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The toxicity of molybdate to freshwater and marine organisms. II. Effects assessment of molybdate in the aquatic environment under REACH

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ABSTRACT

The REACH Molybdenum Consortium initiated an extensive research program in order to generate robust PNECs, based on the SSD approach, for both the freshwater and marine environments. This activity was part of the REACH dossier preparation and to form the basis for scientific dialogues with other national and international regulatory authorities.

Chronic ecotoxicity data sets for the freshwater and marine environments served as starting point for the derivation of PNECs for both compartments, in accordance with the recommended derivation procedures established by the European Chemicals Agency (ECHA). The $HC_{5,50\%}$s that were derived from the generated Species Sensitivity Distributions were 38.2 mg Mo/L and 5.75 mg Mo/L for the freshwater and marine water compartment, respectively. Uncertainty analysis on both data sets and available data on bioaccumulation at high exposure levels justified an assessment factor of 3 on both $HC_{5,50\%}$ leading to a $PNEC_{freshwater}$ of 12.7 mg Mo/L and a $PNEC_{marine}$ of 1.92 mg Mo/L.

As there are currently insufficient ecotoxicological data available for the derivation of PNECs in the sediment compartment, the equilibrium partitioning method was applied; typical K_D-values for both the freshwater and marine compartments were identified and combined with the respective PNEC, leading to a $PNEC_{sediment}$ of 22,600 mg/kg dry weight and 1980 mg/kg dry weight for freshwater and marine sediments, respectively. The chronic data sets were also used for the derivation of final chronic values using the procedures that are outlined by the US Environmental Protection Agency for deriving such water benchmarks. Comparing PNECs with FCVs showed that both methodologies result in comparable protective concentration levels for molybdenum in the environment.

1. Introduction

According to EU Regulation No 1907/2006 concerning the Registration, Evaluation, Authorization and Restriction of Chemical substances (REACH), the registration dossiers for high-volume compounds have to comply with the data requirements that are outlined in Annexes VII–X of the REACH legislation. In order to meet the acceptance criteria for such dossiers, the predicted no-effect concentrations (PNECs) for different environmental compartments need to be addressed, either by proposing a PNEC or by providing a rationale for not proposing such a value. Should only a limited ecotoxicological data set be available, the assessment factor method is followed for PNEC derivation. However, when sufficient data are available, European Chemicals Agency (ECHA) guidance allows the derivation of a PNEC by means of the scientifically more robust statistical extrapolation method (ECHA, 2008). This method is based on a species sensitivity distribution (SSD) that can be developed when at least 10 chronic data points representing at least 8 different taxonomic groups are available.

In an effort to generate robust PNECs for both the freshwater and marine environments, i.e., based on the SSD approach, an extensive research program was initiated by the IMOA REACH Molybdenum Consortium as part of the REACH dossier preparation for molybdenum and molybdenum compounds. As a first step, the International Molybdenum Association (IMOA) commissioned a thorough evaluation of all existing chronic toxicity data for molybdate [MoO_4^{2-} is the biological and environmental relevant form of molybdenum, De Schamphelaere et al., 2010] in the aquatic environment. Based on the outcome of this review a testing program was conducted that aimed to generate the data necessary to:

- develop SSDs for molybdate, for both the freshwater and marine compartment,
- derive $HC_{5,50\%}$s (Hazardous Concentration that affects 5% of the population) which serve as a reference value for setting water quality benchmarks in the aquatic environment.

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This publication describes the effects assessment that was conducted with the aquatic data generated in this research program and published by De Schamphelaere et al. (2010; freshwater compartment) and Heijerick et al. (accepted for publication), and which formed part of the REACH Chemical Safety Reports (CSRs) for molybdenum and molybdenum compounds. This effects assessment covers both PNECs for the water column, as well as the PNECs for the sediment compartment.

In addition, the same data set was also used for the derivation of Final Chronic Values, following the methodology as outlined by the United States Environmental Protection Agency (US EPA) (Stephan et al., 1985), and which were compared to the $HC_{5,50\%}$s derived in this study.

2. Effects assessment for the freshwater and marine aquatic compartment

Table 1 presents a concise overview of the available ecotoxicological data that were categorized as 'reliable' (Klimisch 1) or 'reliable with restrictions' (Klimisch 2) based on the quality criteria for evaluating data as outlined by Klimisch et al. (1997). These data were previously described and discussed in detail by De Schamphelaere et al. (2010) and Heijerick et al. (accepted for publication). Chronic no-effect levels – expressed as EC_{10}s – ranged from 43.2 mg Mo/L (rainbow trout Oncorhynchus *mykiss*) to 241.5 mg Mo/L (duckweed *Lemna minor*) for the freshwater compartment, but covered a wider span of concentration levels in the marine compartment with values situated between 4.4 mg Mo/L (mussel *Mytilus edulis*) and 1174 mg Mo/L (American oyster *Crassostrea gigas*). Most data were generated during the aquatic research program commissioned by IMOA. The only reliable study that was taken from the open literature was the 48 h-development study with the mussel *M. edulis* (Morgan et al., 1986); the reported EC_{10} of 4.4 mg Mo/L was not provided by these authors, but was recalculated by Heijerick et al. (accepted for publication) based on available raw data. Most values also represent the outcome of a single study; only for *Daphnia magna*, *Ceriodaphnia dubia*, *Pseudokirchneriella subcapitata* and *Pimephales promelas* the value represents a geometric mean of more than one reliable value (De Schamphelaere et al., 2010).

These data were used for the construction of a freshwater and marine species sensitivity distribution (SSD) from which two different parameters have been derived:

- the 5th percentile (HC_5, Hazardous Concentration affecting 5% of the ecosystem);
- the median 5th percentile, i.e. the $HC_{5,50\%}$ 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 (Aldenberg and Jaworska, 2000).

2.1. Derivation of a freshwater $HC_{5,50\%}$

The freshwater EC_{10}s (Table 1) were log-transformed and different types of distributions were fitted though the dataset using the software package BestFit. Statistical significant distributions included the Normal, Logistic, Gamma, PearsonV and the Inverse Gaussian Distribution on Log-transformed data. Taking into account factors such as statistical goodness-of-fit, ecological relevance and degree of conservatism (i.e., which distributions represent the lowest HC_5s), the Normal Distribution on Log-transformed data was defined as the optimal distribution (Log-Normal Distribution, Fig. 1). The HC_5 and $HC_{5,50\%}$ (±95% CL) associated with this distribution were 39.5 mg Mo/L and 38.2 mg Mo/L (95% CL: 18.7–57.3 mg Mo/L), respectively.

Application of an assessment factor on this $HC_{5,50\%}$ results in the final PNEC for molybdenum in the aquatic environment. The value of this assessment factor depends on the uncertainty analysis that is conducted on the ecotoxicological freshwater dataset and on the outcome of the statistical analysis (ECHA, 2008).

2.2. Uncertainty analysis and $PNEC_{freshwater}$ derivation

An expert discussion on how statistical techniques can be used in the risk assessment process for the environment was held during the London Workshop (2001). The primary objective of this meeting was

Table 1
Overview of reliable chronic EC_{10} values for molybdenum (as molybdate) in the freshwater and marine environment.

Freshwater species[a]	EC10 (mg Mo/L)	Marine species[b]	EC_{10} (mg Mo/L)
Oncorhynchus mykiss (De Schamphelaere et al, 2010)	43.2	*Mytilus edulis* (Morgan et al, 1986)	4.4[c]
Pimephales promelas (De Schamphelaere et al, 2010; GEI, 2009)	60.2	*Acartia tonsa* (Kools and Vanagt, 2009)	7.96
Ceriodaphnia dubia (De Schamphelaere et al, 2010; GEI, 2009)	63.0	*Cyprinodon variegatus* (Parametrix, 2009)	84.1
Pseudokirchneriella subcapitata (De Schamphelaere et al, 2010)	74.3	*Americamysis bahia* (Lehman, 2010)	116[c]
Daphnia magna (De Schamphelaere et al, 2010; GEI, 2009)	89.5	*Phaeodactylum tricornutum* (Kools and Vanagt, 2009)	170
Xenopus laevis (De Schamphelaere et al, 2010)	115.9	*Dendraster excentricus* (Parametrix, 2008)	233.6
Chironomus riparius (De Schamphelaere et al, 2010)	121.4	*Strongylocentrotus purpuratus* (Parametrix, 2010)	325.8
Brachionus calyciflorus (De Schamphelaere et al, 2010)	193.6	*Ceramium tenuicorne* (Le Page et al, 2010)	641[c]
Lymnaea stagnalis (De Schamphelaere et al, 2010)	221.3	*Dunaliella tertiolecta* (Le Page and Hayfield, 2010)	881
Lemna minor (De Schamphelaere et al, 2010)	241.5	*Crassostrea gigas* (Kools and Vanagt, 2009)	1174

[a] Data reported in De Schamphelaere et al (2010).
[b] Data reported in Heijerick et al (accepted for publication).
[c] NOEC-value.

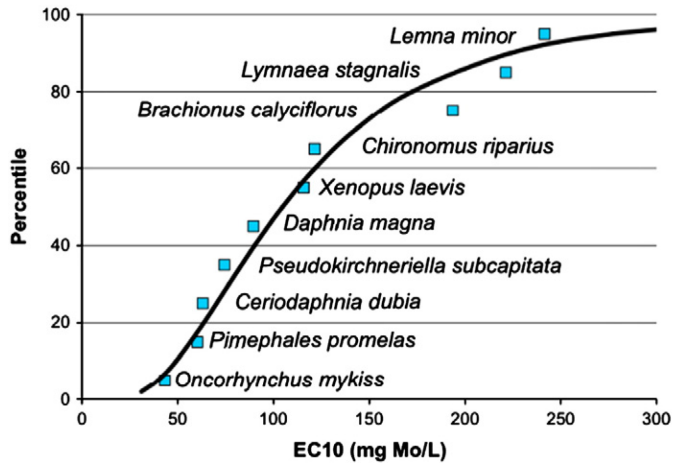

Fig. 1. Species sensitivity distribution of molybdenum (as molybdate) in the freshwater environment.

to evaluate how statistics could be used for the derivation of aquatic PNECs in environmental risk assessments of metals and their compounds. The main conclusions of this workshop were adopted by ECHA (2008), and include their recommendation for the application of an additional assessment factor on the $HC_{5,50\%}$, with an AF between 1 and 5, to be judged on a case-by-case basis. Based on the available chronic EC_{10}/NOEC data, five quality criteria are evaluated when determining the size of the assessment factor. The outcome of this quality analysis is presented in the following sections.

2.2.1. Criterion 1: the overall quality of the database and the end-points covered

The evaluation of the quality of the database is based on the overview of the test endpoints and test medium characteristics that were reported by De Schamphelaere et al. (2010). Only those tests used for the derivation of an $HC_{5,50\%}$ were taken into consideration. This evaluation led to the following conclusions:

- The freshwater Mo-database only covers ecologically relevant endpoints. The selected endpoints are all relevant for potential effects at the population level: growth (biomass, length), population growth rate, reproduction, abnormalities/deformations.
- The molybdenum ecotoxicity database contains chronic exposure data for each trophic level that needs to be included in the SSD (ECHA, 2008). The conclusion whether a specific value represents an acute or chronic effect not only depends on recommended exposure durations from standard ecotoxicity protocols, but should also take into account the life cycle of the test organisms. The chronic exposure periods for standard OECD test species that were exposed to molybdenum, were in line with the durations that are recommended in their respective guidelines: 21 d test with *D. magna* (OECD, 1998), >28 days for early life stage tests with fish (OECD, 1992), 3 d test with *P. subcapitata* (OECD, 2006a) and the 7 d growth test with *L. minor* (OECD, 2006b). The duration and type of tests with fish (*P. promelas*, *O. mykiss*) met the chronic testing criteria as described in ASTM (2001).

Tests with insects can have a duration of up to 240 days (OECD, 2004). The early life stage test with frogs has a duration of 4 days which is in line with the ASTM guideline (ASTM, 1998). The early life stage tests with fish have an exposure duration varying between 30 and 40 days. According to OECD guidelines, early life stage tests with fish (e.g. with *O. mykiss*, *P. promelas*) should have a test duration of at least 30 days which is in accordance with the selected toxicity data for fish in the database. The chronic tests with juveniles/fry have exposure durations between 17 and 330 days. The length is very much dependent of the species of fish and the type of chronic test: sac fry test, growth inhibition test on juveniles or the Fish Early Life Stage test (FELS).

- Sensitive life stages were covered in the database. Almost all chronic toxicity tests were performed using sensitive life stages (e.g. all fish/frog tests used early life stage, the tests with crustaceans were started with newly born organisms, tests with insects were initiated with larvae).
- The EC_{10}/NOEC data were extracted from tests performed in a variety of natural/artificial freshwaters, covering a considerable part of the wide range of the freshwater characteristics (e.g., pH and hardness) that are normally found in European freshwaters. The pH and hardness of the test media that were used, ranged between 6.5–8.2 and 24–250 mg/L as $CaCO_3$, respectively. In Table 2 these ranges are compared to ranges of pH and hardness observed in the EU surface waters. Values were derived from monitoring datasets from various national environmental organizations and institutes. Monitoring data for Belgium were obtained from the Flemish Environment Agency and from the Walloon Scientific Institute for Public Services. German monitoring data included in Table 2 originate from the Wassergütestelle Elbe (Hamburg) and are only representative for the river Elbe (1996, 2000). The monitoring data for the Netherlands were gathered between 1990 and 2000 by the Rijkswaterstaat, the executive organization of the Dutch Ministry of Transport, Public Works and Water Management. The Swedish monitoring data were obtained from the Swedish University of Agricultural Science (1995–2001). The COMMPS (Combined Monitoring-Based and Modeling-Based Priority Setting) database reports data on the physico-chemical characteristics of Spanish freshwaters in 1997. Finally, data for the United Kingdom were generated in the framework of "The Harmonised Monitoring Scheme" a monitoring campaign that has been conducted since 1974 covering England and Wales.

The hardness content in EU freshwaters ranges between 37.4 and 323.3 mg $CaCO_3$/L (range as 10th and 90th percentiles). A typical hardness (50th percentile) of 99.4 mg $CaCO_3$/L was estimated in EU freshwaters. Both range and median value are similar to the values that were observed in the test media (i.e., a median of 98.7 mg $CaCO_3$/L as in the test media). The pH in EU freshwaters ranges between 6.6 and 8.1 (range as 10th and 90th percentiles). A typical pH (50th percentile) of 7.5 was estimated in EU freshwaters. For this parameter a similar range and median were also found in the test media (i.e., a median pH of 7.58 in the test media).

These data confirm that the media used for generating ecotoxicological data for molybdenum are representative of the variability in physicochemical conditions encountered in European surface waters. This is of importance as toxicity of metals in general can be affected by these water properties. In the absence of bioavailability models that can normalize effects data to specific environmental conditions, a variation in test media that reflects the natural environmental variation is the optimal approach to take into account the potential impact of these parameters on the observed toxicity.

Table 2
Comparison of range and median values for hardness and pH in EU-freshwaters and in test media used for the development of the Mo-effects database.

	Test media	EU-surface water
pH	Range: 6.5–8.2 Median: 7.58	10th/90th P: 6.6–8.1 Median: 7.5
Hardness (mg/L as $CaCO_3$)	Range: 24–250 Median: 98.7	10th/90th P: 37.4–323.3 Median: 99.4

2.2.2. Criterion 2: the diversity and representativeness of the taxonomic groups covered by the database

The Mo-database fulfills the recommendations that at least 10 to 15 different NOECs should be available for the derivation of a PNEC based on the statistical extrapolation method. This recommendation is associated with the determination of the assessment factor that leads to the final PNEC, as outlined in the former EU Technical Guidance Document (EC, 2003), as well as stipulated by ECHA (2008). Chronic (no-)effect levels (EC_{10}, NOEC) were identified for ten different species. Chronic endpoints were compiled, and all data are categorized as Klimisch 1 (reliable without restriction). The London Workshop (2001) also defined 8 different taxonomic groups that should be included in the effects database (Table 3), and this guidance was adopted in Chapter R10 of the ECHA Guidance Document (ECHA, 2008). The freshwater chronic effects database for molybdenum includes organisms for each of these categories.

2.2.3. Criterion 3: statistical uncertainties around the 5th percentile estimate, e.g., reflected in the goodness-of-fit or the size of confidence interval around the 5th percentile

Based on the distribution that is fitted through the K1-data set, the HC_5 is derived as the 5th percentile of the SSD. Different types of SSD curve fitting functions and goodness of fit approaches were investigated when estimating the HC_5. The choice of SSD curve fitting and goodness of fit approach impacts the derivation of the HC_5 to some degree, but the impact was no greater than a factor of 1.15 and thus not of large significance. The Log-Normal distribution turned out to be the best fitting curve, and resulted in the most conservative HC_5 compared to other distributions that resulted in a significant fit.

According to the guidelines presented in the TGD (EC, 2003) and ECHA Guidance Chapter 10 (ECHA, 2008) the 50% confidence interval (or median confidence interval) is considered as a reference value for PNEC derivation. This percentile was calculated by conducting an analysis according to the methodology presented by Aldenberg and Jaworska (2000). The resulting $HC_{5,50\%}$ with 95% CL was 38.2 mg Mo/L (95% CL: 18.7–57.3 mg Mo/L).

2.2.4. Criterion 4: comparisons between field and mesocosm studies and the 5th percentile of mesocosm/field studies to evaluate the laboratory to field extrapolation

No field data were available that allow the derivation of threshold concentrations of molybdenum in freshwaters at the field scale.

However, the lack of such data does not imply that field testing would generate lower NOECs. In an effort to generate data that support the no-effect hypothesis at PNEC-levels under field conditions, a long term (120 d) bioaccumulation experiment was conducted with the most sensitive species of the SSD using the final PNEC as the exposure concentration (Regoli et al., 2012). The results of this experiment in relation to the proposed PNEC are discussed further.

Table 3
Minimum taxonomic group requirements for the extrapolation method (London workshop, 2001; ECHA, 2008).

Taxonomic groups	Mo-database
1. Fish (usually tested species like salmons, bluegill, channel catfish, etc.)	*Oncorhynchus mykiss*, *Pimephales promelas*
2. A second family in the phylum Chordata (fish, amphibian, etc.)	*Xenopus laevis*
3. A crustacean (e.g. cladoceran, copepod, ostracod, isopod, amphipod, crayfish etc.)	*Daphnia magna*, *Ceriodaphnia dubia*
4. An insect (e.g. mayfly, dragonfly, damselfly, stonefly, caddis fly, mosquito, midge)	*Chironomus riparius*
5. A family in a phylum other than Arthropoda or Chordata (e.g. Rotifera, Annelida, Mollusca, etc.)	*Brachionus calyciflorus*
6. A family in any order of insect or any phylum not already represented	*Lymnaea stagnalis*
7. Algae	*Pseudokirchneriella subcapitata*
8. Higher plants	*Lemna minor*

2.2.5. Criterion 5: comparison of the $HC_{5,50\%}$ with unbounded NOECs

The evaluation of literature data revealed some studies that resulted in reliable, unbounded NOECs (i.e., highest test concentration did not reveal any significant effect), and therefore these studies were not suited for the derivation of a HC_5/PNEC (De Schamphelaere et al., 2010). Nevertheless these studies have their scientific merit, and the reported findings (i.e., absence of adverse effects) are considered reliable; therefore the reported unbounded NOECs are compared with the $HC_{5,50\%}$ of 38.2 mg Mo/L:

- McConnell (1977) reported an unbounded NOEC of >17 mg/L (nominal value) for the rainbow trout *O. mykiss*. This value is a factor of 2.25 below the $HC_{5,50\%}$ of 38.2 mg Mo/L;
- Ennevor (1993), on the other hand, observed no significant adverse effects on the trout *Oncorhynchus kisutch* at the highest test concentration of 19.5 mg Mo/L, i.e., a factor of 1.96 below the $HC_{5,50\%}$.

Based on these findings can be concluded that an AF>2.25 would result in a PNEC that is also sufficiently protective for the test species that were evaluated in these studies.

2.3. $PNEC_{freshwater}$ derivation

The uncertainty analysis revealed that the effects database for molybdenum fulfills all requirements that were stipulated at the London workshop (2001) for the application of the statistical extrapolation method (SSD-method) when deriving a $PNEC_{freshwater}$:

- High quality (Klimisch 1) no-effects data are available for 10 different species;
- From an ecological point of view all species and evaluated endpoints (growth, reproduction, developmental malformations) are relevant for the aquatic environment;
- The eight different trophic levels that were defined in the London Workshop (2001) are represented in the database;
- Test media are relevant for EU surface water conditions, and the variation in physicochemical parameters (e.g., pH, hardness) is representative for EU freshwaters;
- All tests were performed in artificial test media that contain no (or very few) natural compounds in the water that can form metal complexes. Assuming that the concepts of the FIAM model (Campbell, 1996) are also applicable for molybdate, i.e., that the free ion is the most bioavailable speciation form of a metal, the test media should represent a worst case scenario.
- The HC_5 and $HC_{5,50\%}$ are based on the most conservative (Log-Normal) fitted distribution.

Each of these findings supports the conclusion that the $HC_{5,50\%}$ calculated with this dataset should be sufficiently protective for the aquatic environment. Comparing the $HC_{5,50\%}$ with the lowest EC_{10} of the molybdenum SSD ($HC_{5,50\%}$ of 38.2 mg Mo/L<EC_{10} of 43.2 mg Mo/L; *O. mykiss*) demonstrated that the $HC_{5,50\%}$ does not exceed any of the species-mean EC_{10}s or NOECs that are available for molybdenum.

There are, however, arguments that may promote the application of an additional assessment factor in the $HC_{5,50\%}$ for the determination of the $PNEC_{aquatic}$.

No mesocosm studies with molybdenum are available, therefore creating uncertainty with regard to the lab-to-field translation of Mo related effects. In the EU RAR for nickel, the lack of mesocosm data was one of the arguments to apply a safety factor of 3 on the $HC_{5,50\%}$.

There are two unbounded NOECs taken from reliable experiments that are situated below the $HC_{5,50\%}$ — one of them for a species that is not included in the SSD distribution (*O. kisutch* (Ennevor, 1993)). The difference between the $HC_{5,50\%}$ and these unbounded NOECs is a factor of 2.25 or less.

The 95% confidence limit that is associated with the $HC_{5,50\%}$ is 18.7–57.3 mg Mo/L; the lower 95% CL is a factor of 2.05 below the $HC_{5,50\%}$.

Based on the above observations – and taking sufficient conservatism into account–an Assessment Factor of 3 on the $HC_{5,50\%}$ was put forward for the derivation of an aquatic PNEC, resulting in a $PNEC_{aquatic}$ of 12.7 mg Mo/L; the use of an AF of 3 results in a $PNEC_{aquatic}$ that is below the reported unbounded NOECs.

This PNEC as used as reference value for selecting a range of exposure concentrations that were used in a 120 d bioaccumulation (60 d) and depuration (60 d) experiment using the most sensitive species of the SSD as the test organism (the rainbow trout *Oncorhynchus mykiss*) (Regoli et al., 2012). At the end of the 60 d accumulation period it was found that the internal Mo-concentrations in fish exposed to the highest concentration level of 11.1 mg Mo/L (measured value close to the PNEC) were not statistically different from the control. As such, the outcome of this study provides evidence that internal molybdenum levels are regulated in fish exposed to the $PNEC_{freshwater}$. As long as the internal concentration of molybdenum is regulated within an organism, it is unlikely that significant adverse effects will arise from this element. Taking into account that these findings were obtained by using the most sensitive organism of the SSD, it can be concluded that there is no need to increase the AF of 3 that has been applied on the $HC_{5,50\%}$ for deriving the $PNEC_{freshwater}$.

2.4. Derivation of a marine $HC_{5,50\%}$

The SSD that has been developed for the assessment of molybdenum in the marine compartment is presented in Fig. 2, and was generated with the reliable chronic toxicity effect levels that are reported in Table 1. The marine EC_{10}s were Log-transformed and an initial analysis of the data set with BestFit showed that the Normal Distribution on the Log-transformed data was significant, and resulted in a conservative estimation of the HC_5. Using the RIVM software package ETX, a Log-Normal Distribution was plotted through this data set. The $HC_{5,50\%}$ (±95% CL) associated with this distribution was 5.7 mg Mo/L (95% CL: 0.58–21 mg Mo/L). This value served as reference value for the derivation of the aquatic $PNEC_{marine}$.

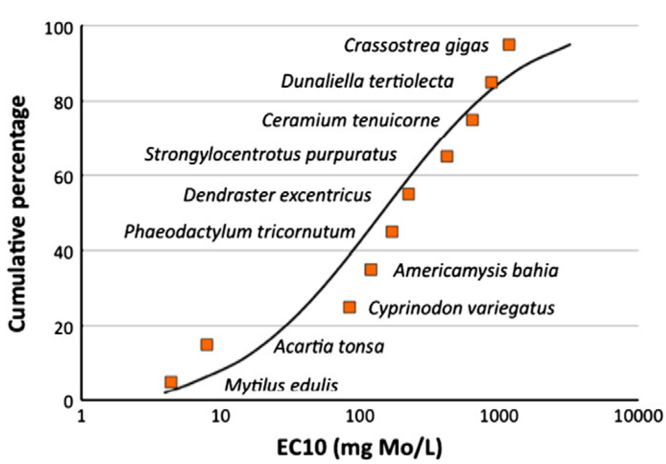

Fig. 2. Species sensitivity distribution of molybdenum (as molybdate) in the marine environment.

2.5. Uncertainty analysis and $PNEC_{marine}$ derivation

The assessment factor that was applied on the $HC_{5,50}$ of 5.7 mg Mo/L was based on the evaluation of the quality and reliability of the test endpoints and test medium characteristics that were reported by Heijerick et al. (accepted for publication). Only those tests that were used for the derivation of a $HC_{5,50\%}$ were taken into consideration. The quality criteria that were used for this assessment were similar to those that were evaluated for the freshwater dataset; the London Workshop 2001 did not specifically address the marine compartment, but the same principles as those that were outlined for the freshwater compartment were applied to the quality assessment of the marine chronic dataset.

2.5.1. Criterion 1: the overall quality of the database and the end-points covered

- The marine Mo-database only covers ecologically relevant endpoints. All selected endpoints are considered relevant for potential effects at the population level: growth (biomass, length), population growth rate, reproduction, abnormalities/deformations.
- Covering 'chronic' exposure times is also achieved for all trophic levels in the marine chronic dataset for molybdenum. As mentioned in Section 2.2, a relevant exposure period is related to the typical life cycle and to the recommended exposure duration from standard ecotoxicity protocols. The exposure periods that were evaluated in the chronic marine tests with molybdenum did meet these criteria. The reported exposure times for algae were 3 days, and 7 days for higher plants, which is in accordance with international guidelines for these species (OECD, 2006a; ISO, 2006, 2008). Larval developments of echinoderms and molluscs were evaluated after a 48 h exposure period. The available guidelines for this test recommend a test duration of 48–96 h, i.e., until the typical D-shaped larval stage (molluscs) or the pluteus larval stage (echinoderm) has been reached in the control (ASTM 2004a,b; USEPA, 1995). The early life stage test with fish had an exposure duration of 28 days which is also in line with ASTM and OECD standard guidelines (ASTM, 2003; OECD, 1992). The exposure duration of tests with the marine invertebrates (*Americamyis bahia*, *Acartia tonsa*) was also in line with the recommendations given in international guidelines for these organisms (ASTM, 2008; ISO, 1999; OECD, 2005; Ward et al, 1979, in: ASTM STP 667).
- All tests were conducted according to standard guidelines that have been published for these organisms, or that are acceptable for regulatory purposes.
- Sensitive life stages were covered in the database. All chronic toxicity tests are performed using sensitive life stages (e.g. fish test used an early life stage, the tests with crustaceans were started with newly hatched organisms, tests with echinoderms and molluscs were conducted with freshly fertilized embryos).
- The EC_{10}/NOEC data were extracted from tests performed in a variety of natural or reconstituted marine waters, covering a considerable part of the salinity that can be encountered in the marine environment (salinity range: 20‰–35‰ (natural filtered seawater)). Unlike for the freshwater compartment, an in-depth analysis of typical marine physicochemical properties was not conducted. On average the pH of the marine environment is situated around 8.0–8.2 (Jacobson, 2005) and shows little variation when compared to the freshwater environment. Differences in pH among different test media are therefore limited, and are not expected to have any major impact on observed chronic toxicity levels. Secondly, the concentration levels of the ions that may affect metal bioavailability (and toxicity) are several orders of magnitude higher than the levels that are observed in the freshwater environment. Relative variation of these ions in the marine environment will therefore be small and not expected to alter or affect the degree of toxicity that is observed.

2.5.2. The diversity and representativeness of the taxonomic groups that are covered by the marine dataset

From the data in Table 1 it can be concluded that the molybdenum database fulfills the recommendation of 10 to 15 different EC_{10}/NOECs. This recommendation is associated with the determination of the assessment factor that leads to the final PNEC. The EC_{10}s/NOECs for 10 different species were identified, and all data are categorized as Klimisch 1 (reliable without restriction) (Klimisch et al., 1997).

The London Workshop (2001) defined 8 different taxonomic groups for the freshwater environment that should be included in the effects database, and this guidance was adopted in ECHA Guidance Document, Chapter R10 (ECHA, 2008). It was ensured that the marine data set also covered 8 different trophic levels that were relevant for the marine environment, and that had additional ecological and/or economic value: fish, copepod, mysid, echinoderm (2), mollusc (2), diatom, microalga, and macroalga.

The EU TGD (2003) and ECHA (2008) both stated that the greater species diversity in the marine environment (including some taxa that only occur in the marine environment) implies a broader distribution of sensitivities of species and a higher uncertainty in extrapolation. In an effort to capture this broader range of sensitivity in the SSD, it is essential that taxonomic groups that only occur in the marine environment are included. The current marine chronic dataset includes a number of species that only occur in 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).

2.5.3. Statistical uncertainties around the 5th percentile estimate, as reflected in the goodness-of-fit or the size of confidence interval around the 5th percentile

According to the guidelines presented in the TGD (EC, 2003) and ECHA Guidance Document, Chapter 10 (ECHA, 2008) the 50% confidence interval (or median confidence interval) is considered in deriving the PNEC. This percentile was calculated by conducting an analysis according to the methodology presented by Aldenberg and Jaworska (2000).

2.5.4. Comparisons between field and mesocosm studies and the 5th percentile to evaluate the laboratory to field extrapolation

To our knowledge, no field studies that investigate the long-term impact of elevated molybdenum levels in the marine environment have been reported. The availability of field-generated no-effect levels for organisms (or the ecosystem in general) allows to compare $HC_{5,50\%}$ generated under laboratory conditions with the actual effects in the environment. As such, the results of field studies represent relevant information that has a direct impact on the selection of an appropriate AF on the $HC_{5,50\%}$ for PNEC setting.

2.6. $PNEC_{marine}$ derivation

The uncertainty analysis revealed that the effects database for molybdenum fulfills all requirements that were stipulated at the London Workshop (2001) for the application of the statistical extrapolation method:

- High quality (Klimisch 1) no-effects data are available for 10 different species;
- From an ecological point of view all species and evaluated endpoints (growth, reproduction, developmental malformations) are relevant for the marine environment;
- Eight different trophic levels are included in the data set, each representing a trophic level that is typical for the marine environment; moreover, several species belong to taxonomic groups that do not occur in the freshwater environment.
- Each of the used test media is relevant for the marine environment
- All tests were performed under laboratory conditions in media which are considered to promote metal bioavailability (i.e., worst case conditions)
- All reported effects data (EC_{10}, NOEC) are based on measured molybdenum values.
- The $HC_{5,50\%}$ is based on the (Log-normal) distribution.

As for the freshwater environment, no mesocosm studies with molybdenum have been available for the marine environment, therefore creating uncertainty with regard to the lab-to-field translation of molybdenum related effects.

Overall, there is a large similarity between the quality of the freshwater and marine dataset: Both datasets were in line with the criteria of the London Workshop (2001), relevant endpoints and test species were used, measured effects levels were the result of tests that were performed according to international guidelines, and in both cases the $HC_{5,50\%}$ was derived from a Normal distribution that was fitted through the Log-transformed data set. Finally, no mesocosm data were available for both environmental compartments. Consequently, the same Assessment Factor of 3 that was used for deriving the $PNEC_{freshwater}$ was used for the calculation of the $PNEC_{marine}$, resulting in a value of 1.9 mg Mo/L.

This value is about one order of magnitude below the $PNEC_{freshwater}$ of 12.7 mg Mo/L. It is noteworthy that this difference of a factor of 10 is in line with the additional safety factor of 10 that the ECHA guidance (2008) imposes for deriving a $PNEC_{marine}$ using the $PNEC_{freshwater}$ as the starting point (i.e., in the absence of chronic marine data: $PNEC_{marine} = PNEC_{freshwater} / 10$). As mentioned before the rationale behind this additional assessment factor is the hypothesis that the larger variation in marine taxonomic groups results in an overall expansion of the sensitivity spectrum. Our findings indicate that, at least for molybdenum, there is such a difference in sensitivity between the freshwater and marine environment.

3. Setting water quality criteria according to the US EPA methodology

The US EPA takes a different approach for the derivation of so-called Final Acute and Chronic reference Values (FAV, FCV). In short, the US EPA calculation method (Stephan et al., 1985) only takes into account the four lowest no-effect values and the number of available data points in the acute or chronic ecotoxicity data set. The FCV can also be derived by dividing the FAV by a Final Acute-to-Chronic Ratio (FACR). The FCV is then used to determine the Criterion Continuous Concentration (CCC) which is derived by dividing the FCV by 2. Similar to the requirements set by ECHA (2008), the data set should represent at least eight families, including two fish families, amphipod/crayfish and molluscs. However, algal species and higher plants (e.g., green alga, duckweed) are not included, and available no-effect levels for these taxonomic groups are discarded when deriving the FCV. As both the freshwater and marine datasets for molybdenum do not comply with the 8-family criterion when micro-alga, a macro-alga or a higher plant are not taken into account, it is currently not possible to derive a freshwater or marine CRV for molybdenum that would be acceptable for regulatory purposes. However, for the sole purpose of comparing the outcome of the US EPA method with the European approach for setting water quality criteria, the US EPA calculation method was applied on the complete chronic freshwater and marine datasets.

The freshwater FCV — taking all chronic freshwater data into account ($n = 10$), was 38.8 mg/L, and is based on the EC_{10}s for the fish *O. mykiss* and *P. promelas*, the invertebrate *C. dubia* and the green alga *P. subcapitata*. This value is almost identical to the $HC_{5,50\%}$ of 38.2 mg/L that was generated with the same data set. When no algal and higher plant data are used ($n = 8$) the $FCV_{freshwater}$ becomes

34.8 mg Mo/L. For this calculation the *P. subcapitata* datapoint was replaced by the EC_{10} determined for the cladoceran *D. magna*. The observed decrease of the FCV from 38.8 to 34.8 mg Mo/L can be attributed to the increased uncertainty that has been introduced by reducing "n" from 10 to 8, and by the larger difference between the lowest and highest EC_{10} that are used for the calculation of the FCV.

For the marine environment the preliminary FCV_{marine} is 1.14 mg Mo/L when considering the complete data set (n = 10). This value is derived with the no-effect levels that were determined for the mussel *M. edulis*, the copepod *A. tonsa*, the fish *C. variegatus* and the mysid *A. bahia*. Removing micro- and macro-algal data from the dataset had no impact on the critical values, but reduced the size of the data set. This reduction caused the FCV_{marine} to drop to 0.75 mg Mo/L. The former value is about a factor of 5 below the marine $HC_{5,50\%}$. This difference increases when algal data are not considered (factor of 7.6).

A larger variation among the four values that are used for the derivation and a decrease of the size of the data are two significant parameters that significantly lower the FCV. For the freshwater data set the values used for FCV-derivation ranged between 43.3 mg Mo/L and 74.3 mg Mo/L (factor of 1.7 difference), whereas a much wider range was noted for the marine data, i.e., between 4.4 mg Mo/L and 120 mg Mo/L (difference of a factor of 27.3). With the USA-methodology, an increased variation results in a FCV that is situated well below the lowest EC_{10}. When applying the SSD-methodology, the larger variation is not translated to a lower $HC_{5,50\%}$, but results in a wider 95%-Confidence Interval (C.I.) for this parameter (0.58–21.0 mg Mo/L). Note that this confidence interval does cover the FCV that was calculated for the marine environment. The 95% C.I. for the $PNEC_{freshwater}$ — based on a data set with markedly less variation among the different EC_{10}s, was 18.7–57.3 mg Mo/L.

The data that are currently available seem to indicate that the US EPA method would most likely result in water quality benchmarks that comparable to the PNECs that were calculated with the statistical extrapolation method (SSD-method). In order to meet the minimal data requirements for deriving a FCV based on chronic data, additional testing is required for both aquatic compartments. It remains, however, debatable whether there is an ecological justification for discarding algal data from the data set when it comes to setting water quality criteria. Lewis (1995) stated that the environmental relevance of results from standard laboratory algal toxicity tests is unclear, and OECD (1984) and ISO (1987) mentioned that the effects data from algal tests should not be used to predict environmental impact but only to provide the likelihood of effect or no effect. US EPA guidelines for effluent toxicity tests, on the other hand, suggested that "safe levels" can be established with algal toxicity data (Weber et al, 1988, 1989). In addition, Blanck et al. (1984) and Wangberg and Blanck (1988) reported that the ecological realism in the assessment of substances would increase by using a battery of algal species from a broad range of taxonomic representation groups.

4. PNEC derivation for the sediment compartment

According to ECHA REACH Guidance Chapter 10 (ECHA, 2008) the $PNEC_{sediment}$ can be derived in two ways:

- Results of tests with sediment living organisms.
- Using the equilibrium partitioning method (EPM) when only toxicity data (results of tests or non-test methods) for aquatic (pelagic) organisms are available.

The estimation of the $PNEC_{sediment}$ with equilibrium partitioning method is based on the assumption that the sensitivity of pelagic and sediment living organisms is comparable, but that in sediment the availability of the substance is reduced due to sorption to the sediment. This implies the use of partitioning calculations, assuming that equilibrium is obtained. It should be noted that EPM only considers uptake via the water phase. For highly adsorbing chemicals, however, the uptake via other exposure pathways like ingestion or direct contact with sediment becomes more important, depending on the organism used for testing. Uptake via the gut is likely to play an increasingly important role for compounds with a log Kow greater than 5 or with a correspondingly high adsorption or binding behavior. In such cases, the equilibrium partitioning method can only be used in a modified way. In order to allow for uptake of substances via ingestion of sediment, an additional factor of 10 is applied to the PEC/PNEC ratio (PEC: Predicted Environmental Concentration) for such substances. While log Kow is a good predictor for the bioaccumulation for certain types of organic compounds (e.g., non-polar organic substances), it is irrelevant for inorganic substances such as inorganic metal compounds (UNECE, 2005). For metals, the partition coefficient (log K_D) between the water and sediment phases may give a better indication on the potential impact of ingestion on the outcome of the EPM. In the case of molybdenum the log K_D is relatively low (typical value of 3.25, see further) and hence there is no need for the application of an additional safety factor on the $PNEC_{sediment}$ for molybdenum that was derived with this method.

For molybdenum, no reliable acute or chronic toxicity data for the sediment compartment were identified in the open literature or in the gray literature. Liber et al. (2011) exposed amphipods (*Hyalella azteca*) and midge larvae (*Chironomus dilutus*) for 10 days (acute exposure) to Mo-spiked sediments, but no effects were noted at the highest test concentrations (≥3.74 g Mo/L and ≥3.59 g Mo/L for *H. azteca* and *C. dilutus*, respectively). It was therefore decided to derive an EPM based $PNEC_{sediment}$ using the aquatic PNECs as a starting point.

The following formulas are applied for the estimation of the $PNEC_{sediment}$:

- Conversion of a Mo partitioning coefficient ($K_{D,sed-water}$) from L/kg to m³/m³:

$$K_{D,sed-water}\left(m^3/m^3\right) = 0.8 + \left[0.2 \times \left(K_{D,sed-water}(L/kg) \times 2500\right)/1000\right].$$

- Determination of the $PNEC_{sediment}$:

$$PNEC_{sediment} = \left(K_{D,sed-water}/RHO_{sed}\right) \times PNEC_{aquatic} \times 1000$$

with $PNEC_{aquatic}$ expressed as mg/L, RHO_{sed} = 1300 kg/m³ (bulk density of sediment) and the $K_{D,sed-water}$ expressed as m³/m³. The $PNEC_{sediment}$ is then expressed as mg/kg wet weight, but can be converted to a dry weight based PNEC, using a conversion factor of 2.6 (ECHA, 2010).

For the freshwater compartment, a median $K_{D,sediment}$ of 1778 L/kg (Log K_D: 3.25) was estimated with data generated in the FOREGS monitoring campaign (>800 coupled data of baseline concentration levels in water and sediment).

The FOREGS Geochemical Baseline Mapping Programme was approved in 1996 by the Forum of European Geological Surveys' Directors (FOREGS); its main aim was to provide high quality, multi-purpose environmental geochemical baseline data for Europe. The results of this program were published by De Vos et al. (2006) and Salminen et al. (2005). Raw data were used to derive typical (median) Mo levels in water and sediment for each country (Heijerick and Van Sprang, 2008; MoCon, 2010). Assuming equilibrium between the water and sediment compartments, typical country-specific K_D values could be determined. These K_D are shown in Fig. 3, and using the distribution that gives the best fit through these data, the regional K_D of 1778 L/kg was determined.

This value was put forward as a typical partition coefficient for molybdenum between the water and sediment compartment. A marine $K_{D,sed\text{-}water}$ was estimated by assuming equilibrium between the reasonable ambient PEC for marine water and marine sediment as reported in the Chemical Safety Report for sodium molybdate (MoCon, 2010). The proposed $K_{D,sed\text{-}water}$ value for this compartment was 1037 mg L/kg (Log K_D: 3.02).

Applying the aforementioned formula with these K_D values $PNEC_{sed,freshwater}$ and $PNEC_{sed,marine}$ of 22.6 g Mo/kg dw and 1.98 g Mo/kg dw, respectively, were derived. Both PNECs are based on an aquatic PNEC that already include an AF of three, and there is no rationale for the introduction of an additional AF.

In order to place the derived PNEC levels in a relevant ecological context, the different PNEC levels are compared with the reasonable worst case (RWC) ambient Predicted Environmental Concentrations (PECs) that were reported in the Chemical Safety Report for molybdenum (MoCon, 2010). An overview of the PNEC and PEC levels for the different environmental compartments is given in Table 4, together with the PNEC/PEC ratio; the latter value represents the factor of increase in Mo-levels that is allowed before adverse effects are observed.

Firstly, only small differences are noted between the ratios for the water columns and sediment compartment. The identical ratio for the marine compartment is a logical result from the fact that the K_D value that was used for deriving a $PNEC_{sediment}$ is based on the marine PECs that are reported in Table 4. For the freshwater compartment, however, the K_D value was determined on baseline levels of molybdenum in water and sediment, whereas the RWC-ambient PEC is based on several other monitoring data sets. Nevertheless similar PNEC/PEC ratios were noted within the freshwater compartment (5522 and 5994).

Secondly, the ratios in the marine environment were more than one order of magnitude lower than those observed in freshwaters. This large difference is the combined result of a broader range of sensitivity towards molybdenum in the marine environment and of higher environmental concentrations of molybdenum in the aquatic environment.

The results of the bioaccumulation experiment as reported by Regoli et al. (2012) support the ecological reliability of these large differences; Mo levels that are more than three orders of magnitude higher than ambient levels are still efficiently regulated in the most sensitive aquatic organisms of the SSD (*O. mykiss*). Assuming a similar degree of sensitivity between pelagic and benthic organisms, the calculated $PNEC_{sediment}$ of 22.6 g/kg dw is also considered as sufficiently protective as the difference between the PNEC/PEC ratio for the water column and sediment is almost negligible (5522 versus 5994).

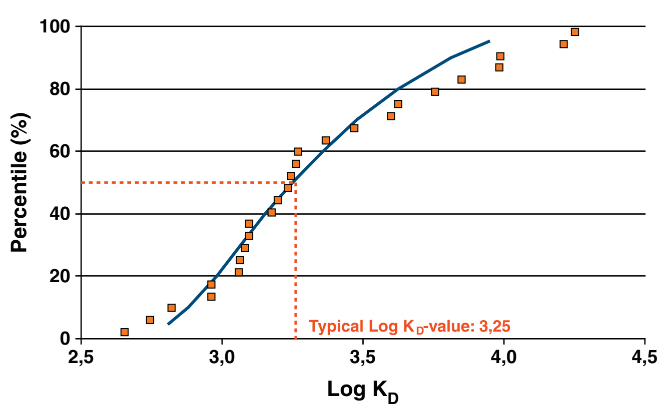

Fig. 3. Distribution of typical, country-specific Log-K_D-values, based on background concentrations in water and sediment (data from FOREGS Geochemical Baseline Programme).

Table 4
Overview of Predicted No-Effect Concentrations (PNECs) and reasonable worst case (RWC)-ambient Predicted Exposure Concentrations (PECs) of molybdenum in the aquatic compartment (freshwater/marine; water column/sediment).
Source: MoCon (2010).

	PNEC	RWC-ambient PEC	PNEC/PEC ratio
Freshwater compartment			
Water column	12.7 mg Mo/L	0.0023 mg Mo/L	5522
Sediment phase	22,600 mg Mo/kg dw	3.77 mg Mo/L	5994
Marine compartment			
Water column	1.91 mg Mo/L	0.0136 mg Mo/L	140
Sediment phase	1980 mg Mo/kg dw	14.1 mg Mo/kg dw	140

5. Conclusion

The statistical extrapolation methodology for deriving a PNEC for molybdenum in the aquatic compartment was successfully applied with the chronic ecotoxicity data that were identified for both the freshwater and marine environment. Based on the uncertainty assessment that was conducted on these data sets, an assessment factor of 3 was applied on the generated $HC_{5,50\%}$s, resulting in a PNEC of 12.7 mg/L and 1.91 mg Mo/L for the freshwater and marine environment, respectively. The broader range of effects levels in the marine environment not only resulted in a lower $HC_{5,50\%}$, but also widened the 95%CI that was associated with the $HC_{5,50\%}$. The same data were also used for the derivation of a Final Chronic Value, hereby following the methodology as outlined by US EPA (Stephan et al., 1985). Marginal differences were noted between the freshwater $HC_{5,50\%}$ and the FCV, but the earlier reported wider range of effect levels in the marine environment resulted in a FCV_{marine} that was a factor of 3 below the $PNEC_{marine}$.

Due to the lack of relevant data for the sediment compartment, a $PNEC_{sediment}$ could only be determined with the equilibrium partitioning method. Sediment PNECs were 22.6 g Mo/kg dw and 1.98 g Mo/kg dw for freshwater and marine sediment, respectively. The ecological relevance of the freshwater PNEC was demonstrated by the bioaccumulation data that are reported in Regoli et al. (2012); the most sensitive organism of the freshwater SSD (rainbow trout *O. mykiss*) was still able to regulate internal Mo-levels when exposed for 60 d to the PNEC concentration, and did so with the absence of any other adverse effect on behavior, growth or development.

As such, it can be concluded that the PNEC-levels that are reported in this study—and which were also reported in the EU REACH Chemical Safety Report for molybdenum and other molybdenum compounds, are sufficiently protective for freshwater and marine ecosystems.

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