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The chronic toxicity of molybdate to marine organisms. I. Generating reliable effects data

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ABSTRACT

A scientific research program was initiated by the International Molybdenum Association (IMOA) which addressed identified gaps in the environmental toxicity data for the molybdate ion (MoO_4^{2-}). These gaps were previously identified during the preparation of EU-REACH-dossiers for different molybdenum compounds (European Union regulation on Registration, Evaluation, Authorization and Restriction of Chemical substances; EC, 2006). Evaluation of the open literature identified few reliable marine ecotoxicological data that could be used for deriving a Predicted No-Effect Concentration (PNEC) for the marine environment. Rather than calculating a $PNEC_{marine}$ using the assessment factor methodology on a combined freshwater/marine dataset, IMOA decided to generate sufficient reliable marine chronic data to permit derivation of a PNEC by means of the more scientifically robust species sensitivity distribution (SSD) approach (also called the statistical extrapolation approach). Nine test species were chronically exposed to molybdate (added as sodium molybdate dihydrate, $Na_2MoO_4 \cdot 2H_2O$) according to published standard testing guidelines that are acceptable for a broad range of regulatory purposes. The selected test organisms were representative for typical marine trophic levels: micro-algae/diatom (*Phaeodactylum tricornutum*, *Dunaliella tertiolecta*), macro-alga (*Ceramium tenuicorne*), mysids (*Americamysis bahia*), copepod (*Acartia tonsa*), fish (*Cyprinodon variegatus*), echinoderms (*Dendraster exentricus*, *Strongylocentrotus purpuratus*) and molluscs (*Mytilus edulis*, *Crassostrea gigas*). Available NOEC/EC_{10} levels ranged between 4.4 mg Mo/L (blue mussel *M. edulis*) and 1174 mg Mo/L (oyster *C. gigas*).

Using all available reliable marine chronic effects data that are currently available, a $HC_{5,50\%}$ (median hazardous concentration affecting 5% of the species) of 5.74 (mg Mo)/L was derived with the statistical extrapolation approach, a value that can be used for national and international regulatory purposes.

1. Introduction

According to EU Regulation No. 1907/2006 concerning the Registration, Evaluation, Authorization and Restriction of Chemical substances (REACH) (EC, 2006), the registration dossier for high-volume compounds should comply with the data requirements outlined in Annexes VII–X of the REACH legislation. In order to meet the acceptance criteria for such dossiers, predicted no-effect concentrations (PNECs) for different environmental compartments need to be addressed, either by proposing a PNEC-value or by providing a rationale for not proposing such a value. Though the derivation (or waiving) of a PNEC for the marine environment is required, according to Annexes VII–X there is no need to provide or generate acute/chronic marine eco-toxicological data; according to the ECHA (European Chemicals Agency) guidance document (Chapter R.10), a marine PNEC can be derived from freshwater data only (ECHA, 2008).

This approach, however, encompasses an additional assessment factor (AF) of at least 100 on the lowest No Observed Effect Concentration (NOEC) if no chronic data for typical marine species are included in the data set; greater species and taxonomic diversity in the marine environment, compared to freshwaters, implies a broader distribution of sensitivities of species and a higher uncertainty in extrapolation. Reducing the AF of 100 to 50 or 10 can be achieved when chronic data for typical marine taxonomic groups are available (e.g., molluscs, echinoderms).

ECHA guidance on PNEC-derivation allows the derivation of a PNEC by means of the scientifically more robust statistical extrapolation method (ECHA, 2008) when sufficient data are available. This method is based on a species sensitivity distribution (SSD) that can be developed when at least 10 chronic data points representing 8 (or more) different taxonomic groups are available.

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In an effort to generate a robust $PNEC_{marine}$ based on the SSD approach, a marine research program was initiated by the REACH Molybdenum–Consortium as part of the REACH dossier preparation for molybdenum and molybdenum compounds. Molybdenum is an essential element that is a component of several metalloenzymes (Hays and Swenson, 1985; Murray et al., 2000) and acts a co-factor in several physiological processes (e.g. oxidation/detoxification of other compounds, production of uric acid, metabolizing of sulfur- and nitrogen-containing amino acids that are present in DNA/RNA). It is an important micronutrient in both animal and plant nutrition (Deosthale, 1990). Elevated environmental concentration and/or uptake may potentially cause adverse effects. De Schamphelaere et al. (2010) published a data set with chronic toxicity data for 10 different aquatic organisms; effect levels, however, were several orders of magnitude higher than ambient concentrations levels in the aquatic environment. Van Gestel et al. (2011) also reported terrestrial chronic toxicity data for different terrestrial invertebrate species. Elevated dietary intake of molybdenum, specifically by ruminants (cattle, sheep; Underwood 1971), may result in copper deficiency due to the formation of thiomolybdates in the rumen of these animals (Suttle and Field, 1968). These thiomolybdates then form insoluble complexes with copper available from the diet, thus leading to copper deficiency. The underlying mechanisms that cause the observed toxicity of molybdate in the aquatic environment, however, are currently not fully understood.

As a first step, IMOA commissioned a thorough evaluation of all existing chronic toxicity data for molybdate in the marine environment. Based on the outcome of this review a testing program was conducted aimed at generating the data necessary to 1) develop a marine species sensitivity distribution for molybdate, and 2) derive a $HC_{5,50\%}$ (hazardous concentration that affects 5% of the species) which serves as a reference value for setting water quality criteria in the marine environment. Results of the eco-toxicological testing program, the literature review and $HC_{5,50\%}$ derivation are presented in this paper.

2. Materials and methods

2.1. Literature review

Studies provided by industry and relevant publications that were identified in open literature were collected and evaluated according to the criteria of quality as outlined by Klimisch et al. (1997). In short, this evaluation procedure allows an objective classification of toxicity data in four different categories (Klimisch 1 to 4), whereby only data placed in Category 1 (reliable without restrictions) and Category 2 (reliable with restrictions) can be used for risk assessment purposes as stipulated in the REACH legislation. Data that are categorized as Klimisch 3 (not reliable) or Klimisch 4 (not assignable to any of the previous categories due to lack of information) are considered not useful.

This Klimisch-scoring takes both the relevance and reliability of the reported test results into account. The term relevance refers to the biological and ecological relevance, relevance of the test substance and test medium, and relevance of the exposure period. Reliability, on the other hand, focuses on parameters like adequate description of the test method and analyses, information on test acceptability criteria, and presence of an effect–concentration relationship.

2.2. Selection of test substance — sodium molybdate

The rationale for using the highly soluble sodium molybdate as reference test substance for the evaluation of molybdenum compounds has been presented by De Schamphelaere et al. (2010). In short, the simple $[MoO_4]^{2-}$ ion is the only relevant Mo-species that will be released by different molybdenum containing substances or compounds under environmental relevant conditions (Cruywagen, 2000; Cruywagen et al., 2002; Greenwood and Earnshaw, 1987).

2.3. Selection of test organisms

For the freshwater compartment detailed guidance is available on test organisms/taxonomic groups that should be represented in a dataset for SSD purposes (ECHA, 2008). With regard to the marine environment, however, such specific guidance is not provided. The final selection of marine test species took into account the general principles that were adopted in RIP 3.2, Chapter R10 (ECHA, 2008) for the freshwater compartment (e.g., presence of at least eight different taxonomic groups). The selection was also based on the availability of test guideline protocols and on the experience of the testing facilities with the specific organisms and testing procedures. Chronic tests were conducted with the following taxonomic groups:

- Fish: *Cyprinodon variegatus*
- Mysid: *Americamysis bahia*
- Micro-alga: *Dunaliella tertiolecta*
- Macro-alga: *Ceramium tenuicorne*
- Copepod: *Acartia tonsa*
- Diatom: *Phaeodactylum tricornutum*
- Mollusc: *Crassostrea gigas*
- Echinoderms: *Strongylocentrotus purpuratus*, *Dendraster excentricus*

These nine test organisms, together with the mussel *Mytilus edulis* for which a reliable data point was identified in open literature (see Results section), represent eight taxonomic groups that are relevant and typical for the marine environment. Echinoderms, for instance, can only be found in the marine environment, and more than 93% of all mysid species live in the marine environment (Porter et al., 2008).

Chronic toxicity tests were conducted at four testing facilities: Grontmij/Aquasense (The Netherlands), ABC Laboratories (Columbia, USA), Brixham Environmental Laboratory (Brixham, UK) and Parametrix Environmental Research Lab (Albany, Oregon). Testing facilities were selected based on their previous experience with conducting chronic toxicity tests with marine species, and with the chronic toxicity testing procedures that were assigned to these laboratories. Grontmij/Aquasense performed the tests with the copepod *A. tonsa*, the diatom *P. tricornutum*, and the oyster *C. gigas*. ABC Laboratories conducted tests with the mysid *A. bahia*. Brixham performed the tests with the micro-alga *D. tertiolecta* and the macro-alga *C. tenuicorne*. Parametrix conducted tests with the sheepshead minnow (*C. variegatus*), the sea urchin *S. purpuratus* and the sand dollar *D. excentricus*.

2.4. Test procedures — general information

The chronic toxicity experiments were conducted according to internationally accepted standard testing protocols (i.e., EPA, ASTM, or ISO) or according to test procedures that were in line with those protocols. A detailed overview about origin of the test organisms, test media, test design, exposure duration, test conditions and endpoints recorded are provided in Annex 1. The test substance was provided by the International Molybdenum Association (sodium molybdate dihydrate; CAS number: 10102-40-6, EINECS 231-551-7; Lot/batch No.: 43006L with expiration date 31-12-2009). The salt was a solid, white crystalline powder reported to have an analytical purity of 99.9% (dihydrate form) based on an analyzed molybdenum content of 39.6%. The salt was stored at room temperature (20 °C ± 5 °C) in dry and dark conditions.

The general principles essential for generating high quality data were applied to all tests. Dissolved molybdate levels were analytically measured and reported effect levels (NOECs, EC_{10}-levels) are based on these measured values. Samples for dissolved molybdate (test initiation and termination, filtered through 0.45 μm) were taken from all treatments and measured by ICP-MS or flame Atomic Absorption Spectrophotometry according to standard analytical procedures like US-EPA Method 219.1 (U.S. Environmental Protection Agency, 1979/1983). Physicochemical parameters that are relevant for a correct assessment of chronic metal toxicity were monitored before and during the exposure period (e.g., temperature, pH, hardness, dissolved oxygen content), and steps were taken to ensure that variation of these parameters during the exposure period remained within acceptable boundaries. Finally, criteria for test acceptability were met in each specific test (e.g., growth rate criteria, reproduction criteria, survival criteria, etc.).

2.5. Data treatment/data analysis

Computing the NOECs for the different endpoints and test organisms included one or more of the following statistical methods and tests: Shapiro–Wilk's Test for normality, Levene's Test for homogeneity of variance, *T*-test with Bonferroni Adjustment, Steel's Many–One RankTest, Bartlett's Test, Wilcoxon Rank Sum Test with Bonferroni Adjustment, Dunnett's one-tailed or Multiple Comparison, one-tailed Fisher's Exact test with Hochberg's familywise adjustment for significance. The appropriate test for a one-way analysis of variance (ANOVA) was selected after evaluating the data for normality and homogeneity of variance. Statistical computer packages like CETIS (Comprehensive Environmental Toxicity Information System, McKinleyville, CA, USA), SAS (Statistical Analysis Software, Cary, NC, USA) or TRAP (Toxicity Relationship Analysis Program, Duluth, MN, USA) were used for this type of analysis.

Different methods were used for the determination of EC_x values, i.e., Linear Interpolation (*A. tonsa*, *C. tenuicorne*, *C. gigas*, *P. tricornutum*), Piecewise Linear (or treshold sigmoid) Regression Analysis (*C. variegatus*, *D. tertiolecta*, *S. purpuratus*), Maximum Likelihood Probit (*D. excentricus*), and Trimmed Spearman–Karber methods (*C. variegatus*, *D. excentricus*, *A. bahia*).

3. Results

Table 1 gives an overview of the NOEC, LOEC (Lowest Observed Effect Concentration), and EC_{10} values that were generated for the different test species. The main test properties for the different species are summarized in Appendix A. An overview of the main results is given below for each tested organism. Reported (no-) effect concentrations are based on measured Mo-levels.

A. tonsa (reported in Kools and Vanagt, 2009): NOEC and EC_{10} values were reported for reproduction (F0, F1 generations) and development (F1-generation). The 5d-EC_{10} for $F0_{reproduction}$ was considered unreliable as the calculated value was an extrapolated value well below the lowest test concentration, the 95% confidence interval ranged over more than 3 orders of magnitude. Additionally, the 5d-EC_{10} was about 2 orders of magnitude below the NOEC. Reliable NOEC, EC_{10} and EC_{50} values were generated for 20d-

Table 1
Overview of the generated chronic effect levels (EC_{10}, NOEC and LOEC), expressed as mg $Mo_{dissolved}$/L for nine test species.

Species	Test concentrations (mg Mo/L)	Endpoint	EC10	NOEC	LOEC
			mg $Mo_{dissolved}$/L		
A. tonsa	Nominal: 0 (control); 10; 32; 100; 320; 560; 1000 Measured dissolved: <1; 11; 26; 79; 270; 500; 940	Development F1 generation (20 d)	7.96 (95%CL: 0–906)	26	79
A. bahia	Nominal: 0 (control); 3.8; 7.5; 15; 30; 60; 120 Measured dissolved: <DL[a], 3.75; 7.44; 14.5; 28.8; 58.3; 116	Growth, reproduction, survival (28 days)	>116	≥116	>116
P. tricornutum	Nominal: 0 (control); 56; 100; 180; 320; 560; 1000 Measured dissolved: <DL[a]; 48; 85; 150; 310; 500; 910	Growth rate (72 h)	169.9 (95%CL: 145.7–192.2)	150	310
D. tertiolecta	Nominal[b]: 0 (control); 320; 560; 1000; 1800; 3200 Measured dissolved$_{start}$: <0.1; 113; 188; 353; 566; 1010 Measured dissolved$_{end}$: 0.3; 109; 194; 360; 616; 868	Growth rate (72 h)	881 n.d.	938.3 (mean measured)	>938.3 (mean measured)
C. tenuicorne	Nominal: 0 (control); 320; 560; 1000; 1800; 3200 Measured dissolved$_{start}$: 0.02; 111; 197; 314; 639;1110 Measured dissolved$_{end}$: 0.30; 137; 232; 341; 644; 1280	Growth rate (length) (7 days)	274 (95%CL: 0–507)	641	1190
D. excentricus	Nominal: 0 (control); 16; 31; 63; 125; 250; 500 Measured dissolved: <DL[a]; 14.5; 28.0; 56.7; 113.2; 217.9; 438.4	Development (Proportion normal) (48 h)	233.6 (95%CL: 231.5–235.8)	217.9	438.4
S. purpuratus	Nominal: 0 (control); 31.3; 62.5; 125; 250; 500; 1000; 2000 Measured dissolved: 2.3; 40.2; 80.2; 171.4; 264.5; 460.4; 910.7; 2087.9	Development (combined proportion normal) (48 h)	325.8 (95%CL: 269.2–394.3)	80.2[c]	171.4[c]
C. gigas	Nominal: 0 (control); 32; 100; 180; 320; 560; 1000; 1800; 3200 Measured dissolved: <DL[a]; 35; 110; 200; 360; 610; 1100; 1900; 3500	Development (48 h)	1174 (95%CL: 577–1534)	1100	1900
C. variegatus	Nominal: 0 (control); 62.5; 125; 250; 500; 1000 Measured dissolved: 5.7; 63.9; 111.8; 210.2; 444.3; 823.8	Biomass — dry wt (28 days)	84.1 (95%CL: 35.5–199.2)	<63.9	63.9

[a] <DL: detection limit value not specified in the original report.
[b] Levels expressed as sodium molybdate dihydrate.
[c] Endpoint: proportion normal.

$F1_{development}$ (26 mg Mo/L, 7.96 mg Mo/L (95%CL: 0.14–27.3 mg Mo/L) and 87.2 mg Mo/L (95%CL: 24.2–211 mg Mo/L), respectively) and for 30d-$F1_{reproduction}$ (79 mg Mo/L, 14.9 mg Mo/L (95%CL: 13.9–16.8 mg/L) and 219 mg Mo/L (95%CI: 27–477 mg Mo/L), respectively). $F1_{development}$ was the most sensitive endpoint, and represents the number of adults that developed from nauplii.

C. gigas (reported in Kools and Vanagt, 2009): A clear concentration-dependent effect of molybdate on the development of oyster larvae of *C. gigas* was noted during the 48 h exposure period, resulting in a NOEC, EC_{10} and EC_{50} of 1100 mg Mo/L, 1174 mg Mo/L (95%CL: 577–1534 mg Mo/L) and 2297 mg Mo/L (95%CL: 2025–2242 mg Mo/L), respectively.

P. tricornutum (reported in Kools and Vanagt, 2009): A clear decrease in cell growth was noted with increasing Mo-levels in the test media: growth rate (r) was close to zero at the two highest test concentrations, whereas a slight (though not significant) growth rate stimulation was noted at the lowest test concentration. Derived NOEC, E_rC_{10} and E_rC_{50} were 150 mg Mo/L, 169.9 mg Mo/L (95%CL: 145.7–192.2 mg Mo/L) and 356.9 mg Mo/L (95%CL: 329.3–382.0 mg Mo/L), respectively.

D. tertiolecta (reported in Le Page and Hayfield, 2010): Effect–concentration relationships for both biomass and growth rate were based on daily measurements of cell particle density for each treatment. For growth rate, the 72 h-NOEC and 72 h-EC_{10} were 938 and 881 mg Mo/L, respectively. For the less relevant endpoint "biomass", these values were 838 and 513 mg Mo/L, respectively. The increase in biomass for the parent inoculum culture was monitored over its 4 d growth period prior to the start of the test and was considered to be in an exponential growth phase. An acceptable overall factor increase in cell particle density (considered equivalent to biomass) of 82 was noted (i.e. an approximate daily factor of 9.3). The microscopic observations, made at the end of the test, showed that algal cells sampled from each test concentration appeared normal in comparison to the cells observed from the control replicate.

C. tenuicorne (reported in Le Page et al., 2010): A greatest growth rate (r) inhibition of 38% was noted for the highest test concentration (1190 mg Mo/L; mean measured concentration). Consequently, the growth rate data permitted the calculation of an E_rC_{10}, but not the derivation of a reliable E_rC_{50} (extrapolation well beyond the highest test concentration). The calculated 7d-NOE_rC and 7d-E_rC_{10} were 641 mg Mo/L and 274 mg Mo/L (95%CL: 0–507 mg Mo/L), respectively. An acceptable variability in the growth rate was reported, and variability in the exposure concentrations did not exceed that in the control. However, due to the variability that was present and the shape of the dose response curve produced for the concentrations selected, the confidence interval calculated for the reported EC_{10} value was wide and encompassed zero. Le Page et al. (2010) recommended that the EC_{10} should be treated with caution and concluded that the alternative valid endpoint (i.e. NOEC) was a more reliable endpoint.

A. bahia (reported in Lehman, 2010): After 28 days of exposure, mean survival of F0 mysids was 97% in the dilution-water control and ranged from 90% to 98% in the disodium molybdate treatments. Brood pouches were first evident on day 10 of the exposure in the control and all test-substance treatments. After 28 days of exposure there were no statistically significant differences for F0 male/female mysid mean body lengths at any of the test substance treatments as compared to the control. A similar finding was noted for mysid mean dry weights in any of the test substance treatments as compared to the control. The mean day of first-brood release by F0 mysids was 15.5 days (SD: 1.17) in the dilution-water control and ranged from 15.6 days (SD: 1.08) to 16.1 days (SD: 1.81) for the different Mo-treatments. There was also no statistically significant difference for mean number of total young produced per female in any of the tests substance treatments as compared to the control, and no effect of increased Mo-levels on survival of the F1 generation (96 h) was observed.

No statistically significant differences between any of the treatments and the control were identified. This finding was observed for each of the evaluated endpoints. The NOEC and LOEC values were 116 and >116 mg Mo/L, respectively.

C. variegatus (reported in Parametrix Environmental Research Laboratory, 2009): A difference in the time of egg hatching (time-to-hatch) was observed in the highest test concentration. The overall mean days-to-hatch was 5.7 ± 0.5. An insoluble precipitate that had formed in the highest concentration appeared to be coating the eggs. This precipitate coating likely contributed to the effect noted on embryo hatchability and embryo survival. No further research has been conducted to determine the chemical composition of the precipitate. Laboratory observations indicated that embryos that did not hatch contained dead larval fish that appeared fully developed. Embryonic survival was significantly reduced in the highest concentration relative to control organisms resulting in a NOEC of 444.3 mg Mo/L. The LC_{10} and LC_{50} for this endpoint was 468.9 mg Mo/L (95% CL: 329.5–667.2 mg Mo/L) and 696.1 mg Mo/L (95%CL: 638.3–759.3 mg Mo/L), respectively.

Larval survival was determined based upon the number of organisms that survived in the larval stage (after thinning). Following hatching, there was a significant effect on larval survival at the highest concentration, and a statistically significant effect on organism dry weight per surviving organism at the lowest concentration, resulting in a NOEC of <63.9 mg Mo/L. The effective concentration to reduce organism mean dry weight by 10% relative to control (EC_{10}) was 84.1 mg Mo/L (95% CL: 35.5–199.2 mg Mo/L). There were also significant effects on mean weight per original organism (biomass — a combined endpoint for lethality and growth). The biomass for the three highest treatments was significantly reduced relative to controls resulting in a NOEC of 111.8 mg Mo/L. The effective concentration to reduce organism biomass by 10% relative to control performance (EC_{10}) was 392.0 mg Mo/L (308.5–498.2 mg Mo/L).

Dendraster excentricus (reported in Parametrix Environmental Research Laboratory, 2008): The proportion of normal larvae, and the proportion of surviving larvae were calculated for each treatment replicate. Observations were calculated as 1) proportion combined survived and normal developed, and 2) proportion survived. The 48 h-NOEC for survival was 438.4 mg Mo/L. For proportion of normal eggs/larvae, the NOEC, EC_{10} and EC_{50} were 217.8 mg/L, 233.6 mg Mo/L (95%CL: 231.5–235.8 mg Mo/L) and 309.6 mg Mo/L (95%CL: 308.8–310.3 mg Mo/L), respectively. Treating the data in a different way, that is deriving (no-)effect levels for number of normal eggs/larvae and combined proportion of normal eggs/larvae, resulted in values that were slightly higher.

S. purpuratus (reported in Parametrix Environmental Research Laboratory, 2010): A significant effect on survival of the test organisms was noted at the highest test concentration of 2087.9 mg Mo/L, resulting in a $NOEC_{survival}$ of 910.7 Mo/L. As for the endpoint 'development', observations were calculated as 1) proportion combined survived and normal developed, and 2) proportion normal developed. The first method resulted in a 48-h NOEC, EC_{10} and EC_{50} of 264.5 mg Mo/L, 325.8 mg Mo/L (95%CL: 269.2–394.3 mg Mo/L) and 411.5 mg Mo/L (95%CL: 348.5–485.9 mg Mo/L), respectively. The second approach led to similar effect levels, i.e., 80.2 mg Mo/L, 483.0 mg Mo/L (95%CL: 421.9–552.9 mg Mo/L) and 576.2 mg Mo/L (95%CL: 512.2–648.2 mg Mo/L) for the 48-h NOEC, EC_{10} and EC_{50}, respectively.

Table 2 gives an overview of the (open) literature data on acute/chronic toxicity of molybdenum in the marine environment and their respective Klimisch scoring. With regard to chronic exposure, no useful data were identified for primary producers (algae). For the primary and secondary consumers however data of Klimisch 1

Table 2
Overview of marine toxicity data for molybdenum (as molybdate) in open literature and in industry reports (K1: Klimisch 1, reliable; K2: Klimisch 2, reliable with restrictions; K3: Klimisch 3, not reliable).

Species	Mo-salt	Effect level (mg Mo/L)	Quality label	Reference
(Micro-)algae (primary producers)				
Gymnodinium splendens	Ammonium molybdate	48 h-MTL: 2–35 48 h-NTL: 1–30	K3	Wilson and Freeburg (1980)
Glenodinium halli	Ammonium molybdate	48 h-MTL: 2–25 48 h-NTL: 1–20	K3	Wilson and Freeburg (1980)
Isochrysis galbani	Ammonium molybdate	48 h-MTL: 10–>300 48 h-NTL: 20–>300	K3	Wilson and Freeburg (1980)
Thalassiosira pseudonana	Ammonium molybdate	48 h-MTL: 1–100 48 h-NTL: 0.5–80	K3	Wilson and Freeburg (1980)
Invertebrates (crabs, shrimps, mysids)				
Carcinus maenas (shore crab)	Ammonium molybdate	48 h-TL_m: >254 48 h-TL_m: 1018	K1 K1	Abbott (1977)
Euparagus bernhardus	Ammonium molybdate	24 h-TL_m: 127–254 48 h-TL_m: 191–254	K3	Abbott (1977)
Allorchestes compressa	Ammonium molybdate	96 h-LC_{50}: 247.1(215.8–282.9)	K3	Ahsanullah (1982)
Mysidopsis bahia	Molybdenum trioxide	96 h-LC_{50}: 180 (160.6–201.8)	K2	Carr (1987)
Penaeus duorarum	Sodium molybdate	96 h-LC_{50}: 1849	K1	Knothe and Van Riper (1988)
Mysidopsis bahia	Sodium molybdate	96 h-LC_{50}: 1045	K1	Knothe and Van Riper (1988)
Fish (secondary consumers)				
Morone saxatilis	Sodium molybdate	96 h-LC_{50}: >79.8	K2	Dwyer et al. (1992)
Oncorhynchus tshawwytscha	Sodium molybdate	96 h-LC_{50}: > 1000	K3	Hamilton and Buhl (1990)
Oncorhynchus kisutch	Sodium molybdate	96 h-LC_{50}: > 1000	K3	Hamilton and Buhl (1990)
Cyprinodon variegatus	Sodium molybdate	96 h-LC_{50}: 2587	K1	Knothe and Van Riper (1988)
Molluscs, starfish (secondary consumers)				
Asterias rubens (starfish)	Ammonium molybdate	24 h-TL_m: 127–254	K3	Abbott (1977)
Venerupis pullastra (pullet-shell)	Ammonium molybdate	24 h-TL_m: 254–509	K3	Abbott (1977)
Crassostrea virginica	Sodium molybdate	96 h-LC_{50}: 1375	K1	Knothe and Van Riper (1988)
Mytilus edulis	Ammonium molybdate	48 h-EC_{50}: 147 (127–169) 48 h-EC_{10}: 4.40[a]	K1	Morgan et al. (1986)

[a] Not reported but derived from published raw data.

standard were available. It should be noted that data of Klimisch 3 standard are not considered useful for PNEC-derivation within a regulatory context, but the information in these publications may still have scientific merit.

4. Discussion

4.1. Evaluation of the data generated

Chronic effect levels for the nine tested species (Table 1) were between 7.96 mg Mo/L (lowest EC_{10} for *A. tonsa*; test conducted by Kools and Vanagt, 2009) and 1174 mg Mo/L (EC_{10} for *C. gigas*; test conducted by Kools and Vanagt, 2009), which is a difference of a factor of 147.5 between the most and the least sensitive species. By including the only reliable literature data point identified for molybdate in the marine environment, a 48 h-EC_{10} of 4.4 mg Mo/L for the mussel *M. edulis* (Morgan et al., 1986; see Table 2), the factor of difference becomes 267.

The ranking in sensitivity of tested species, from most to least sensitive, is as follows: *M. edulis* > *A. tonsa* > *C. variegatus* > *A. bahia* > *P. tricornutum* > *D. excentricus* > *S. purpuratus* > *C. tenuicorne* > *D. tertiolecta* > *C. gigas*.

It is noteworthy that different types of molluscs are found on either side of the sensitivity spectrum: the mussel *M. edulis* proved to be more sensitive than the oyster *C. gigas*, and this by more than a factor of 250. This large variation in response toward metal exposure among molluscs is though not uncommon. The REACH Chemical Safety Report for lead (http://apps.echa.europa.eu/registered/registered-sub.aspx) reported more than 100 fold difference between the chronic toxicity of the oyster *C. gigas* and the mussel *Mytilus trossolus*; *C. gigas* being the least sensitive marine organism that was included in the Pb-species sensitivity distribution, and *M. trossolus* the most sensitive.

Both echinoderms (*D. excentricus*, *S. purpuratus*) belong to the lesser sensitive organisms that were evaluated, with EC_{10}s of 233.6 and 325.8 mg Mo/L, respectively. All tested marine micro- and macro-algal species also belong to those species that show a greater tolerance to elevated Mo-levels.

4.2. Existing literature data on Mo-toxicity in the marine environment

From Table 2 it can be concluded that prior to the studies reported herein, only one reliable chronic study was available in open literature, namely the study conducted by Morgan et al. (1986). In short, adult blue mussels (*M. edulis*) were field collected, kept in unfiltered seawater and were brought to gonadal maturation. Spawning of the mussels was induced and fertilization was accomplished within 1 h of spawning initiation. Twelve Mo-levels were evaluated, using ammonium molybdate as the test substance. Tests were conducted in filtered, UV-sterilized seawater at 26 ppt salinity, pH 8.4 ± 0.2; an incubation temperature of 19 ± 1 °C and dissolved oxygen levels >70% saturation. The exposure period started within 2 h of fertilization and was terminated 48 h later (preservation of larvae in 5% buffered formalin). Larvae which failed to transform to the fully shelled, straight hinged, 'D' shaped prodissoconch 1 stage were considered abnormal. Results were analyzed to express abnormal development in terms of a 48 h-EC_{50} by computational methods (Binomial Probability, Moving Average or Probit analysis) and confirmed by graphical log-probit analysis

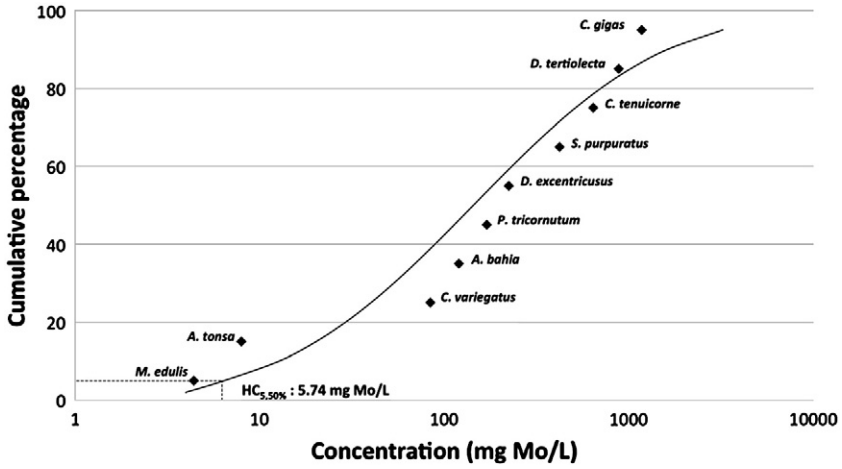

Fig. 1. Species sensitivity distribution and $HC_{5,50\%}$ derivation for molybdate in the marine environment.

(APHA, 1980). The reported 48 h-EC_{50} by Morgan et al. (1986) was 147 mg/L (95%CL: 127–169 mg/L).

According to the ASTM Guideline E724 "Conducting Static Acute Toxicity Tests Starting with Embryos of four species of Saltwater Bivalve Molluscs", a 24 h-exposure test should be used for the evaluation of acute toxicity, whereas the 48 h period is considered more appropriate for the evaluation of chronic toxicity. Morgan et al. (1986), however, only reported a 50% effect level (acute endpoint) on development (chronic parameter) for a 48 h-exposure period (chronic duration). As the raw data were included in the original paper, it was possible to derive the 48 h-EC_{10} (chronic endpoint) for this experiment, based on confirmed nominal concentrations. A log-normal distribution was fitted through the data, and a 48 h-EC_{10} of 4.40 mg Mo/L was derived (see Table 2).

Reliable acute toxicity data were available for two test organisms used in this study for chronic molybdenum toxicity determination: *A. bahia* and *C. variegatus*. Both Carr (1987) and Knothe and Van Riper (1988) investigated the acute toxicity of molybdenum toward the mysid shrimp *A. bahia* (the previous name of *Mysidopsis bahia* was used in the original report). Knothe and Van Riper (1988) reported a 96 h-LC_{50} of 1045 mg Mo/L, using sodium molybdate as the test substance. This acute value is about 10 times higher than the chronic EC_{10} of 116 mg Mo/L reported by Lehman (2010). Results of another acute 96 h toxicity test with this species were reported by Carr (1987) who performed this test in the framework of a study "Development of saltwater criteria advisories", commissioned by US Environmental Protection Agency. This latter study used molybdenum trioxide as test substance and the derived nominal 96 h-LC_{50} was 180 mg Mo/L, i.e., one order of magnitude below the acute toxicity that was determined by Knothe and Van Riper (1988) for this species. It should be noted that the pH of the test solutions was not measured/reported by Carr (1987). Studies performed under freshwater conditions have demonstrated that addition of high amounts of MoO_3 (i.e., >10–50 mg/L) may decrease the pH of the test solution below the tolerance limit for aquatic life (i.e., pH<4) (ECTX, 2007a,b,c). This drop in pH is a consequence of the transformation of molybdenum trioxide into molybdate ions. Quantitative data on the amount of MoO_3 that must be added to seawater in order to observe a significant pH-drop are currently not available. However, given the fact that the amount of administered MoO_3 at LC_{50}-level (180 mg Mo/L) exceeds the amount of MoO_3 causing a significant pH-drop in freshwater by approximately one order of magnitude, it remains unclear whether the observed 50% mortality at 180 mg/L is related to Mo in the test solution or whether it is caused by a decrease of the pH below the tolerance limit of *A. bahia* for this parameter. Therefore the lower acute toxicity level of 180 mg Mo/L from this study above is not considered relevant for assessing molybdenum toxicity.

The acute toxicity of molybdenum to the sheepshead minnow *C. variegatus* was evaluated in a second test that was conducted by Knothe and Van Riper (1988). The acute 96 h-LC_{50} of 2587 mg Mo/L for this organism was a factor of 30 higher than the chronic EC_{10} of 84.1 mg Mo/L that is reported in Table 1. These investigators also determined the acute toxicity of two other marine species. A 96 h LC_{50} of 1849 mg Mo/L was found for the pink shrimp *Penaeus duorarum*, and a 96 h LC_{50} of 1375 mg Mo/L was reported for the American oyster *Crassostrea virginica*. The latter value is somewhat higher than the chronic 48 h-EC_{10} of 1174 mg Mo/L that was determined for another *Crassostrea* sp. (*C. gigas*, see Table 1).

The final reliable acute value that was found in open literature was the unbounded 96 h-LC_{50} of >79.8 mg Mo/L that was reported by Dwyer et al. (1992) for the striped bass *Morone saxatilis*.

Several other publications (Abbott, 1977; Ahsanullah, 1982; Anderson and Mackas, 1986; Hamilton and Buhl, 1990; Wilson and Freeburg, 1980) reported acute toxicity levels of molybdenum for various marine species, but the data reported were not considered reliable and/or relevant for various reasons: test methodology was poorly described, field organisms with unknown history/age/health were used as test organisms, interaction of other adverse effects (e.g., low pH) could not be excluded, test concentrations were not measured or properly described, no standard test substance was used (e.g., mine tailings), the test medium was not representative for the marine environment, and the endpoint/end parameter was not suited for risk assessment purposes.

4.3. Derivation of a species sensitivity distribution

A species sensitivity distribution (SSD) has been developed for the assessment of molybdate in the freshwater compartment, using the reliable species-specific chronic toxicity effect levels that have been identified in Tables 1 and 2. All toxicity tests were performed using sodium molybdate as the test substance, and only the most sensitive end parameters were taken into account.

Fig. 1 presents the SSD that was fitted though the ten species-specific no-effect levels that were determined. Using the software package BestFit, the log-normal distribution was defined as the optimal distribution.

The $HC_{5,50\%}$ (±95%CL) that was associated with this distribution was determined with ETX, a statistical computer program developed by the Netherlands National Institute for Public Health and the Environment (RIVM), and which calculates this endpoint (+95% confidence intervals) by means of a log-normal distribution which is fitted through the data set. The $HC_{5,50\%}$ is the median 5th percentile with 5%–95%-confidence interval. This confidence interval is calculated using a Monte Carlo analysis on the generated distribution (2000 simulations) and is based on the selection of random samples of model input parameters according to the respective assigned probability distribution. The outcome of this analysis allows the derivation of the $HC_{5,50\%}$ with 5%–95% confidence interval. According to TGD (EC, 2003) and ECHA (2008) guidance, this parameter serves as the reference point for final PNEC-setting. The $HC_{5,50\%}$ (±95%CL) that was associated with the generated species sensitivity distribution (Fig. 1) was 5.74 mg Mo/L (95%CL: 0.58–21 mg Mo/L).

Similar work to that presented in the current study has been conducted and reported by De Schamphelaere et al. (2010), but with focus on the freshwater compartment. These authors derived $HC_{5,50\%}$ for molybdenum of 38.2 mg Mo/L in the freshwater environment.

As mentioned in the introduction the ECHA guidance document (Chapter R.10) states that the larger diversity among species and taxonomic groups in the marine environment, compared to freshwaters, may result in a broader distribution of sensitivities of species. The marine data that are presented in this study confirm this hypothesis for molybdenum. The range of EC_{10}/NOEC values for the freshwater compartment (43.2–241.5 mg Mo/L; De Schamphelaere et al., 2010) was noticeable smaller than the range of observed EC_{10}/NOECs in the marine environment (4.4–1174 mg Mo/L). Secondly, the lowest chronic value for the freshwater environment (43.2 mg Mo/L) for the rainbow trout *Oncorhynchus mykiss* was approximately one order of magnitude higher than the lowest marine values for *M. edulis* (4.4 mg Mo/L; Morgan et al., 1986) and *A. tonsa* (7.96 mg Mo/L; Kools and Vanagt, 2009). On the other side of the spectrum however, the highest freshwater chronic value of 241.5 mg Mo/L for duckweed *Lemna minor* was almost one order of magnitude lower than the highest marine chronic value of 1174 mg Mo/L for the oyster *C. gigas* (Kools and Vanagt, 2009). As mentioned in the Introduction, ECHA guidance allows the derivation of marine no-effect levels based on freshwater data, but in that case an additional assessment factor of 10 has to be applied on the freshwater ecotoxicity reference value. It is noteworthy that this factor of 10 is in line with the observed difference between the marine and freshwater $HC_{5,50\%}$.

Marine PNEC values for other metals have been disseminated by ECHA. The values originate from the REACH dossiers that have been submitted in 2010 by various metal commodities associations. For metals such as copper, zinc and lead the PNEC-values represent the $HC_{5,50\%}$ divided by an assessment factors ranging from 1 to 5, with the size of the AF depending on the outcome of an uncertainty analysis on the marine effects data set. The reported PNECs for Cu, Zn and Pb are 5.2, 6.1 and 3.4 μg/L, respectively. These PNEC-values are more or less three orders of magnitude lower than our $HC_{5,50\%}$ of 5.74 mg Mo/L. The relative low toxicity of molybdenum toward the marine environment in comparison with other metals confirms the previous findings that were also noted for the freshwater environment. Marine PNECs for some other metals that were released on the ECHA website (reference date: July 2011) are 0.067 μg Hg/L, 1.14 μg Cd/L, 2.36 μg Co/L, 2.5 μg V/L and 11.3 μg Sb/L. It should be noted that the PNECs for these other metals may be subject to change over time if new reliable ecotoxicological data become available. Indeed, new data could alter the reference value in both the assessment factor method (chronic ERV) or the statistical extrapolation method ($HC_{5,50\%}$). Generating additional data could also result in the application of a more/less conservative assessment factor on the $HC_{5,50\%}$, or could even trigger a change in the applied methodology for deriving a PNEC (assessment factor vs statistical extrapolation).

5. Conclusion

Chronic toxicity tests were conducted with nine different marine aquatic organisms, using sodium molybdate as test substance. Based on the quality criteria as defined by Klimisch et al. (1997), the chronic no-effect levels taken from these experiments were found to be reliable without restrictions (Klimisch 1 data points). Chronic effect levels for the tested species ranged from 7.96 mg Mo/L (*A. tonsa*) to 1174 mg Mo/L (*C. gigas*), which is a maximum difference of a factor of 147 among tested organisms. One reliable chronic value was identified from open literature, and this for the mussel *M. edulis* (Morgan et al., 1986). From the raw data that were provided in the original publication a 48 h-EC_{10} of 4.4 mg Mo/L (endpoint: embryonal development) could be calculated. This species proved to be more sensitive toward molybdenum than any of the other tested organisms. The high sensitivity of *Mytilus* sp. to metals compared to other marine organisms was previously observed for copper and lead. The $HC_{5,50\%}$, taken from the species sensitivity distribution was 5.7 mg Mo/L. A comparison of the available chronic data and $HC_{5,50\%}$ for molybdenum that are available for the freshwater and marine environment is in line with the general assumption that the marine ecosystem, with its greater diversity of species and taxonomic groups, is more sensitive than the freshwater environment toward contaminants. However, when considering the toxicity of metals in the marine environment, molybdenum can be categorized as one of the least toxic metals with a $HC_{5,50\%}$ situated in the mg/L range, which is several orders of magnitude higher than the reasonable worst-case ambient Mo-concentration of 13.6 μg/L in marine waters as reported in the Chemical Safety Report for Molybdenum and molybdenum compounds (http://apps.echa.europa.eu/registered/registered-sub.aspx). The data that are presented in this paper are intended to form the basis for the derivation of a scientifically-sound PNEC or other water quality standards for the marine compartment.

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Appendix A. Overview of the chronic toxicity test conditions with sodium molybdate for nine different test species

	Acartia tonsa	Crassostrea gigas	Phaeodactylum tricornutum	Dunaliella tertiolecta	Ceramium tenuicorne	Americamysis bahia	Cyprinodon variegatus	Strongylocentrotus purpuratus	Dendraster excentricus
Testing facility	Grontmij/Aquasense	Grontmij/Aquasense	Grontmij/ Aquasense	Brixham Env. Lab.	Brixham Env. Lab.	ABC Laboratories	Parametrix Env.Research Lab.	Parametrix Env.Research Lab.	Parametrix Env. Research Lab.
Origin	Organisms obtained from Guernsey Sea Farms	Organisms obtained from Guernsey Sea Farms	In-house culture, algal strain from Microbiotests, Belgium	In-house culture CCAP 19/27, Culture Collection of Algae and Protozoans, Argyle, UK	In-house culture, originally obtained from ITM (Stockholm Univ.), kept under axenic conditions	Established in-house culture with a known history	Test organisms obtained from Aquatic Biosystems Inc (CO., USA)	Adults obtained from Marine Pollution Studies Lab (Carmel, Ca, USA)	Marinus Inc, Garden Grove, CA.
Culture medium	Natural filtered seawater	Natural filtered seawater	Artificial seawater with nutrients	MM/5 Medium, based on ISO 10253/2006	Natural seawater + added nutrients (ISO-guideline)	Laboratory saltwater (commercial sea salt mix (Christal Sea Marine mix) added to laboratory freshwater; salinity 20 ± 2 ppt)	Reconstituted water; MilliQ water + Red Sea Salt; salinity of 28‰	Yaquina Bay seawater, Oregon, adjusted to salinity of 30.0‰	Yaquina Bay seawater, Oregon, adjusted to salinity of 30.0‰
Test protocol	ISO 14669; OECD, 2005; Ward et al., 1979 (in ASTM STP 667)	ASTM E724-98 (2004)	ISO 10253 (Marine Algal Growth Inhibition Test with *P. tricornutum*)	International Standard: ISO 10253:2006	International Standard: ISO/DIS 10710 :2009	ASTM E1191-03a, 2008.	PERL protocol based on ASTM Method E1241 (2003)	ASTM E1563-98, 2004	US-EPA/600/ R-95/136, 1995 ASTM E1563-95, 2004
Test duration	30 days	48 hours	72 hours	72 hours	7 days	14 days	28 days	48 hours	48 hours
Test medium	Natural filtered seawater	Natural filtered seawater	Artificial seawater with nutrients	MM/5 Medium, based on ISO 10253/2006	Natural seawater + added nutrients (ISO-guideline)	Laboratory saltwater (commercial sea salt mix (Christal Sea Marine mix) added to laboratory freshwater; salinity 20 ± 2‰)	Reconstituted water; MilliQ water + Red Sea Salt; salinity of 28‰	Yaquina Bay seawater, Oregon, adjusted to salinity of 30.0‰	Yaquina Bay seawater, Oregon, adjusted to salinity of 30.0‰ with MilliQ
Endpoints	Reproduction (F0, F1 generation) and development (F1 generation)	Development of *in vitro* fertilized eggs which were obtained after temperature-induced spawning	Growth rate	Growth rate	Growth rate based on length	Survival, time to brood, length after 14/ 28d, # young/female	Survival and growth (dry wt)	Development	Development
Tested life stage	Eggs from age-synchronised adults and their first generation	Freshly fertilized egg	Algal cells originating from a 4d-old preculture	Algal cells originating from a 4d-old pre-culture in exponential growth phase	Cutted algal tips from 2d old cultures	<24 h-old larvae	freshly-fertilized eggs	Embryos (1 h post – fertilization)	Embryos (2 h post- fertilization), originating from 5 females and 2 males
Tested concentrations (nominal)	10 to 1000 mg Mo/L (6 concentrations)	32 to 3200 mg Mo/L (6 concentrations)	56 to 1000 mg Mo/L (6 concentrations)	320 to 3,200 mg/L ($Na_2MoO_4.2H_2O$) (5 concentrations)	320 to 3,200 mg/L ($Na_2MoO_4.2H_2O$) (5 concentrations)	3.8 to 120 mg Mo/L (6 concentrations)	62.5 to 1,000 mg Mo/L (5 concentrations)	31.5 to 2,000 mg/L (7 concentrations)	16 to 500 mg Mo/L (6 concentrations)
Renewal frequency	Static renewal (3 times/week)	No renewal	No renewal	No renewal	No renewal	Flow-through	Flow-through	No renewal	No renewal
# Replicates/ conc.	3	4	3 (control: 6)	3 (control: 6)	4	3 2 chambers/replicate	4	4	4
# Individuals/ replicate	F0: 50–80 eggs F1: 100	± 300	± 10,000 cells/mL	± 10,000 cells/mL	2 algal tips/replicate	30 (15/chamber)	20 freshly-fertilized eggs	26.1 embryos/mL	24.2 embryos/mL
Test conditions	Loading of <1 adult animal/mL test solution, 19.7-20.5 °C, pH 8.0-8.2, 16 L:8D photoperiod	20 mL per replicate, 19.7-20.9 °C, pH 7.85-8.06; 16 L:8D photoperiod	50 mL per replicate, 22.0-23.4 °C, pH 8.0-8.1, 24 L:0D photoperiod	100 mL per replicate, 20 ± 2 °C, pH 8.0-8.5, 24 L:0D, orbital shaking (100 rpm), 8,000-10,000 lux,	10 mL per replicate, 21.8-21.9 °C, pH: 8.1-8.3, 14 L:10D (70 ± 7 μmol/m^2/s)	± 32 L per replicate (+ quartz sand as substrate), 25 ± 2 °C, 14 L:10D photoperiod with 15/30 min transition periods)	200-300 mL per replicate, 25.1 ± 0.4 °C, pH 7.6-8.0, 16 L:8D	10 mL per replicate, 15.0 ± 1.0 °C, pH 8.0-8.1, 16 L:8D	10 mL per replicate, 15.9 ± 0.1 °C, pH 8.0 ± 0.1, 16 L:8D

(*continued on next page*)

Appendix A (*continued*)

	Acartia tonsa	Crassostrea gigas	Phaeodactylum tricornutum	Dunaliella tertiolecta	Ceramium tenuicorne	Americamysis bahia	Cyprinodon variegatus	Strongylocentrotus purpuratus	Dendraster excentricus
Feeding regime	Mixture of marine algae	None	None	"cool-white" illumination None	No feeding during the test period	Newly hatched brine shrimp, at least twice/day *at libitum*	Concentrated suspension of brine shrimp nauplii; added volume dependent on the amount of uneated food left over 1 h after feeding	Not relevant	Not relevant

	Mytilus edulis
Authors (ref.)	Morgan et al., 1986
Origin	collected from Woodlands, Indian Arm, British Columbia
Culture medium	Natural filtered seawater
Test protocol	Not specified; in line with ASTM Guideline E724
Test duration	48 h
Test medium	Natural filtered seawater
Endpoints	Development
Tested life stage	Freshly fertilized eggs from spawning-induced mussels (2 hours after fertilization)
Tested concentrations (nominal)	1 to 560 mg Mo/L (12 concentrations)
Renewal frequency	Static
# Replicates/conc.	3
# Individuals/replicate	F0: 50–80 eggs F1: 100
Test conditions	Loading of <1 adult animal/mL test solution, 19.7-20.5 °C, pH 8.0-8.2, 16 L:8D photoperiod
Feeding regime	Mixture of marine algae

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