Addendum to Report ZEH-WA-01

Development and validation of biotic ligand models for predicting chronic zinc toxicity to fish, daphnids and algae.

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Following additional experiments have been conducted since the final report of ZEH-WA-01 (28th February, 2003) was released:

- Chronic (30d) rainbow trout toxicity tests at Ca concentrations of 0.2, 1 and 4 mM
- A chronic (30d) rainbow trout toxicity test using an additional natural water (Brisy)
- Chronic (21d) toxicity tests with *Daphnia magna* in 6 natural waters and in one synthetic water (reference test)

The results of these experiments are described in the following three sections.

1. Additional Ca-tests with rainbow trout and refinement of chronic Zn-BLM

The additional Ca-tests allowed deriving final chronic BLM-constants. This must be considered an important improvement as in the ZEH-WA-01 report some assumptions were taken to derive the Ca-competition constant, resulting in a range of possible log K's instead of one log K (see Table 3.18 in ZEH-WA-01). In this "addendum"-report we will only focus on the refinement of the chronic Zn-BLM for fish and not on the acute Zn-BLM (which we also described in ZEH-WA-01, but which is of less relevance for the risk assessment).

It is noted that the Table numbers below are not starting from 1. They are given the numbers of corresponding Tables in ZEH-WA-01 and they can be considered as extensions of the latter ones. The raw data are given in the annexes.

Table 3.6 Average physico-chemistry of the test-media used for the BLM-development

Code	pH	Hardness[a] (mg $CaCO_3$/L)	Ca (mg/L)	Mg (mg/L)	Na (mg/L)	K (mg/L)	SO_4 (mg/L)	Cl (mg/L)	Alkalinity (mg $CaCO_3$/L)
CA-0.2	7.61	29.1	9.14	1.53	18.3	2.11	11.1	16.2	47.1
CA-1	7.58	102	38.2	1.65	17	1.92	11.7	69.2	52.4
CA-2 *	7.87	190	73.9	1.38	15.7	1.51	12.3	144	66.8
CA-4	7.68	396	156	1.68	17.1	1.99	11.1	270	44.1

[a] Hardness is calculated from measured Ca and Mg levels
* test already performed in ZEH-WA-01

Table 3.7 Effect concentrations of Zn (µg/L) used for the BLM-development. Numbers between brackets indicate 95% confidence limits

Code	96h-LC50	30d-LC50	30d-LC10	30d-NOEC
CA-0.2	194 (152 - 248)	159 (110-228)	34.5 (14.5-82.4)	78.9
CA-1	904 (608 - 1343)	759 (568-1014)	171 (91-324)	169
CA-2 *	1470 (910-2300)	1240 (760-2040)	290 (96-880)	885
CA-4	2280 (1460 - 3560)	1860 (1230-2820)	337 (113-1010)	786

* test already performed for ZEH-WA-01

As anticipated in ZEH-WA-01, we have now indeed observed a clear decrease of chronic Zn-toxicity with increased Ca-concentrations, i.e. about a factor of 10 with an increase from 0.2 to 4 mM Ca. The 30d-LC50 data have been used to calculate speciation and to estimate the BLM constants for chronic toxicity (Tables 3.8, 3.13 and 3.15).

Table 3.8 Chemical speciation in the chronic toxicity tests used for the BLM-development. Ca^{2+}, Mg^{2+}, Na^{+} and H^{+} are given as chemical activity (in M); 30d-LC50 is given as Zn^{2+}-activity (in M)

Code	Ca^{2+}	Mg^{2+}	Na^{+}	H^{+}	30d-LC50
CA-0.2	1.65E-04	4.57E-05	7.58E-04	2.45E-08	1.54E-06
CA-1	6.55E-04	4.66E-05	6.90E-04	2.63E-08	7.22E-06
CA-2*	1.19E-03	3.68E-05	6.25E-04	1.35E-08	1.03E-05
CA-4	2.33E-03	4.13E-05	6.62E-04	2.09E-08	1.56E-05

* test already performed in ZEH-WA-01

Table 3.13 Slopes (S) and intercepts (I) of regression equations for Ca for chronic toxicity; r^2 is the correlation coefficient for the regression; R is the ratio slope/intercept.

	S	I (M)	r^2	R_{Ca} (M^{-1})	Log R_{Ca}
Data points used: CA-0.2, CA-1, CA-2 and CA-4					
Chronic	6.16E-03	1.97E-06	0.95	3.12E+03	3.5

Tabel 3.15 Stability constants (K, in M^{-1}) for the competing cations for the chronic BLM (30-day mortality) calculated with Eq. 8. Data used to calculate the constants are given for each cation investigated: the R-value (in M^{-1}; see Tables 3.9, 3.10, 3.11 and 3.13) and the mean activity of the other competing cations (in M) in the tests used to derive the R-values (see Table 3.8).

$Cation_X$	R_X	$(Ca^{2+})_X$	$(Mg^{2+})_X$	$(Na^{+})_X$	$(H^{+})_X$	K_{XBL}	Log K_{XBL}
Ca^{2+}	3.12E+03	NA	4.26E-05	6.84E-04	2.13E-08	3.99E+03	3.6
Mg^{2+} *	7.12E+02	1.49E-04	NA	7.27E-04	1.98E-08	1.29E+03	3.1
Na^{+} *	1.47E+02	1.55E-04	5.01E-05	NA	3.39E-08	2.60E+02	2.4
H^{+} *	7.36E+05	1.48E-04	4.48E-05	4.25E-03	NA	2.11E+06	6.3

NA = not applicable

* test already performed in ZEH-WA-01

The constants of the refined chronic rainbow trout Zn-BLM are given in Table 3.18. Table 3.18 demonstrates that the competition constants are well within the 90% confidence interval estimated in ZEH-WA-01. Figure 3.1 and Table 3.19 give the predictive capacity of the BLM for the different synthetic test media. All LC10s and LC50s are predicted within < factor of 2.

Table 3.18 Old (ZEH-WA-01 report, given as 90% confidence interval) and new (present report) BLM-constants for chronic zinc toxicity to juvenile rainbow trout

	Old	**New**
Log K_{ZnBL}[a]	5.31	5.5
Log K_{CaBL}	3.35 – 4.01	3.6
Log K_{MgBL}	3.04 – 3.31	3.1
Log K_{NaBL}	2.33 -2.61	2.4
Log K_{HBL}	6.24 – 6.52	6.3
$f_{ZnBL}^{50\%}$	0.189 -104	0.246±0.070
$f_{ZnBL}^{10\%}$	0.067 - 0.034	0.0938±0.038

[a] Log K_{ZnBL} was originally set to the same value as reported in Heijerick et al. (2002) for the acute Zn-BLM for *D. Magna*, but the new model has the same value as the one reported by Santore et al. (2002) for the acute Zn-BLM for fish

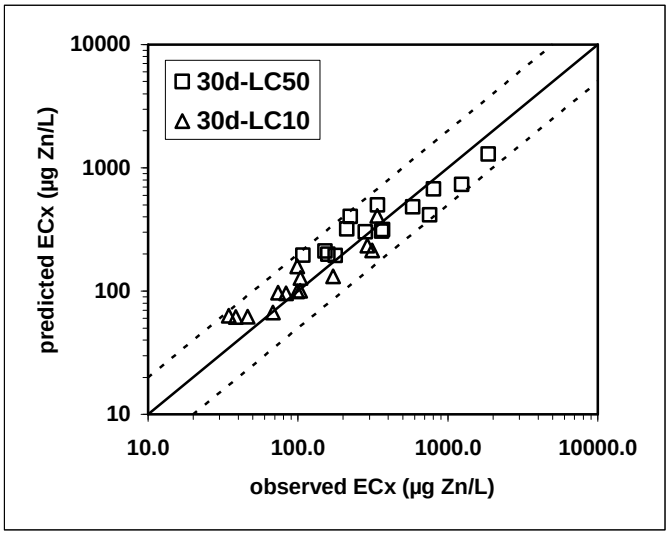

Figure 3.1 Observed and predicted 30d-LC50s and 30d-LC10s for test media used for the development of the chronic BLM.

Tabel 3.19 Observed and predicted 30d-LC50s and 30d-LC10s of Zn (µg/L). Numbers between brackets indicate 95% confidence limits.

Code	Observed 30d-LC50	Predicted 30d-LC50	Observed 30d-LC10	Predicted 30d-LC10
RF-B	177 (132-238)	194	38.4 (19.7-74.5)	62
RF-CA4	>1530	NC	NC	NC
RF-MG3	583 (504-676)	482	NC	NC
RF-NA5	282 (196-450)	302	83.2 (24.9-278.1)	96
MG-B	108 (81-143)	195	46.1 (25.5-83.6)	62
MG-0.2	151 (121-189)	211	67.9 (33.4-138.0)	67
MG-1	212 (183-247)	319	103 (77-139)	101
MG-2	224 (185-247)	403	104 (70-156)	128
MG-3	339 (269-428)	501	99.1 (58.7-167.5)	159
PH-5.5	802 (630-1023)	676	312 (184-531)	214
PH-6.5	368 (214-632)	315	99.1 (30.3-324.3)	100
PH-7.5	361 (226-579)	306	73.6 (23.4-231.7)	97
CA-B*	159 (110-228)	198	34.5 (14.5-82.4)	63
CA-1	759 (568-1014)	416	171 (91-324)	132
CA-2*	1240 (760-2040)	736	290 (96-880)	233
CA-4*	1860 (1230-2820)	1291	337 (113-1010)	409

NC = not calculated as no EC10 could be derived for these tests
* new experiments

2. Test with rainbow trout in an additional natural water: validation of the refined BLM using surface waters

An additional test with a spiked natural surface water was conducted. To that end, as in ZEH-WA-01, a surface water (Brisy), was concentrated in the field using reverse osmosis. In the laboratory, the surface water was re-diluted and Ca and Mg concentrations were adjusted to those measured in the original surface water. The physico-chemistry and the ecotoxicity test results for this test water and for the other natural waters are given in Tables 3.21 to 3.22.

Table 3.21 Average physico-chemistry during the toxicity tests in reconstituted natural waters used for the rainbow trout BLM-validation.

Code	pH	DOC (mg/L)	Hardness [a] (mg $CaCO_3$/L)	Ca (mg/L)	Mg (mg/L)	Na (mg/L)	K (mg/L)	SO_4 (mg/L)	Cl (mg/L)	Alkalinity (mg $CaCO_3$/L)
ANK*	7.76	22.9	104	32.0	5.77	34.2	0.74	56.3	93.4	49.1
MAR*	8.13	6.22	176	49.0	13.1	100	0.89	153	158	68.5
VOY*	6.80	3.92	28.2	9.54	1.06	16.7	0.87	14.1	22.8	17.1
BIH*	6.15	4.25	23.3	7.09	1.38	5.59	0.26	7.84	10.83	1.7
BRISY	7.08	2.84	31.5	8.05	2.77	5.9	0.48	13.5	10.4	14.3

[a] Hardness is calculated from measured Ca and Mg levels (mg $CaCO_3$/L)
* tests described in ZEH-WA-01

Table 3.22 Effect concentrations of Zn (µg/L) in the natural waters. Numbers between brackets indicate 95% confidence limits.

Code	30d-LC50	30d-LC10	30d-NOEC
ANK*	1970 (1460-2670)	902 (483-1683)	771
MAR*	1850 (1410-2410)	578 (322-1038)	696
VOY*	406 (263-624)	185 (99-346)	324
BIH*	582 (394-859)	259 (123-548)	370
BRISY	337 (292-389)	219 (111-432)	199

* tests described in ZEH-WA-01

Tables 3.23 and Figure 3.2 present the predictive capacity of the refined chronic Zn-BLM.

Table 3.23 Observed and predicted 30d-LC50s and 30d-LC10s of Zn (µg/L). Numbers between brackets indicate 95% confidence limits. Predictions were carried out with and without DOC assumed.

Code	Observed 30d-LC50	Predicted 30d-LC50	Predicted 30d-LC50 (no DOC)	Observed 30d-LC10	Predicted 30d-LC10	Predicted 30d-LC10 (no DOC)
ANK	1970 (1460-2670)	2014	434	902 (483-1683)	1147	141
MAR	1850 (1410-2410)	1284	800	578 (322-1038)	587	258
VOY	406 (263-624)	404	211	185 (99-346)	159	68
BIH	582 (394-859)	473	265	259 (123-548)	169	84
BRISY	337 (292-389)	328	184	219 (111-432)	134	60

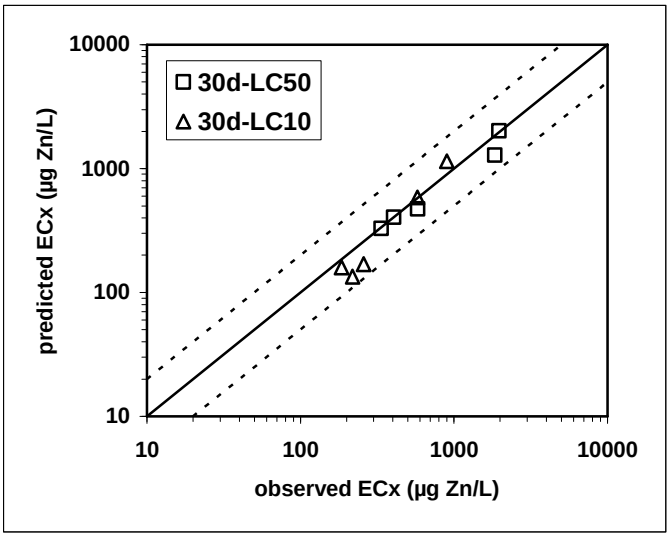

Figure 3.2 Observed and predicted 30d-LC50s and 30d-LC10s for natrual waters.

The results in Table 3.23 and Figure 3.2 Clearly demonstrate that all LC50s and LC10s are predicted with an error of less than factor 2. Comparing the predictions with DOC and those without DOC, it is observed that DOC accounts for a factor of 2 to 8 decrease of toxicity (when LC10s are considered). Hence, DOC must be considered a significant parameter in evaluating the risks of zinc in natural waters.

3. Chronic Zn toxicity to *Daphnia magna* in natural waters

Six natural surface waters were sampled. Tests were conducted with these non-manipulated samples and not with reconstituted samples as for the fish tests (i.e. reverse osmosis concentration followed by dilution in laboratory). An additional test was conducted in synthetic test water with increased Ca (2 mM) and Mg (0.5 mM). The physico-chemistry of these waters is summarized in the Table 4.12. Since validation tests were not reported in ZEH-WA-01, the table numbers below start from 4.12 (Table 4.11 was the last Table in the chapter on the *Daphnia* model in ZEH-WA-01). Table 4.12 gives the physico-chemistry of the natural waters.

Table 4.12 Physico-chemistry of synthetic and natural waters used for the BLM-validation.

Water	pH	DOC (mg/L)	Hardness [a] (mg $CaCO_3$/L)	Ca (mg/L)	Mg (mg/L)	Na (mg/L)	K (mg/L)	SO_4 (mg/L)	Cl (mg/L)	Alkalinity[b] (mg $CaCO_3$/L)
Synthetic	7.15	0.3 [b]	250	80.2	12.2	17.7	3.0	48.0	73.8	33.2
Ankeveen	6.77	17.3	123	38.3	6.5	17.4	6.1	127	50.0	12.3
Brisy	7.34	2.53	26.4	5.0	3.4	8.8	2.1	9.5	23.0	13.6
Markermeer	8.03	7.49	189	52.7	14.0	87.3	8.7	109.0	318	127
Regge	8.00	9.87	183	60.1	8.0	52.9	10.4	63.0	144	165
Rhine	8.22	2.30	196	61.0	10.7	55.4	5.0	57.0	215	159
Voyon	8.40	4.17	122	37.1	7.1	10.0	1.3	20.0	21	125

[a] Hardness is calculated from measured Ca and Mg levels (mg $CaCO_3$/L)
[b] background DOC concentration of deionized water in our laboratory (~300µg/L)

Table 4.13 gives the observed and predicted 21d-EC50s and 21-day NOECs obtained in these tests. Figure 4.2 visualizes the observed versus predicted EC50s and NOECs.

Table 4.13 Observed and predicted 21d-EC50 and 21d-NOEC of Zn to *Daphnia magna* (in µg Zn/L).

Water	Observed EC50	Predicted EC50	Observed NOEC	Predicted NOEC
Synthetic	299	237	155	165
Ankeveen	536	569	491	428
Brisy	112	177	94.5	136
Markermeer	313	679	244	540
Regge	473	824	251	664
Rhine	242	497	143	370
Voyon	171	450	72.7	354

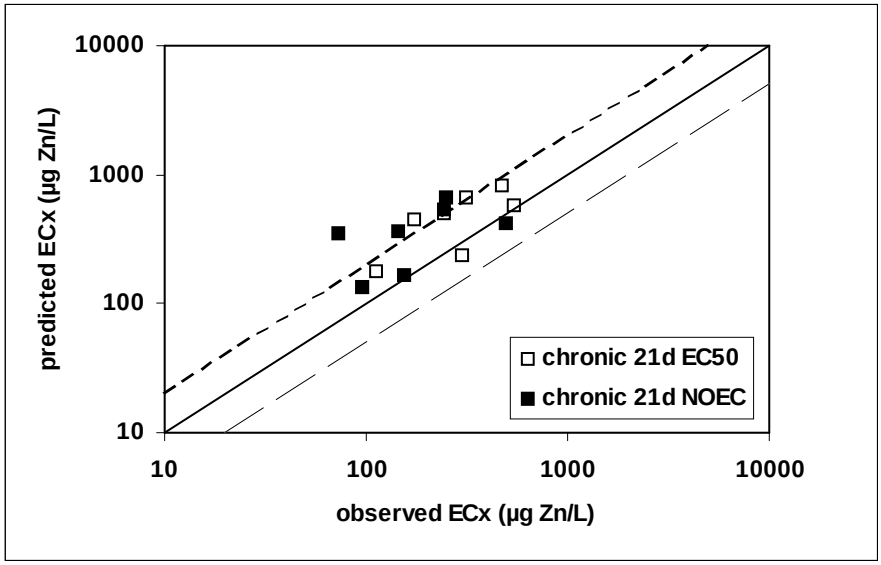

Figure 4.2 Observed and predicted EC50 and NOEC of Zn for *Daphnia magna* in natural surface waters.

Tables 4.13 and Figure 4.2 indicate that 3 of the 6 EC50s, and 2 of the 6 NOECs are predicted within a factor 2. The EC50 and NOEC in synthetic water (increased hardness) were predicted within a factor 2 of the observed values. The reason for this underestimation of toxicity, especially at the NOEC-level, needs further investigation.

ANNEXES

Table 3B.4 Results of the additional Ca-tests (2nd test series) with rainbow trout. * =significantly different from control; dissolved Zn concentrations in bold are NOECs

CODE	Dissolved Zn (±SE) (µg/L)	96hours mortality (%)	30days mortality (%)	p (dunnet) 30d-mortality	r_3 (±SE) (d^{-1})	p t-test (r3)
CA-0.2	<5.0	0	0		0.0434 (±0.0020)	
	32.7 (±0.4)	8.3	16.7	0.305	0.0430 (±0.0056)	0.978
	78.9 (±0.6)	25.0	25.0	0.124	0.0284 (±0.0073)	0.412
	166 (±4)	41.7	50.0	0.004*	0.0195 (±0.0177)	0.210
	346 (±8)	72.7	72.7	<0.001*	0.0161 (±0.0011)	0.006*
	773 (±18)	83.3	100.0	<0.001*		
CA-1	<5.0	0	0		0.0391 (±0.0044)	
	77 (±2)	0.0	0.0	0.808	0.0321 (±0.0063)	0.601
	170 (±3)	10.0	10.0	0.313	0.0305 (±0.0048)	0.448
	363 (±9)	26.7	26.7	0.009	0.0301 (±0.0069)	0.536
	848 (±36)	46.7	53.3	<0.001*	0.0418 (±0.0068)	0.844
CA-4	<5.0	6.7	6.7		0.0357 (±0.0047)	
	346 (±8)	10.0	10.0	0.713	0.0467 (±0.0069)	0.551
	786 (±10)	0.0	20.0	0.231	0.0327 (±0.0076)	0.884
	1670 (±10)	58.3	58.3	<0.001*	0.0498 (±0.0049)	0.410
	3580 (±260)	50.0	50.0	<0.001*	0.0395 (±0.0043)	0.787
	7323	100.0	100.0	<0.001*		

*Table 3B.5 Results of the test in the 5th natural water (Brisy) with rainbow trout. * =significantly different from control; dissolved Zn concentrations in bold are NOECs*

CODE	Dissolved Zn (±SE) (µg/L)	96hours mortality (%)	30days mortality (%)	p (dunnet) 30d-mortality	r_3 (±SE) (d^{-1})	p t-test (r3)
Brisy	<5.0	0	0		In progress	
	37.5 (±12.2)	0	0	0.787	In progress	
	199 (±12)	6.7	6.7	0.636	In progress	
	346 (±15)	48.3	55.2	0.005*	In progress	
	927 (±36)	87.5	100	<0.001*	In progress	