Molybdenum Consortium

An initiative of the International Molybdenum Association

POSSESSION OF, OR ACCESS TO THIS DOCUMENT, IN NO WAY CONFERS A 'PERMISSION TO REFER' UNDER REACH.

Ecotoxicity of the Molybdate Ion (MoO_4^{2-}) to Eight Freshwater Species

September 2008

Prepared by Gent University, Belgium for:

International Molybdenum Association (**IMOA**)
4 Heathfield Terrace, Chiswick,
London, W4 4JE, UK

Faculty of Bioscience Engineering

Laboratory of Environmental Toxicology and Aquatic Ecology

MOLYTOX

Ecotoxicity of molybdate ion (MoO_4^{2-}) to eight freshwater species

Karel A.C. De Schamphelaere
Lien T.H. Nguyen
Colin R. Janssen

DRAFT FINAL REPORT
Prepared for IMOA

September 3, 2008

Possession of or access to this report in no way confers a 'right to refer' under REACH

Principle investigators: Colin Janssen and Karel De Schamphelaere
Ghent University (UGent)
Laboratory of Environmental Toxicology and Aquatic Ecology
J. Plateaustraat 22 – 9000 Gent
Tel. : 09-264.37.75 or 09-264.37.64 - Fax. : 09-264.37.66
E-mail: 0HColin.Janssen@Ugent.be;
1HKarel.Deschamphelaere@UGent.be

UNIVERSITEIT GENT

1. Aim

The aim of this study was to determine the chronic toxicity of molybdate ion (MoO_4^{2-}), added as Na_2MoO_4 to eight freshwater species, i.e. the aquatic plant *Lemna minor*, the rotifer *Brachionus calyciflorus*, the cladocerans *Daphnia magna* and *Ceriodaphnia dubia*, the snail *Lymnaea stagnalis*, the frog *Xenopus laevis*, and the midge *Chironomus riparius*, and the green alga *Pseudokirchneriella subcapitata*. The results of the test with *P. subcapitata* have already been reported in detail in an earlier report (De Schamphelaere and Janssen, 2007). This report is added as Annex 1. The methods used and the results for the other species are discussed in detail below.

2. Materials and methods

2.1. Ecotoxicity test procedures

Chronic toxicity experiments with all species adhered to internationally accepted standard testing protocols such as from OECD, ASTM or APHA, except for the snail *Lymnaea stagnalis*. For *L. stagnalis* no internationally accepted testing protocols are currently available and therefore we followed the methodology reported by De Schamphelaere et al. (2008). Details about origin and culturing of the test organisms, test media, test design, exposure duration, end-points recorded and test conditions are given in Table 1.

2.2. Chemical analyses

Samples for total Mo (test initiation) and dissolved Mo (test initiation and termination, filtered through 0.45 μm) were taken from all treatments and were measured by ICP-MS. pH was recorded at regular intervals. No observed effect concentrations (NOEC, see 2.3) are reported on the basis of measured dissolved Mo concentrations at test initiation.

Table 1 Details of the ecotoxicity testing methods for seven freshwater species

	Lemna minor	***Brachionus calyciflorus***	***Daphnia magna***	***Ceriodaphnia dubia***
Origin	In house culture	Cysts obtained from Microbiotests NV (Nazareth, Belgium)	In house culture	In house culture
Culture medium	Modified SSI (Annex 2.1)	Cysts were hatched in EPA-MH medium (Annex 2.2)	City tap water (Ghent, Belgium), filtered over activated carbon and biofilter (pH ~ 7.6, hardness ~ 200 mg $CaCO_3$/L)	City tap water (Ghent, Belgium), filtered over activated carbon and biofilter (pH ~ 7.6, hardness ~ 200 mg $CaCO_3$/L)
Test protocol	OECD TG221 (OECD, 2006)	ROTOXKIT F Chronic (Microbiotests NV); conforms to APHA 8420 (APHA, 1998)	OECD TG211 (OECD, 1998)	EPA-821-R-02-013. Test method 1002.0. (USEPA, 2002)
Test duration	7 days	48 hours	21 days	7 days
Test medium	Modified SSI (Annex 2.1)	EPA-MH (Annex 2.2)	Modified M4 (Annex 2.3)	City tap water (Ghent, Belgium) (see above)
Endpoints	Growth rate based on frond number	Population growth rate	Survival, reproduction	Survival, reproduction
Life stage tested (start test)	Colonies with 2-4 fronds	2 hours post-hatch	<24h old neonates	<24h old neonates
Nominal concentrations Min-max (spacing factor)	25 - 1600 mg/L (2)	46 – 2200 (mg/L) (2.2)	10-1000 mg/L (1.8)	5.6-560 mg/L (1.8)
Static / Semi-static	Static	Static	Semi-static	Semi-static
Renewal frequency	No renewal	No renewal	3x per week	Daily
# replicates/concentration	3	8	10	10
Volume/replicate	100 mL	1 mL	50 mL	15 mL
#Individuals/replicate	Total of 12 fronds	1	1	1
Temperature	24°C	24°C	20°C	24°C
Lightintensity (light:dark)	85-125 µE $m^{-2}s^{-1}$ (24L:0D)	Incubation in darkness	10-20 µE $m^{-2}s^{-1}$ (16L:8D)	10-20 µE $m^{-2}s^{-1}$ (16L:8D)
Feeding	Not applicable	At test initiation: $2 \cdot 10^6$ cells/mL (*P. subcapitata*)	A 3:1 mixture (cell number basis) of *P. subcapitata* and *Chlamydomonas reinhardtii*; daily feeding; 250 µg, 500 µg, 750 µg dry wt in week 1, 2, and 3, respectively	Daily feeding with a mixture of YCT (12 mg solids/L) and *P. subcapitata* ($2 \cdot 10^5$ cells/mL)

Table 1 Details of the ecotoxicity testing methods for seven freshwater species (continued)

	Lymnaea stagnalis	***Xenopus laevis***	***Chironomus riparius***
Origin	University of Amsterdam (see De Schamphelaere et al., 2008 for more details)	UGent stock	UGent culture
Culture medium	Maintained/acclimated for two weeks in test medium prior to testing		EPA moderately hard medium (Annex 2.2)
Test protocol	No standard protocol available, methodology of De Schamphelaere et al. (2008) was followed.	ASTM E1439-98 (ASTM, 1998)	OECD 218 (OECD, 2004)
Test duration	28 days	4 days	14 days
Test medium	Modified AFNOR (Annex 2.4)	FETAX (Annex 2.5)	EPA moderately hard medium (Annex 2.2)
Endpoints	Survival and growth rate (length, wet wt)	Survival and malformation	14-d survival, growth (dry wt)
Life stage tested (start test)	4-week old juveniles (mean length 1.08 cm, mean wet wt 70 mg)	Embryos in early gastrula (stage 10)	48-hour old larvae
Nominal concentrations Min-max (spacing factor)	50-800 mg/L (2)	25-400 mg/L (2)	25-1600 mg/L (2)
Static / Semi-static	Semi-static	Semi-static	Semi-static
Renewal frequency	2x per week	daily	2x per week
# replicates/concentration	8	2 (4 control replicates)	5 (survival) or 10 (growth)
Volume/replicate	100 mL	10 mL	250 mL (+quartz sand as substrate)
#Individuals/replicate	1	25	10
Temperature	20°C	24°C	20°C
Light-intensity (light:dark)	1000 lux (12L:12D)	10-20 $\mu E\ m^{-2}s^{-1}$ (12L:12D)	1000 lux (16L:8D)
Feeding	60 mg fresh lettuce per snail at every renewal (=*ad libitum*)	No feeding	TetraMin®, daily 0.25 mg/larva (d0-d7) 0.50 mg/larva (d8-d14)

2.3. Data analysis

Before calculation of the NOEC's and EC10's, raw biological recordings needed to be treated in some cases. This was the case for *L. minor*, *L. stagnalis*, and *B. calyciflorus*. For *L. minor* the growth rate was calculated from the initial frond number and the front numbers recorded on day 2, 4, and 7 of the test. Following OECD Test Guideline 221, the growth rate in each replicate of each treatment was calculated as the slope of the linear regression of the natural logarithm of the frond number versus time. The same principle was applied to calculate the length and biomass based growth rate of *L. stagnalis* and the population growth rate of *B. calyciflorus*.

The determination of the NOEC for each of the endpoints and organisms considered were calculated using the methods recommended by OECD (2005). The Jonkheere-Terpstra trend test was always used unless specified otherwise. Briefly, this statistical test, tests if a significant trend is observed of the observed endpoint vs. the concentration. It starts with calculating p-values for the whole dataset (all concentrations). If $p>0.05$, the NOEC is unbounded, i.e. $\geq$ the highest tested concentration. If $p<0.05$, the trend is considered significant. Next, the data from the highest concentration are eliminated from the dataset and the p-value is calculated for the dataset up to the second highest concentration. If $p>0.05$, the second highest concentration is the NOEC. If $p<0.05$, the procedure continues by eliminating more concentrations from the dataset (from high to low). This iterative procedure stops at the concentration where the trend becomes not significant ($p>0.05$) and this concentration is considered the NOEC. All hypothesis testing was conducted one-sided at the α level of 0.05 as recommended by OECD (2005). Hypothesis testing was performed with SPSS16® software. NOEC's are reported as dissolved concentrations measured at the start of the test. The EC_{10}'s and their 95% confidence interval (CI) were calculated based on dissolved concentrations at the start of the test using the standard log-logistic concentration-response model unless noted otherwise. Calculations were performed using Statistica® software.

3. Results

3.1. Lemna minor

Table 2 presents the results of the ecotoxicity test with *L. minor*, including measured total and dissolved concentration of Mo and the mean and the standard deviation of the growth rate. Frond counts, the growth rate in all replicates and recorded pH values are given in Annex 3A. The growth rate in the control is 0.332 d^{-1}, which gives a doubling time (calculated following OECD TG211 (OECD, 2006) of the frond number of 2.1 days. According to OECD TG221, which requires a doubling time < 2.5 days, the test is therefore considered valid. Total Mo deviated by less than 10% from the nominal concentration and more than 90% of the total Mo was dissolved. The $NOEC_{dissolved}$ is 24.7 (1% reduction of growth rate compared to control), the $LOEC_{dissolved}$ is 51.7 mg/L (7% reduction of growth rate). The EC10 is 241.5 mg/L (95% CI: 183.6-317.7 mg/L).

Table 2 Results of the 7-day toxicity test with *Lemna minor*

$Mo_{nominal}$ (mg/L)	Mo_{total} (mg/L) (t=0)	$Mo_{dissolved}$ (mg/L) (t=0)	$Mo_{dissolved}$ (mg/L) (t=7d)	μ (d^{-1}) mean	μ (d^{-1}) stdev	p[b]
Control[a]	<DL	<DL	<DL	0.332	0.005	
25	26.6	24.7	24.9	0.333	0.006	0.402
50	54.6	51.7	52.3	0.310	0.012	0.042
100	106	101	103	0.305	0.018	0.006
200	197	191	208	0.283	0.012	<0.001
400	393	380	410	0.251	0.007	<0.001
800	807	774	814	0.182	0.003	<0.001
1600	1574	1521	1575	0.056	0.012	<0.001

[a] Control treatment (no added Mo)
[b] p-value for the Jonkheere-Terpstra step-down trend test up to this concentration

3.2. Brachionus calyciflorus

Table 3 presents the results of the ecotoxicity test with *B. calyciflorus*, including the measured dissolved concentration of Mo and the mean and the standard deviation of the growth rate. The number of individuals counted on day 2 and the growth rate in all replicates is given in Annex 3.2. The mean growth rate in the control is 0.734 d^{-1} which is above the minimum acceptable growth rate of 0.7 d^{-1} as required by APHA (1998). The test is therefore considered valid. The $NOEC_{dissolved}$ is 244 mg/L (0 % reduction of growth rate compared to

control), the $LOEC_{dissolved}$ is 508 mg/L (41% reduction of growth rate). The EC10 is 193.6 mg/L (95% CI: 49.6-756.3 mg/L).

Table 3 Results of the 2-day toxicity test with *Brachionus calyciflorus*

$Mo_{nominal}$ (mg/L)	$Mo_{dissolved}$ (mg/L) (t=0)	μ (d^{-1}) mean	μ (d^{-1}) stdev	p[b]
Control[a]	<DL	0.734	0.201	
46	55	0.713	0.112	
100	116	0.777	0.227	
220	244	0.736	0.138	0.422
460	508	0.437	0.300	0.028
1000	1109	0.423	0.282	<0.001
2200	2301	0.199	0.222	<0.001

[a] Control treatment (no added Mo)
[b] p-value for the Jonkheere-Terpstra step-down trend test up to this concentration

3.3. Daphnia magna

Table 4 presents the results of the ecotoxicity test with *Daphnia magna*, including the measured total and dissolved concentration of Mo, the survival, and the mean and the standard deviation of the reproduction (R). The measured pH in new and old test media, as well as the total number of juveniles produced in each replicate are given in Annex 3.3. Survival in the control was 100% and the mean reproduction in the control was 68.3. Both are higher than the minimum survival of 80% and reproduction of 60, required by OECD TG 211 (OECD, 1998) for a valid test.

The total Mo concentration in all treatments is within 12% of the nominal concentration. The dissolved Mo concentration represents >94% of the total concentration. The $NOEC_{dissolved}$ for survival is 325 mg/L, the $LOEC_{dissolved}$ for survival is 569 mg/L. The response of reproduction versus concentration is not monotonous. An initial increase of the reproduction up to 85.9 juveniles at 33.8 mg/L is observed. At higher concentrations, the reproductive output again decreased. Since the response was not monotonous and the variances among treatments were not homogenous (Levene's test, $p<0.05$), the Mann Whitney U test with Bonferroni-Holm correction was applied, following recommendations by OECD (2005). As such, the $NOEC_{dissolved}$ was 112 mg/L (13% lower reproduction compared to control) and the $LOEC_{dissolved}$ was 196 mg/L (52% lower reproduction than control). Given the significant stimulation of reproduction at low Mo, the hormesis model of Van Ewijk and Hoekstra (1993)

was fitted to the concentration response data, resulting in an EC10 for reproduction of 105.6 mg/L (95% CI: 91.5-121.8 mg/L).

Table 4 Results of the 21-day toxicity test with *Daphnia magna*

$Mo_{nominal}$ (mg/L)	Mo_{total} (mg/L) (t=0)	$Mo_{dissolved}$ (mg/L) (t=0)	$Mo_{dissolved}$ (mg/L) (t=21d)	Survival	R mean	R Stdev	p[b]
Control[a]	<DL	<DL	<DL	10/10	68.3	13.2	
10	11.2	10.8	10.7	9/9	67.4	11.2	0.478
18	19.5	19.1	19.4	10/10	77.6	11.7	ND[c]
32	35.7	33.8	35.5	10/10	85.9	14.6	ND[c]
56	61.6	60	62.1	10/10	72.9	9.6	ND[c]
100	109	112	111	8/8	59.8	8.2	0.092 (0.092)
180	197	196	200	9/10	32.4	8.4	<0.001 (<0.002)
320	329	325	336	10/10	4.8	3.5	<0.001 (<0.003)
560	576	569	563	0/10	-	-	-
1000	1068	1063	ND[d]	0/10	-	-	-

[a] Control treatment (no added Mo)
[b] p-value for the comparison of reproduction with the control (Mann-Whitney U test); Bonferoni-Holm adjusted p-value between parentheses
[c] not determined (reproduction higher than in control)
[d] not determined (all adults had died at day 21)

3.4. Ceriodaphnia dubia

Table 5 presents the results of the ecotoxicity test with *Ceriodaphnia dubia*, including the measured total and dissolved concentration of Mo, the survival, and the mean and the standard deviation of the reproduction. The measured pH in new and old test media, a few other chemical characteristics of the test media as well as the total number of juveniles produced in each replicate are given in Annex 3.4. Total Mo was within 6% of the nominal added concentration for all treatments. Dissolved Mo represented more than 90% of the total Mo. Survival in the control was 100% and the mean reproduction was 18.4. Both are higher than the minimum survival of 80% and reproduction of 15 required by USEPA (2002) for a valid test. The $NOEC_{dissolved}$ for survival is 177 mg/L, the $LOEC_{dissolved}$ for survival is 302 mg/L. The $NOEC_{dissolved}$ for reproduction was 97.3 mg/L (18% lower reproduction compared to control) and the $LOEC_{dissolved}$ was 177 mg/L (52% lower reproduction than control). The EC10 for reproduction is 78.2. mg/L (95%CI: 49.8-122.7 mg/L).

Table 5 Results of the 7-day toxicity test with *Ceriodaphnia dubia*.

$Mo_{nominal}$ (mg/L)	Mo_{total} (mg/L) (t=0)	$Mo_{dissolved}$ (mg/L) (t=0)	$Mo_{dissolved}$ (mg/L) (t=21d)	Survival	R mean	R stdev	p[b]
Control[a]	<DL	<DL	<DL	10/10	18.4	6.9	
5.6	5.9	5.3	5.0	10/10	19.2	4.3	ND[c]
10	10.6	10.1	9.8	10/10	19.8	3.3	ND[c]
18	17.9	17.3	17.0	10/10	20.1	6.8	ND[c]
32	33.8	32.4	31.4	10/10	18.1	5.6	ND[c]
56	57.8	55.9	54.6	10/10	18.0	7.6	0.337
100	100	97.3	95.6	10/10	15.1	6.0	0.076
180	183	177	172	10/10	8.8	5.8	<0.001
320	325	302	299	4/10	3.0	2.6	<0.001
560	557	550	535	1/10	1.0	-	<0.001

[a] Control treatment (no added Mo)
[b] p-value for the Jonkheere-Terpstra step-down trend test up to this concentration
[c] not determined (trend analysis stops at the first concentration where p>0.05)

3.5. Lymnaea stagnalis

Table 6 presents the results of the ecotoxicity test with *L. stagnalis*, including the measured total and dissolved concentration of Mo and the mean and the standard deviation of the length and biomass growth rate. The measured pH in new and old test media and the raw length and wet weight recordings (and calculated growth rates) in each replicate are given in Annex 3.5. Total Mo was within 6% of the nominal added concentration for all treatments. Dissolved Mo represented more than 93% of the total Mo. Since there is no standard test protocol for *L. stagnalis* there are no standard test validity criteria either. However, survival in the control was 100%. The biomass growth rate in the control was 7.1% per day, which is in line with earlier reported growth rates of 4-week old *L. stagnalis* (De Schamphelaere et al., 2008). These data suggest that test organisms were "normal" during toxicity testing. Survival was 100% at all Mo concentrations. Hence, a $NOEC_{dissolved}$ for survival ≥808 mg/L is derived. Length and biomass growth rate were equally sensitive endpoints, with a $NOEC_{dissolved}$ of 200 mg/L (6% and 5% lower length and biomass growth rate compared to control, respectively) and a $LOEC_{dissolved}$ of 388 mg/L. The EC10 for length growth rate is 221.3 mg/L (95%CI: 173.3-282.5 mg/L) and the EC10 for biomass growth rate is 221.8 mg/L (95%CI: 180.4-272.8 mg/L). Both endpoints are equally sensitive.

Table 6 Results of the 28-day toxicity test with *Lymnaea stagnalis*

$Mo_{nominal}$ (mg/L)	Mo_{total} (mg/L) (t=0)	$Mo_{dissolved}$ (mg/L) (t=0)	$Mo_{dissolved}$ (mg/L) (t=21d)	Mean length growth rate (%/d)	Stdev length growth rate (%/d)	p[b]	Mean Biomass growth rate (%/d)	Stdev biomass growth rate (%/d)	p[b]
Control[a]	<DL	<DL	<DL	2.15	0.21		7.14	0.50	
50	52.9	49.0	53.4	2.17	0.16	ND[c]	7.47	0.55	ND[c]
100	106	101	102	2.04	0.19	ND[c]	7.02	0.64	ND[c]
200	208	200	ND[d]	2.02	0.28	0.151	6.75	0.77	0.143
400	404	388	382	0.80	0.34	<0.001	3.03	0.97	<0.001
800	833	808	ND[d]	0.10	0.06	<0.001	0.15	0.25	<0.001

[a] Control treatment (no added Mo)
[b] p-value for the Jonkheere-Terpstra step-down trend test up to this concentration
[c] not determined (trend analysis stops at the first concentration where p>0.05)
[d] not determined (sample lost)

3.6. Xenopus laevis

Table 7 presents the results of the ecotoxicity test with *X. laevis*, including the measured total and dissolved concentration of Mo and the number of dead and malformed embryos. The number of dead and malformed embryos per replicate is given in Annex 3.6. Total Mo was within 3% of the nominal added concentration for all treatments. Dissolved Mo represented more than 90% of the total Mo. Only 1 of 101 embryos in the control died during the test; all other embryos (99%) reached stage 46 of the embryo development, which is more than the 90% required for a valid test according to the ASTM E1439-91 standard (ASTM, 1998). For the survival endpoint, the $NOEC_{dissolved}$ is 177 mg/L (2% mortality) and the $LOEC_{dissolved}$ is 369 mg/L (8% mortality). For the endpoint malformation, the $NOEC_{dissolved}$ is 22.4 mg/L (8% malformation) and the $LOEC_{dissolved}$ is 44.6 mg/L. The EC10 for survival is 415.4 mg/L (95%CI: 313.2-550.8 mg/L) and the EC10 for malformation is 115.9 mg/L (34.4-390.5).

Table 7 Results of the 4-day toxicity test with *Xenopus laevis*

$Mo_{nominal}$ (mg/L)	Mo_{total} (mg/L) (t=0)	$Mo_{dissolved}$ (mg/L) (t=0)	$Mo_{dissolved}$ (mg/L) (t=21d)	#embryos tested	# dead (96h)	p[b]	# mal-formed (96h)	p[b]
Control[a]	<DL	<DL	<DL	100	1		1	
25	24.2	22.4	25.1	50	0	ND[c]	4	0.067
50	48.7	44.6	50.8	50	0	ND[c]	5	0.024
100	97.1	87.3	96.3	50	0	ND[c]	4	0.011
200	194	177	192	50	1	0.470	6	0.004
400	389	369	344	50	4	0.048	11	<0.001

[a] Control treatment (no added Mo)
[b] p-value for the Jonkheere-Terpstra step-down trend test up to this concentration
[c] not determined (trend analysis stops at the first concentration where p>0.05)

3.7. Chironomus riparius

Table 8 presents the results of the ecotoxicity test with *C. riparius*, including the measured total and dissolved concentration of Mo, the 14d-survival and the 14d-growth (dry weight). Measured pH and raw survival and weight data are given in Annex 3.7. Total Mo was within 3% of the nominal added concentration for all treatments. Dissolved Mo represented more than 92% of the total Mo. There are no standard test validity criteria for the endpoints recorded (i.e. survival and growth), but survival in the control was 88% and this is in line with acceptability criteria of most chronic ecotoxicity test protocols (typically >80% survival is required). Neither survival nor growth (dry wt) exhibit a monotonous concentration-response relationship. Hence, the Jonkheere-Terpstra step-down test cannot be applied to these data. Furthermore not all conditions for allowing parametric ANOVA are met. For the survival data, variances are not homogenous among treatments (p=0.035). For the growth data the standard deviations are significantly positively correlated with the means (r=0.78, p=0.022). Hence, following recommendations by OECD (2005), the non-parametric Mann Whitney U test with Bonferroni-Holm correction was applied for inferring statistical differences among control and Mo treatments. A $NOEC_{dissolved}$ for survival ≥1564 mg/L is derived. A $NOEC_{dissolved}$ for growth of 393 mg/L is derived (35% lower dry wt), with a $LOEC_{dissolved}$ of 794 mg/L. The EC10 for survival could not be determined due to the highly non-monotonous nature of the concentration-response. The EC10 for growth (dry wt) was determined by fitting the hormesis model of Van Ewijk and Hoekstra (1993) to the concentration-response data and was found to be 121.4 mg/L (95%CI: 60.9-241.8 mg/L).

Table 7 Results of the 14-day toxicity test with *Chironomus riparius*

$Mo_{nominal}$ (mg/L)	Mo_{total} (mg/L) (t=0)	$Mo_{dissolved}$ (mg/L) (t=0)	$Mo_{dissolved}$ (mg/L) (t=21d)	Mean survival (%)	Stdev survival (%)	p[b]	Mean dry wt (µg/ org)	Stdev dry wt (µg/ org)	p[b]
Control[a]	<DL	<DL	<DL	88.0	17.9		798	268	
25	24.8	22.9	25.6	76.0	12.6	0.197	789	181	0.961
50	50.1	47.6	51.7	76.0	12.6	0.200	956	241	ND[c]
100	99.2	95.8	103	68.0	9.6	0.085	774	182	0.733
200	199	191	204	84.0	12.9	0.512	444	214	0.016 (0.048)
400	410	393	410	68.0	12.6	0.086	521	159	0.040 (0.080)
800	821	794	845	66.0	27.5	0.108	346	158	0.002 (0.010)
1600	1642	1564	1624	58.0	9.6	0.033 (0.066)	153	75	0.005 (0.020)

[a] Control treatment (no added Mo)
[b] p-value for the comparison of survival or dry wt with the control (Mann-Whitney U test); Bonferoni-Holm adjusted p-value between parentheses
c not determined (mean dry wt higher than in control)

3.8. Pseudokirchneriella subcapitata

Applying the Jonkheere-Terpstra test to the earlier-reported concentration-response data for *P. subcapitata* (see De Schamphelaere et al., 2007, Annex 1) revealed a $NOEC_{dissolved}$ of 62.3 mg/L (1% reduction of growth rate) and a $LOEC_{dissolved}$ of 132 mg/L (5% reduction of growth rate). The EC10 values were reported earlier (see Annex 1).

4. Conclusion

Table 9 gives a concluding overview of the $NOEC_{dissolved}$ and the EC10 values for the most sensitive endpoint for all eight species investigated.

Table 9 Overview of the NOEC and EC10 values for the molybdate ion for eight freshwater species

	Exposure duration (d)	**Endpoint**	**$NOEC_{dissolved}$ (mg Mo/L)**	**$EC10_{dissolved}$ (mg Mo/L)**
Lemna minor	7	Growth rate	24.7	241.5
Brachionus calyciflorus	2	Population growth rate	244	193.6
Daphnia magna	21	Reproduction	112	105.6
Ceriodaphnia dubia	7	Reproduction	97.3	78.2
Lymnaea stagnalis	28	Biomass growth rate	200	221.8
Xenopus laevis	4	Embryo malformation	22.4	115.9
Chironomus riparius	14	Growth	393	121.4
Pseudokirchneriella subcapitata	3	Growth rate	62.3	283.8

5. Cited literature

APHA. 1998. Standard method 8420. In: Clesceri, L.S., Greenberg, A.E., Eaton, A.D. (Eds.) Standard Methods for the Examination of Water and Wastewater, 20th ed., American Public Health Association, Washington, DC, USA.

ASTM. 1998. Standard Guide for Conducting the Frog Embryo Teratogenesis Assay with *Xenopus* (FETAX). ASTM-E1439-98.

De Schamphelaere KAC, Janssen CR. 2007. Toxicity of molybdate ion (MoO_4^{2-}) to the green alga *Pseudokirchneriella subcapitata*. Research report prepared for IMOA, 28 September 2007.

De Schamphelaere KAC, Koene JM, Heijerick DG, Janssen CR. 2008. Reduction of growth and haemolymph Ca levels in the freshwater snail *Lymnaea stagnalis* chronically exposed to cobalt. Ecotoxicology and Environmental Safety 71:65-71.

Microbiotests. 2008. ROTOXKIT F CHRONIC. Chronic toxicity test for freshtwater. 48h reproduction inhibition test based on the rotifer *Brachionus calyciflorus*. Standard operational procedure. Microbiotests, Nazareth, Belgium (www.microbiotests.be).

OECD. 1998. Test Guideline 211. *Daphnia magna* Reproduction Test. Original Guideline. Organization for Economic Cooperation and Development, Paris, France.

OECD. 2004. Test Guideline 219. Sediment-water chironomid toxicity test using spiked sediment. Original Guideline. Organization for Economic Cooperation and Development, Paris, France.

OECD. 2005. Guidance Document on the Statistical Analysis of Ecotoxicity Data. Environmental Health and Safety Publications; Series on Testing and Assessment; Environment Directorate; Organisation for Economic Co-operation and Development, Paris.

OECD. 2006. Test Guideline 221. *Lemna* sp. Growth Inhibition Test. Original guideline. Organization for Economic Cooperation and Development, Paris, France.

USEPA. 2002. Test method 1002.0. Daphnid, *Ceriodaphnia dubia*, survival and reproduction test. In: "Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms, Fourth Edition. United States Environmental Protection Agency, EPA-821-R-02-013.

Van Ewijk PH, Hoekstra JA. 1993. Calculation of the EC50 and its confidence interval when subtoxic stimulus is present. Ecotoxicology and Environmental Safety 25:25-32.

ANNEXES

ANNEX 1: COPY OF THE REPORT ON ALGAL TOXICITY TESTING WITH MOLYBDATE ION

1. Aim

The aim of this study was to determine the chronic toxicity of molybdate ion (MoO_4^{2-}), added as $Na_2MoO_4^{2-}$ and based on measured concentrations of total and dissolved Mo^{6+}, to the green alga *Pseudokirchneriella subcapitata* according to the standard OECD test protocol No. 201 ('algal growth inhibition test'; OECD, 2006)

2. Materials and Methods

Standard 72-hour chronic growth inhibition tests were conducted according to the draft revised OECD guideline No. 201 (OECD, 2006) with *Pseudokirchneriella subcapitata* (strain CCAP 278/4 from the Culture Collection of Algae and Protozoans, Argyl, United Kingdom). Tests were conducted in standard OECD test medium (nominal hardness 25 mg $CaCO_3$/L) (OECD, 2006). A control (no added Mo) and eleven spiked Mo concentrations were investigated in triplicate. , Initial cell densities were 10^4 cells/mL. Cell density was monitored daily using a Coulter Counter. Growth rate (μ) was calculated according to OECD (2006) on the basis of the cell density measurements. Samples for total Mo (test initiation) and dissolved Mo (test initiation and termination, filtered through 0.45 μm) were taken from all treatments and were measured by ICP-MS. pH was recorded daily. At the end of the test the EC10 was calculated – on the basis of measured dissolved Mo concentrations at test initiation - using a log-normal concentration-response model. Mo concentrations were log-transformed and a normal distribution was fitted through the data set using the statistical computer package Best Fit® (Palisade Decision Tools). Using Monte-Carlo analysis (bootstrapping) the EC10 and its 95% confidence interval were determined.

3. Results

The test validity criteria values according to OECD are reported in Table 1. All validity criteria were fulfilled over the 72 hour exposure period. pH values measured over the course of the experiment are reported in Table 2. pH always remained within 1 pH unit, as recommended by OECD. Hence, the test fulfilled all validity criteria and the results are reported in Table 2 (concentration-response data) and Table 3 (effect concentrations).

Table 1 Test validity for the 72-hour exposure of *P. subcapitata*

	Criterion	Value for this test	Valid? (YES/NO)
Mean control μ (d^{-1})	>0.92	1.38	YES
Factor increase of cell density over 72 hours	>16	64.0	YES
Coefficient of variation of control μ (%)	<7	1.6	YES
Coefficient of variation of mean sectional μ (%)	<35	14.8	YES

Table 2 pH values measured during the test in all treatments

$Mo_{nominal}$ (mg/L)	Start	24h	48h	72h
Control[a]	7.46	7.40	7.54	8.01
2.76	7.51	7.45	7.74	8.13
5.99	7.53	7.48	7.73	8.27
13.2	7.44	7.54	7.71	8.30
27.6	7.46	7.52	7.69	8.30
59.9	7.42	7.55	7.71	8.24
132	7.50	7.60	7.82	8.21
276	7.50	7.63	7.75	8.04
599	7.47	7.64	7.69	7.88
1320	7.55	7.66	7.65	7.77
2760	7.54	7.66	7.66	7.65
5990	7.48	7.68	7.71	7.70

Table 3 Raw results of the 72-hour toxicity test with *P. subcapitata*

$Mo_{nominal}$ (mg/L)	Mo_{total} (mg/L) (t=0)	$Mo_{dissolved}$ (mg/L) (t=0)	$Mo_{dissolved}$ (mg/L) (t=72h)	μ (d^{-1}) Rep 1	μ (d^{-1}) Rep 2	μ (d^{-1}) Rep 3	μ (d^{-1}) mean	μ (d^{-1}) stdev
Control[a]	<DL	<DL	<DL	1.401	1.380	1.357	1.379	0.022
2.76	2.94	2.86	2.79	1.389	1.384	1.415	1.396	0.017
5.99	6.29	6.00	6.69	1.335	1.423	1.377	1.378	0.044
13.2	13.5	12.9	13.7	1.402	1.380	1.413	1.398	0.017
27.6	29.4	28.8	27.6	1.377	1.391	1.372	1.380	0.010
59.9	64.6	62.3	61.1	1.370	1.337	1.379	1.362	0.022
132	133	132	135	1.351	1.312	1.318	1.327	0.021
276	272	270	265	1.212	1.249	1.224	1.228	0.019
599	611	608	584	1.140	1.142	1.143	1.141	0.002
1320	1330	1325	1344	0.981	0.995	1.040	1.005	0.031
2760	2723	2680	2646	0.266	0.262	0.282	0.270	0.011
5990	5734	5669	5523	0.077	0.052	0.050	0.060	0.015

[a] Control treatment (no added Mo)

Table 4. 72-hour EC10 of Mo (mg dissolved Mo/L) and 95% confidence intervals for *P. subcapitata*

	Value	Lower confidence limit	Higher confidence limit
E_rC10	283.8	58.7	446.2

4. Cited literature

OECD. 2006. Test Guideline 201. 201 Freshwater Alga and Cyanobacteria, Growth Inhibition Test. Updated Guideline. Organization for Economic Cooperation and Development, Paris, France.

Annex 2.1 Composition of the modified SSI-culturing and test medium for *Lemna minor*.

Final test medium: 1 L deionised water, containing:

- 10 mL of stock solution I
- 5 mL of stock solution II
- 5 mL of stock solution III
- 5 mL of stock solution IV
- 1 mL of stock solution V
- 5 mL of stock solution VI
- 1 mL of stock solution VII (MOPS)
- pH adjustment to 6.5 ± 0.2

Substance	Concentration in stock solution (g/L)	Substance	Concentration in stock solution (g/L)
Stock solution I		***Stock solution V***	
$NaNO_3$	8.5	H_3BO_3	1.0
KH_2PO_4	1.3	$MnCl_2.2H_2O$	0.20
Stock solution II		$Na_2.MoO_4.2H_2O$	0.010
$MgSO_4.7H_2O$	15.0	$ZnSO_4.7H_2O$	0.050
Stock solution III		$CuSO_4.5H_2O$	0.0050
$CaCl_2.2H_2O$	7.2	$Co(NO_3)_2.6H_2O$	0.010
Stock solution IV		***Stock solution VI***	
Na_2CO_3	4.0	$FeCl_3.6H_2O$	0.17
		Na_2-EDTA.$2H_2O$ (1)	0.28
		Stock solution VII	
		MOPS (buffer)	490

(1): EDTA was replaced with 32 µg/L of Humic Acid (Sigma-Aldrich)

Annex 2.2 Composition of EPA moderately hard water (EPA-MH) for the toxicity test with *Brachionus calyciflorus*

Compound	Concentration
$NaHCO_3$	96 mg/L
$CaSO_4 \cdot 2H_2O$	60 mg/L
$MgSO_4$	60 mg/L
KCl	4 mg/L
pH	Adjusted to pH 7.5 with KOH

Annex 2.3. Composition of M4 medium for the toxicity test with *D. magna*

Note: EDTA was omitted from the solution and replaced with 4 mg/L of DOC collected by reverse osmosis from an unpolluted natural water.

Trace elements

Separate stock solutions (I) of individual trace elements are first prepared in water of suitable purity, e.g. deionised, distilled or reverse osmosis. From these different stock solutions (I) a second single stock solution (II) is prepared, which contains all trace elements (combined solution), i.e:

Stock solution(s) I (single substance)	Amount added to water mg/l	Concentration (related to medium M4)	To prepare the combined stock-solution II add the following amount of stock solution I to water ml/l	
			M 4	M 7
H_3BO_3	57 190	20 000-fold	1.0	0.25
$MnCl_2$•4 H_2O	7 210	20 000-fold	1.0	0.25
LiCl	6 120	20 000-fold	1.0	0.25
RbCl	1 420	20 000-fold	1.0	0.25
$SrCl_2$•6 H_2O	3 040	20 000-fold	1.0	0.25
NaBr	320	20 000-fold	1.0	0.25
Na_2MoO_4 •2 H_2O	1 260	20 000-fold	1.0	0.25
$CuCl_2$•2 H_2O	335	20 000-fold	1.0	0.25
$ZnCl_2$	260	20 000-fold	1.0	1.0
$CoCl_2$•6 H_2O	200	20 000-fold	1.0	1.0
KI	65	20 000-fold	1.0	1.0
Na_2SeO_3	43.8	20 000-fold	1.0	1.0
NH_4VO_3	11.5	20 000-fold	1.0	1.0
Na_2EDTA•2 H_2O	5 000	2 000-fold	-	-
$FeSO_4$•7 H_2O	1 991	2 000-fold	-	-
Both Na_2EDTA and $FeSO_4$ solutions are prepared singly, poured together and autoclaved immediately. This gives:				
21 Fe-EDTA solution		1 000-fold	20.0	5.0

Annex 2.3. Composition of M4 medium for the toxicity test with *D. magna* (Continued)

M4 and M7 media

M4 and M7 media are prepared using stock solution II, the macro-nutrients and vitamins as follows:

	Amount added to water mg/l	Concentration (related to medium M4)	Amount of stock solution added to prepare medium ml/l	
			M 4	M 7
Stock solution II (combined trace elements)		20-fold	50	50
Macro nutrient stock solutions (single substance)				
$CaCl_2$•2 H_2O	293 800	1 000-fold	1.0	1.0
$MgSO_4$•7 H_2O	246 600	2 000-fold	0.5	0.5
KCl	58 000	10 000-fold	0.1	0.1
$NaHCO^3$	64 800	1 000-fold	1.0	1.0
Na_2SiO_3•9 H_2O	50 000	5 000-fold	0.2	0.2
$NaNO_3$	2 740	10 000-fold	0.1	0.1
KH_2PO_4	1 430	10 000-fold	0.1	0.1
K_2HPO_4	1 840	10 000-fold	0.1	0.1
Combined Vitamin stock	-	10 000-fold	0.1	0.1
The combined vitamin stock solution is prepared by adding the 3 vitamins to 1 litre water, as shown below:				
	mg/l			
Thiamine hydrochloride	750	10 000-fold		
Cyanocobalamine (B_{12})	10	10 000-fold		
Biotine	7.5	10 000-fold		

The combined vitamin stock is stored frozen in small aliquots. Vitamins are added to the media shortly before use.

N.B: To avoid precipitation of salts when preparing the complete media, add the aliquots of stock solutions to about 500 - 800 ml deionized water and then fill it up to 1 litre.

N.N.B. The first publication of the M4 medium can be found in Elendt, B.P. (1990). Selenium deficiency in crustacea; an ultrastructural approach to antennal damage in *Daphnia magna* Straus. Protoplasma, 154, 25-33.

Annex 2.4. Composition of modified AFNOR medium for the toxicity test with *L. stagnalis*

Major ions	**Concentration**	**Trace metals[a]**	**Concentration**
$NaHCO_3$	201.5 mg/L (=2.4 mM)	Cu	1.0 µg/L
$MgCl_2$	38.1 mg/L (= 0.4 mM)	Zn	3.0 µg/L
$CaCl_2$	111 mg/L (= 1 mM)	Co	1.0 µg/L
K_2SO_4	26.1 mg/L (=0.15 mM)		

[a] added as chloride salts

Annex 2.5 Composition of FETAX medium for the toxicity test with *Xenopus laevis*

Compound	Concentration
NaCl	625 mg/L
$NaHCO_3$	96 mg/L
KCl	30 mg/L
$CaCl_2$	15 mg/L
$CaSO_4.2H_2O$	60 mg/L
$MgSO_4$	75 mg/L
Measured pH	7.8

Annex 3.1 Raw data of the toxicity tests with *Lemna minor*

pH measured during *L. minor* testing

Nominal Mo (mg/L)	pH day 0	pH day 7
Control	6.51	6.75
25	6.53	6.63
50	6.54	6.60
100	6.56	6.60
200	6.58	6.63
400	6.61	6.65
800	6.65	6.71
1600	6.68	6.78

Frond counts and growth rate per replicate for *L. minor* testing

Nominal Mo (mg/L)	replicate	Fronds day 0	Fronds day 2	Fronds day 4	Fronds day 7	μ (d^{-1})
Control	1	12	20	47	120	0.337
Control	2	12	19	47	112	0.330
Control	3	12	20	48	112	0.328
25	1	12	23	53	117	0.330
25	2	12	24	53	127	0.340
25	3	12	24	52	118	0.329
50	1	12	25	56	109	0.318
50	2	12	24	41	98	0.296
50	3	12	22	50	104	0.314
100	1	12	23	55	111	0.324
100	2	12	21	39	89	0.288
100	3	12	21	50	95	0.304
200	1	12	25	42	83	0.272
200	2	12	22	41	85	0.281
200	3	12	24	48	95	0.297
400	1	12	24	41	75	0.259
400	2	12	18	35	65	0.248
400	3	12	20	35	66	0.245
800	1	12	20	27	45	0.184
800	2	12	18	27	43	0.183
800	3	12	17	27	41	0.179
1600	1	12	14	17	19	0.067
1600	2	12	14	16	18	0.058
1600	3	12	13	15	16	0.043

Annex 3.2 Number of individuals counted on day 2 and population growth rates for *Brachionus calyciflorus*

Number of individuals on day 2 per replicate

Nominal Mo (mg/L)	rep1	rep2	rep3r	rep4	rep5	rep6	rep7	rep8
control	7	5	5	6	4	5	3	2
46	4	4	3	3	5	5	5	5
100	6	3	5	5	8	7	5	2
220	3	5	4	5	3	4	6	6
460	4	1	3	5	3	1	2	3
1000	4	2	1	1	3	3	4	3
2000	1	3	2	2	1	1	2	1

Population growth rate per replicate

Nominal Mo (mg/L)	rep1	rep2	rep3r	rep4	rep5	rep6	rep7	rep8
control	0.973	0.805	0.805	0.896	0.693	0.805	0.549	0.347
46	0.693	0.693	0.549	0.549	0.805	0.805	0.805	0.805
100	0.896	0.549	0.805	0.805	1.040	0.973	0.805	0.347
220	0.549	0.805	0.693	0.805	0.549	0.693	0.896	0.896
460	0.693	0.000	0.549	0.805	0.549	0.000	0.347	0.549
1000	0.693	0.347	0.000	0.000	0.549	0.549	0.693	0.549
2000	0.000	0.549	0.347	0.347	0.000	0.000	0.347	0.000

Annex 3.3 pH and reproduction (number of produced juveniles) per replicate in *Daphnia magna* tests

pH measured during *Daphnia magna* tests (minima and maxima recorded)

Nominal Mo (mg/L)	new medium min	new medium max	old medium min	old medium max
Control	7.4	8.0	7.1	7.4
10	7.6	8.1	7.2	7.5
18	7.6	8.1	7.2	7.4
32	7.6	8.0	7.2	7.5
56	7.5	8.0	7.2	7.6
100	7.5	8.1	7.3	7.6
180	7.5	8.2	7.5	7.7
320	7.6	8.2	7.7	7.8
560	7.7	8.1	7.7	7.8
1000	8.1	8.2	7.8	7.8

Total reproduction after 21 days of exposure in *Daphnia magna* tests

Nominal Mo (mg/L)	rep1	rep2	rep3	rep4	rep5	rep6	rep7	rep8	rep9	rep10
Control	65	71	58	63	55	58	90	83	85	55
10	78	♂	66	80	57	76	62	48	62	78
18	78	85	64	78	59	75	90	99	72	76
32	93	64	72	109	84	77	98	74	103	85
56	79	60	70	71	70	65	68	81	94	71
100	65	50	61	51	57	75	♂	63	56	♂
180	34	21	39	43	26	27	†	24	34	44
320	6	0	0	6	1	6	7	6	11	5
560	†	†	†	†	†	†	†	†	†	†
1000	†	†	†	†	†	†	†	†	†	†

♂: male individual

†: individual died during the 21-day exposure

Annex 3.4 Chemistry and reproduction during *Ceriodaphnia dubia* test

pH measured during *C. dubia* tests (minima and maxima recorded)

Nominal Mo (mg/L)	new medium min	new medium max	old medium min	old medium max
Control	7.6	7.8	7.8	7.8
5.6	7.6	7.8	7.8	7.8
10	7.6	7.8	7.8	7.8
18	7.6	7.8	7.8	7.8
32	7.6	7.8	7.8	7.8
56	7.7	7.8	7.7	7.8
100	7.7	7.8	7.9	7.9
180	7.7	7.9	7.9	8.0
320	7.7	7.9	8.0	8.0
560	7.7	7.9	7.9	8.0

Other chemistry variables measured in test medium

Hardness	180 mg $CaCO_3$/L
Cl^-	16.9 mg/L
SO_4^{2-}	64.5 mg/L
Inorganic carbon	16.3 mg/L

Reproduction recorded during *C. dubia* tests per replicate

Nominal Mo (mg/L)	rep1	rep2	rep3	rep4	rep5	rep6	rep7	rep8	rep9	rep10
Control	25	16	9	23	21	20	22	24	20	4
5.6	23	18	21	22	24	20	14	11	23	16
10	26	24	18	16	21	17	16	19	20	21
18	24	24	18	10	14	17	14	25	33	22
32	21	19	15	11	26	20	25	9	15	20
56	28	19	24	11	25	10	18	7	12	26
100	6	21	18	15	17	23	13	21	7	10
180	10	19	12	4	6	6	5	5	3	18
320	†	†	2	4	†	†	†	0	6	†
560	1	†	†	†	†	†	†	†	†	†

†: individual died during the 7-day exposure

Annex 3.5 Raw data of *Lymnaea stagnalis* test

Raw length measurements and length-based growth rate in *Lymnaea stagnalis* test

Nominal Mo (mg/L)	replicate	length day 0 (cm)	length day 7 (cm)	length day 14 (cm)	length day 21 (cm)	length Day 28 (cm)	μ (%/d)
0	1	1.065	1.220	1.380	1.610	1.810	1.91
0	2	1.170	1.360	1.610	1.805	2.050	2.01
0	3	1.015	1.300	1.545	1.700	1.985	2.30
0	4	1.120	1.330	1.570	1.755	1.980	2.02
0	5	1.170	1.340	1.590	1.815	2.000	1.97
0	6	1.080	1.260	1.610	1.820	2.090	2.41
0	7	1.175	1.505	1.750	1.955	2.165	2.12
0	8	1.005	1.320	1.490	1.805	2.030	2.46
50	1	1.040	1.245	1.540	1.865	1.990	2.43
50	2	1.030	1.230	1.395	1.620	1.810	2.00
50	3	1.115	1.410	1.530	1.895	2.160	2.31
50	4	1.045	1.260	1.430	1.670	1.845	2.03
50	5	1.040	1.220	1.430	1.630	1.970	2.24
50	6	1.000	1.095	1.295	1.560	1.770	2.14
50	7	1.010	1.215	1.485	1.680	1.865	2.22
50	8	1.140	1.310	1.580	1.775	1.955	1.97
100	1	1.090	1.320	1.530	1.805	1.900	2.03
100	2	1.090	1.325	1.500	1.740	1.760	1.76
100	3	1.020	1.230	1.385	1.640	1.800	2.03
100	4	1.060	1.335	1.570	1.755	1.975	2.17
100	5	1.110	1.300	1.420	1.660	1.810	1.75
100	6	1.135	1.370	1.580	1.845	2.065	2.14
100	7	1.085	1.340	1.555	1.860	2.030	2.26
100	8	0.990	1.220	1.380	1.570	1.850	2.15
200	1	1.105	1.285	1.515	1.710	1.950	2.03
200	2	1.030	1.295	1.520	1.745	2.045	2.39
200	3	1.110	1.280	1.510	1.755	2.090	2.26
200	4	1.020	1.190	1.415	1.720	1.900	2.30
200	5	1.160	1.385	1.610	1.670	1.900	1.68
200	6	1.100	1.290	1.490	1.670	1.915	1.95
200	7	1.055	1.260	1.370	1.585	1.820	1.89
200	8	1.180	1.345	1.500	1.705	1.860	1.64
400	1	1.030	1.175	1.195	1.345	1.490	1.25
400	2	1.010	1.040	1.030	1.050	1.155	0.40
400	3	1.055	1.175	1.220	1.300	1.400	0.95
400	4	1.070	1.100	1.090	1.160	1.205	0.42
400	5	1.160	1.280	1.270	1.475	1.630	1.17
400	6	1.065	1.145	1.155	1.165	1.235	0.45
400	7	1.185	1.295	1.325	1.520	1.520	0.94
400	8	1.120	1.220	1.235	1.290	1.470	0.86
800	1	1.025	1.075	1.060	1.020	1.075	0.06
800	2	1.190	1.210	1.205	1.235	1.190	0.03
800	3	1.140	1.205	1.225	1.215	1.195	0.15
800	4	1.165	1.230	1.195	1.180	1.210	0.05
800	5	1.125	1.180	1.165	1.170	1.150	0.05
800	6	1.070	1.105	1.130	1.130	1.135	0.20
800	7	1.065	1.095	1.090	1.115	1.090	0.09
800	8	1.010	1.035	1.055	1.060	1.050	0.15

Annex 3.5 Raw data of *Lymnaea stagnalis* test (continued)

Raw wet weight measurements and weight-based growth rate in *Lymnaea* test

Nominal Mo (mg/L)	replicate	weight day 0 (mg)	weight day 7 (mg)	weight day 14 (mg)	weight day 21 (mg)	weight Day 28 (mg)	μ (%/d)
0	1	63.2	113.1	163.1	235.1	399.0	6.31
0	2	78.0	155.2	226.0	363.4	599.5	7.04
0	3	82.7	129.1	212.2	346.3	569.1	6.92
0	4	74.7	138.6	235.5	351.1	536.5	6.96
0	5	77.9	157.9	261.7	440.9	624.6	7.41
0	6	61.0	144.5	236.4	395.4	614.0	8.04
0	7	80.9	173.8	302.6	476.8	651.8	7.40
0	8	111.1	102.6	204.0	421.3	646.5	7.05
50	1	57.2	104.6	203.1	349.8	509.5	7.97
50	2	57.3	109.7	181.8	297.2	414.1	7.07
50	3	75.4	166.9	232.7	459.8	680.8	7.73
50	4	57.8	116.9	197.5	328.5	ND	8.20
50	5	65.8	124.0	211.0	368.3	557.3	7.66
50	6	51.1	89.4	147.9	269.0	407.4	7.51
50	7	60.1	109.7	208.5	311.5	435.2	7.15
50	8	76.6	117.4	216.7	325.8	443.9	6.48
100	1	76.8	149.0	221.2	372.5	465.7	6.46
100	2	75.4	103.3	178.5	306.2	408.6	6.38
100	3	50.4	93.4	130.2	257.1	352.5	7.00
100	4	62.9	126.1	216.9	338.2	489.6	7.27
100	5	75.0	133.6	177.9	326.1	454.1	6.42
100	6	72.6	134.0	217.0	355.4	518.5	7.01
100	7	55.7	126.9	193.1	336.8	ND	8.31
100	8	53.2	109.2	170.5	276.7	428.7	7.29
200	1	72.9	125.3	223.3	359.6	583.4	7.45
200	2	64.1	110.1	196.3	314.2	487.8	7.30
200	3	71.5	121.3	200.9	345.8	565.4	7.40
200	4	52.6	90.7	168.1	285.0	398.1	7.42
200	5	87.5	171.8	248.2	347.5	513.6	6.06
200	6	69.0	126.9	190.5	311.9	457.0	6.69
200	7	67.7	132.4	180.6	280.9	419.8	6.29
200	8	88.4	135.8	186.6	293.5	398.8	5.41
400	1	57.2	98.2	109.1	154.8	200.4	4.23
400	2	55.9	68.2	68.3	85.7	116.2	2.42
400	3	89.3	121.2	123.0	168.7	232.6	3.21
400	4	63.3	70.7	72.0	93.0	123.0	2.29
400	5	75.7	108.5	119.8	187.7	268.1	4.40
400	6	78.3	89.8	84.9	98.9	127.4	1.53
400	7	95.6	125.6	129.5	199.1	230.7	3.18
400	8	73.1	94.9	96.5	126.3	182.7	3.03
800	1	54.9	64.2	61.5	70.4	61.8	0.47
800	2	84.4	92.4	71.1	89.7	88.1	0.08
800	3	88.4	70.4	70.0	69.1	84.0	-0.17
800	4	82.8	92.9	73.9	100.6	85.6	0.21
800	5	92.2	87.7	84.3	92.3	94.6	0.15
800	6	66.6	70.1	69.3	73.3	65.5	0.02
800	7	63.9	65.6	62.4	67.6	61.2	-0.08
800	8	66.2	71.6	70.6	73.4	79.5	0.56

Annex 3.5 Raw data of *Lymnaea stagnalis* test (continued)

pH measured in *Lymnaea stagnalis* test (minima and maxima of recorded values)

Nominal Mo (mg/L)	new medium min	new medium max	old medium min	old medium max
Control	7.8	7.9	6.7	7.5
50	7.8	8.0	6.7	7.7
100	7.8	8.0	6.7	7.7
200	7.8	8.0	6.8	7.7
400	8.0	8.1	7.1	7.8
800	7.9	8.2	7.5	8.0

Annex 3.6: Mortality and malformations in *X. laevis* test observed after 96h of exposure

Nominal Mo (mg/L)	Replicate	Number dead out of 25	Number malformed out of 25
Control	1	0	0
Control	2	0	0
Control	3	0	0
Control	4	1	1
25	1	0	2
25	2	0	2
50	1	0	4
50	2	0	1
100	1	0	2
100	2	0	2
200	1	0	3
200	2	1	2
400	1	1	4
400	2	3	7

[a] *X. laevis* were considered malformed if at least one deformity of the ASTM list was observed.the type of malformations observed were: general malformations of gut, tail, face, eye and brain; as well as abdominal and facial oedema.

Annex 3.7 Raw data of 14-day *Chironomus riparius* test

pH measured during *Chironomus riparius* test (minima and maxima recorded)

Nominal Mo (mg/L)	min	max
Control	7.1	7.6
25	7.0	7.7
50	7.0	7.7
100	6.9	7.7
200	6.9	7.8
400	7.0	7.9
800	7.1	7.9
1600	7.1	8.1

14d-survival of *Chironomus riparius* per replicate (%)

Nominal Mo (mg/L)	rep1	rep2	rep3	rep4	rep5
Control	80	100	60	100	100
25	60	90	80	80	70
50	70	70	90	60	90
100	60	70	60	80	70
200	70	100	80	90	80
400	70	50	80	70	70
800	50	90	30	80	80
1600	50	60	70	70	40

Dry wt of *Chironomus riparius* after 14 days of exposure (mg)

Nominal Mo (mg/L)	rep1	rep2	rep3	rep4	rep5	rep6	rep7	rep8	rep9	rep10
Control	1.24	0.55	0.66	0.46	0.9	0.72	0.90	1	1.08	0.47
25	1.11	0.90	0.68	0.75	0.70	0.55	0.83	†	†	†
50	1.37	0.78	1.09	0.86	1.26	0.88	0.84	0.91	0.61	†
100	0.86	0.51	0.99	0.89	0.88	0.55	0.74	†	†	†
200	0.51	0.09	0.54	0.26	0.8	0.33	0.51	0.51	†	†
400	0.26	0.53	0.66	0.4	0.47	0.61	0.72	†	†	†
800	0.25	0.50	0.23	0.38	0.34	0.64	0.27	0.16	†	†
1600	0.11	0.23	0.20	0.07	†	†	†	†	†	†

† Larva died before the 14th day of exposure