equation for each single vessel of the control and test concentrations:

$$\mu = (\ln \text{ cells/ml } 72 \text{ hrs} - \ln \text{ cells/ml } 0 \text{ hrs}) / 3 \text{ (time in days)}.$$

For the control and test concentrations, an average growth rate along with variance estimates is calculated. The specific growth rate for each test concentration is then related to the average growth rate of the control:

$$\% I_r = \frac{\mu_c - \mu_r}{\mu_c} \times 100$$

where:

- $\% I_r$ = percent inhibition in average specific growth rate;
- μ_c = mean value for average specific growth rate (μ) in the control group;
- μ_r = average specific growth rate (μ) for the test concentration replicate.

Yield

The yield is calculated as the biomass at the end of the test minus the starting biomass for each single vessel of controls and test concentrations. The percent inhibition in yield ($\% I_y$) is calculated for each replicate as follows:

$$\% I_y = \frac{Y_c - Y_T}{Y_c} \times 100$$

where:

- $\% I_y$ = percent inhibition of yield;
- Y_c = mean value for yield in the control group;
- Y_T = value for yield for the treatment replicate.

Normal distribution in the data was confirmed using Levene's test and NOEC was determined by analysis of variance (ANOVA) followed by Tukey/Kramer ($p < 0.05$).

All calculations were performed in Excel.

Reference analysis

The highest and lowest nominal test concentrations were analysed with respect to the measured concentrations and the stability of Citalopram. The analysis was performed using Ultra Performance Liquid Chromatography in combination with tandem mass spectrometry (UPLC-MS/MS) according to the method developed and described by Svahn, O., & Björklund, E. (2016). Samples for analysis were collected at 0 hours and 72 hours exposure.

Results

Freshwater Alga and Cyanobacteria, Growth Inhibition Test – OECD 201

The criteria for the validity of the test according to OECD TG No. 201 were fulfilled with one exception:

- The biomass in the control culture increased by a factor of more than 16 within 72 hours.
- The mean coefficient of variation of average specific growth rates in the control culture did not exceed 35%.
- The coefficient of variation of average specific growth rates during the whole test period in replicate control cultures did not exceed 7%.

No morphological differences in the cells was observed between the control and the various test concentrations.

Growth rate for the control group and the test concentrations (mean $\pm$ coefficient variation) are presented in Table 4 and Table 6. The yield is presented in Table 5 and Table 7. The growth of the algae in the control and in the test concentrations during the 72 hours of exposure is presented in Figure 1 as cells/ml from the cell counting and the growth inhibition (%) is depicted in Figure 2.

Results from the statistical analysis are presented in Table 6 and primary data from the algal growth inhibition test are presented in Appendix 1.

The results indicate that exposure to low concentrations of the test item is stimulating growth of the alga. This could possibly be explained by hormesis, which is defined as a phenomenon in which a harmful substance gives stimulating and beneficial effects to living organisms when the quantity of the harmful substance is small.

Table 4. Growth rate for the control and the loading rate (mean $\pm$ coefficient of variation). Growth inhibition (%) after 72 hours of exposure to the different test concentrations of the test item Citalopram.

Test concentration ($\mu\text{g/l}$)	72 h	
	Specific growth rate (μ) Mean $\pm$ CV (%)	Growth inhibition (%)
Control	1.68 $\pm$ 2.48	-
29.8	1.68 $\pm$ 0.52	0.4
95.4	1.73 $\pm$ 2.58	-2.8
305	1.78 $\pm$ 0.84	-5.8
977	1.65 $\pm$ 5.13	2.1
3125	1.02 $\pm$ 2.32	39.1
10000	0.00 $\pm$ 0.00	100

Table 5. Yield for the control and the loading rate (mean $\pm$ coefficient of variation). Inhibition of yield (%) after 72 hours of exposure to the different test concentrations of the test item Citalopram.

Test concentration $\mu\text{g/l}$	72 h	
	Yield ($\times 10^6$) (Y) Mean $\pm$ CV (%)	Inhibition of yield (%)
Control	1.56 $\pm$ 0.13	-
29.8	1.52 $\pm$ 0.03	2.67
95.4	1.79 $\pm$ 0.13	-15.0
305	2.08 $\pm$ 0.05	-33.4
977	1.42 $\pm$ 0.23	8.64
3125	0.20 $\pm$ 0.07	86.8
10000	0.00 $\pm$ 0.00	100

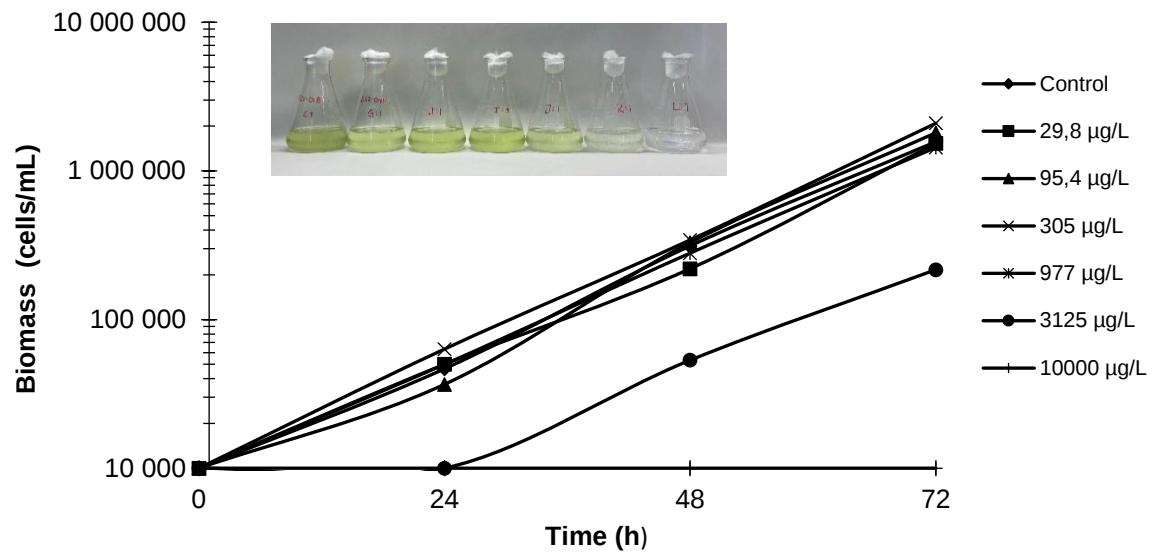


Figure 1. The biomass (cells/ml) in the control and in the test concentrations after 0, 24, 48 and 72 hours of exposure to the test item Citalopram. Concentrations in the legend are nominal. Inserted in the graph is a photograph taken at the end of the 72 hours exposure period. The control (0 µg/L) is to the left in the photograph and the test concentrations are increasing to the right in the photograph with the highest test concentration (10000 µg/L) at the far right.

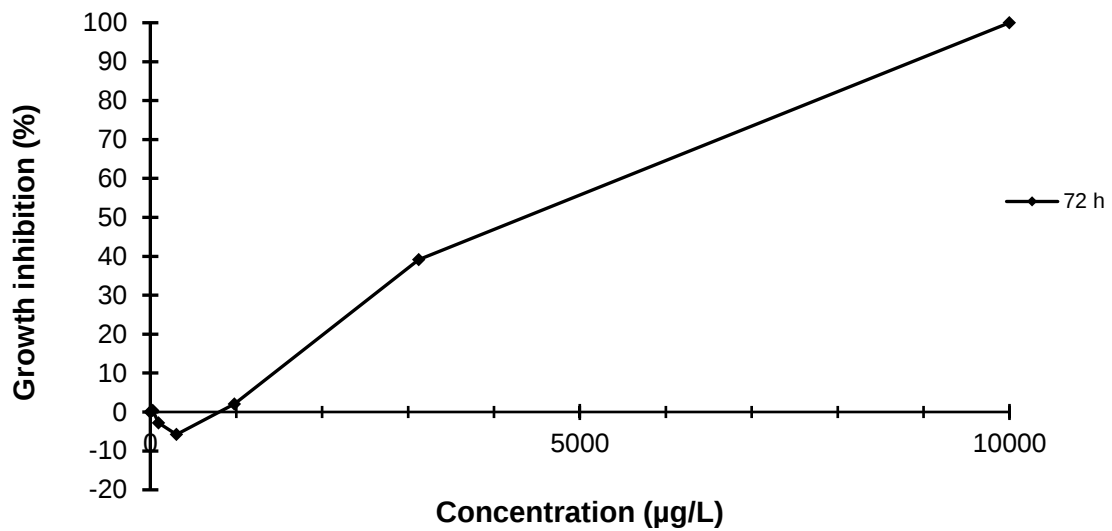


Figure 2. Inhibition (%) of algal growth following 72 hours of exposure of Citalopram at various concentrations.

Table 6. Specific growth rate (μ) of alga following exposure to Citalopram for 72 hours in various concentrations and whether there is a significant difference between control and test concentration.

Specific growth rate of alga after 72 hours exposure to Citalopram							
Test concentrations	Control	29.8	95.4	305	977	3125	10000
	1.71	1.69	1.75	1.77	1.55	1.05	0.00
	1.68	1.67	1.76	1.80	1.70	1.03	0.00
	1.74	1.68	1.68	1.78	1.70	1.00	0.00
	1.67						
	1.62						
	1.67						
Significant difference? (p)	- (-)	No (1.00)	No (0.68)	Yes* (0.05)	No (0.89)	Yes (7.0E-13)	Yes (4.7E-14)

*The specific growth rate in the test concentration is significantly different from the control. However, the difference is due to stimulation of the growth and is not considered a toxic response in this study.

Table 7. Yield (Y, 10^6) of alga following exposure to Citalopram for 72 hours in various concentrations and whether there is a significant difference between control and test concentration.

Yield of alga after 72 hours exposure to Citalopram							
Test concentrations	Control	29.8	95.4	305	977	3125	10000
	1.69	1.56	1.91	2.01	1.04	0.22	0.00
	1.53	1.48	1.95	2.19	1.62	0.21	0.00
	1.86	1.52	1.53	2.05	1.62	0.19	0.00
	1.50						
	1.28						
	1.51						
Significant difference? (p)	- (-)	No (1.00)	No (0.53)	Yes* (0.01)	No (0.93)	Yes (1.0E-07)	Yes (1.2E-08)

*The specific growth rate in the test concentration is significantly different from the control. However, the difference is due to stimulation of the growth and is not considered a toxic response in this study.

Citalopram appears not to have any inhibitory effects on the growth of *Pseudokirchneriella subcapitata* up to a concentration of 977 $\mu\text{g/L}$.

The (nominal) ErC_{50} and ErC_{10} for Citalopram is 3846 $\mu\text{g/L}$ and 1254 $\mu\text{g/L}$ respectively.

Reference analysis

Results from the reference analysis are presented in Table 8 and in Appendix 2. The measured test concentrations indicate that the test substance Citalopram did not degrade during the 72 hours that was the duration of the test.

Table 8. Measured concentrations compared with the nominal test concentration after 0 hours and 72 hours incubation.

Sample	Nominal concentration ($\mu\text{g/L}$)	Measured concentration ($\mu\text{g/L}$)
Citalopram, 0 h	29.8	25.7
Citalopram, 72 h	29.8	24.2
Citalopram, 0 h	10000	10217.6
Citalopram, 72 h	10000	12703.8

References

1. OECD Guideline for testing of chemicals No. 201 “Freshwater Alga and Cyanobacteria, Growth Inhibition Test”. Adopted 2011.
2. SS-EN ISO 10523:2012, Determination of pH.
3. Svahn, O., & Björklund, E. (2016). Increased electrospray ionization intensities and expanded chromatographic possibilities for emerging contaminants using mobile phases of different pH. *Journal of Chromatography B*, 1033, 128-137.

Appendix 1

Protocol for “Freshwater Alga and Cyanobacteria, Growth Inhibition Test”

Titel: Protocol of growth inhibition of micro algae according to OECD TG 201, SS-EN ISO 8692, EU Dir 92/69/EEC		Document no: PROT 034-4	
Approved of: Thomas Olsson	Date: 2010-02-05	Valid from: 2010-02-01	Page of: 1(2)

Date: 220425 to 220428

Project no: E22-018

Test laboratory: Toxicon AB

Test item: Citalopram

Test leader: Anders Walstad

(E22-018-1)

Performance: Susanne Fritz, Anders Walstad

Test species: *Pseudokirchneriella subcapitata*

Clone: NIVA CHL1

Conc. µg/L	pH		Cells/ml (CPML)				(µ) growth rate (h)	% inhibition
	0	72	0 hour	24 hours	48 hours	72 hours		
Control 1	7,91	7,98	10000	30000	340000	1700000	1,71	
Control 2		8,10	10000	40000	340000	1540000	1,68	
Control 3		8,06	10000	40000	410000	1870000	1,74	
Control 4		8,02	10000	80000	210000	1510000	1,67	
Control 5		8,00	10000	30000	190000	1290000	1,62	
Control 6		8,07	10000	60000	390000	1520000	1,67	
mean	7,9	8,0	10000	46667	313333	1571667	1,68	
29,8	7,87	8,11	10000	80000	250000	1570000	1,69	-0,11
		8,07	10000	30000	230000	1490000	1,67	0,93
		8,00	10000	40000	180000	1530000	1,68	0,40
mean	7,9	8,1	10000	50000	220000	1530000	1,68	0,41
95,4	7,91	8,22	10000	40000	270000	1920000	1,75	-4,09
		8,22	10000	30000	320000	1960000	1,76	-4,50
		8,21	10000	40000	400000	1540000	1,68	0,27
mean	7,9	8,2	10000	36667	330000	1806667	1,73	-2,77
305	7,90	8,16	10000	60000	350000	2020000	1,77	-5,10
		8,26	10000	80000	360000	2200000	1,80	-6,79
		8,28	10000	50000	320000	2060000	1,78	-5,49
mean	7,9	8,2	10000	63333	343333	2093333	1,78	-5,79
977	7,91	7,77	10000	20000	190000	1050000	1,55	7,86
		8,07	10000	70000	320000	1630000	1,70	-0,85
		8,17	10000	60000	330000	1630000	1,70	-0,85
mean	7,9	8,0	10000	50000	280000	1436667	1,65	2,05
3125	7,86	7,71	10000	10000	70000	230000	1,05	37,92
		7,67	10000	10000	40000	220000	1,03	38,80
		7,68	10000	10000	50000	200000	1,00	40,69
mean	7,9	7,7	10000	10000	53333	216667	1,02	39,14
10000	7,85	7,63	10000	10000	10000	10000	0,00	100,00
		7,61	10000	10000	10000	10000	0,00	100,00
		7,61	10000	10000	10000	10000	0,00	100,00
mean	7,9	7,6	10000	10000	10000	10000	0,00	100,00
Sign	SF	SF	SF	AWA	AWA	AWA	AWA	AWA

	Graphic	Probit
EC (I) 10:	1253,5	
EC (I) 50:	3846,0	
EC (I) 90:		
NOEC:	977,0	

Observations:

Signature: AWA

Titel: Protocol of growth inhibition of micro algae according to OECD TG 201, SS-EN ISO 8692, EU Dir 92/69/EEC				Document no: PROT 034-4	
Approved of: Thomas Olsson		Date: 2010-02-05		Valid from: 2010-02-01	Page of: 2(2)

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Test laboratory: Toxicon AB

Test item: Citalopram

Test leader: Anders Walstad

(E22-018-1)

Performance: Susanne Fritz, Anders Walstad

Test species: *Pseudokirchneriella subcapitata*

Clone: NIVA CHL1

Conc. µg/L	pH		Cells/ml (CPML)				Yield	% inhibition
	0	72	0 hour	24 hours	48 hours	72 hours		
Control 1	7,91	7,98	10000	30000	340000	1700000	1690000	
Control 2		8,10	10000	40000	340000	1540000	1530000	
Control 3		8,06	10000	40000	410000	1870000	1860000	
Control 4		8,02	10000	80000	210000	1510000	1500000	
Control 5		8,00	10000	30000	190000	1290000	1280000	
Control 6		8,07	10000	60000	390000	1520000	1510000	
mean	7,9	8,0	10000	46667	313333	1571667	1561667	
29,8	7,87	8,11	10000	80000	250000	1570000	1560000	0,11
		8,07	10000	30000	230000	1490000	1480000	5,23
		8,00	10000	40000	180000	1530000	1520000	2,67
mean	7,9	8,1	10000	50000	220000	1530000	1520000	2,67
95,4	7,91	8,22	10000	40000	270000	1920000	1910000	-22,31
		8,22	10000	30000	320000	1960000	1950000	-24,87
		8,21	10000	40000	400000	1540000	1530000	2,03
mean	7,9	8,2	10000	36667	330000	1806667	1796667	-15,05
305	7,90	8,16	10000	60000	350000	2020000	2010000	-28,71
		8,26	10000	80000	360000	2200000	2190000	-40,23
		8,28	10000	50000	320000	2060000	2050000	-31,27
mean	7,9	8,2	10000	63333	343333	2093333	2083333	-33,40
977	7,91	7,77	10000	20000	190000	1050000	1040000	33,40
		8,07	10000	70000	320000	1630000	1620000	-3,74
		8,17	10000	60000	330000	1630000	1620000	-3,74
mean	7,9	8,0	10000	50000	280000	1436667	1426667	8,64
3125	7,86	7,71	10000	10000	70000	230000	220000	85,91
		7,67	10000	10000	40000	220000	210000	86,55
		7,68	10000	10000	50000	200000	190000	87,83
mean	7,9	7,7	10000	10000	53333	216667	206667	86,77
10000	7,85	7,63	10000	10000	10000	10000	0	100,00
		7,61	10000	10000	10000	10000	0	100,00
		7,61	10000	10000	10000	10000	0	100,00
mean	7,9	7,6	10000	10000	10000	10000	0	100,00
Sign	SF	SF	SF	AWA	AWA	AWA	AWA	AWA

Observations:

Signature: *A WA*

Appendix 2

Report from reference analysis.

Sample ID Lab	Sample ID	Citalopram ug/L
Algae test (OECD TG 201)		
22-33-15	ALS E22-018-1 G-0 h 29,8 ug/l 22-04-25	25,7
22-33-16	ALS E22-018-1 L-0 h 10000 ug/l 22-04-25	10217,6
22-33-17	ALS E22-018-1 G-72 h 29,8 ug/l 22-04-28	24,2
22-33-18	ALS E22-018-1 L-72 h 10000 ug/l 22-04-28	12703,8
LOQ (ng/L)		1,2
RS (%)		1,8

Analysis by Ola Svahn (PhD), MoLab, Stridsvagnsvägen 14, Kristianstad (2022-09-28)

Method description: Thawing of frozen samples. Analysis of the samples were performed using chromatography in combination with mass spectrometry (UPLC-MS/MS). The analysis method for citalopram is developed as described by Svahn, O., & Björklund, E. (2016)* and validated according to Standard method 1694 (US EPA, 2007).

*) Svahn, O., & Björklund, E. (2016). *Increased electrospray ionization intensities and expanded chromatographic possibilities for emerging contaminants using mobile phases of different pH. Journal of Chromatography B*, 1033, 128-137.