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Environmental Toxicology

Neonicotinoid Insecticides from a Marine Perspective: Acute and Chronic Copepod Testing and Derivation of Environmental Quality Standards

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Abstract: Neonicotinoid insecticides have become of global concern for the aquatic environment. Harpacticoid copepods are amongst the most sensitive organisms to neonicotinoids. Here, we exposed the brackish copepod *Nitocra spinipes* to four neonicotinoid insecticides, i.e. clothianidin (CLO), imidacloprid (IMI), thiacloprid (TCP) and thiamethoxam (TMX) to investigate acute toxicity on adults (96-h exposure) and effects on larval development (7-d exposure). We used these results in combination with publicly available ecotoxicity data to derive Environmental Quality Standards (EQS). These EQS were ultimately used in a single-substance and mixture risk assessment for the Belgian part of the North Sea. Acute toxicity testing revealed that immobilization is a more sensitive endpoint than mortality, with 96h-EC_{50} values of 6.9, 7.2, 25 and 120 µg L^{-1} for CLO, TCP, IMI and TMX, respectively. In addition, the larval development tests resulted in 7d-NOECs of 2.5, 2.7, 4.2 and >99 µg L^{-1} for CLO, TCP, IMI and TMX,

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respectively. The derived saltwater Annual Average (AA-)EQS were 0.05, 0.0048, 0.002 and 0.016 µg L^{-1} for CLO, TCP, IMI and TMX, respectively. Finally, the risk characterization revealed some exceedances of the AA-EQS in Belgian harbors for IMI (number of exceedances, n = 2/4), TCP (n = 1/4), TMX (n = 1/4) and the mixture of the four neonicotinoids (n = 4/4), but not at the open sea. At the open sea site, the toxic unit sum relative to the AA-EQS was 0.72 and 0.22, suggesting no mixture risk, albeit with a relatively small margin of safety. Including short-term EC_{10} (96h) values of *N. spinipes* for the AA-EQS derivation led to a refinement of the AA-EQS for CLO and TMX, suggesting their use for the AA-EQS derivation since one of the overarching goals of the definition of EQS is to protect species at the population level.

Keywords: neonicotinoids, marine ecotoxicology, copepods, immobilization, acute and chronic testing

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Introduction

The Marine Strategy Framework Directive (MSFD) entered into force in June 2008 and aims to achieve or maintain good environmental status (GES) of the marine environment by the year 2020 (European Parliament and Council 2008b). GES of marine waters is determined by a total of 11 qualitative descriptors of which descriptor 8 asks that "concentrations of contaminants are at levels not giving rise to pollution effects" (European Parliament and Council 2008b). Next to the MSFD, the Water Framework Directive (WFD), which entered into force in October 2000, describes a strategy to fight pollution of water by progressive reduction or phasing-out of discharges and/or emissions (European Parliament and Council 2000). In support of the aims of the WFD, the Environmental Quality Standard (EQS) Directive establishes requirements for the chemical status of surface waters including marine waters by definition of EQS, which are meant to protect sensitive aquatic populations from unintended toxicity (European Parliament and Council 2008a). In addition to 45 listed priority substances and substance groups (European Parliament and Council 2013) a watchlist for potential water pollutants (European Commission 2018) has been compiled by the European Union. The WFD watchlist aims to obtain Union-wide monitoring data on potential water pollutants. In a second stage the European Commission will then decide whether or not EQS should be set for them at an EU level (European Commission 2018). Remarkably, EQS derivation for marine waters does not require any ecotoxicological data for marine test species (European Commission 2011) but can be calculated purely on the basis of standard freshwater species. This might be one reason as to why the availability of marine ecotoxicity data is certainly less than optimal for marine environmental risk assessment (Gustavsson et al. 2017). This is especially concerning since the greater diversity of

saltwater species compared to freshwater species has been recognized for almost a century (Russell and Yonge 1928). Indeed, saltwater environments show more diversity of taxonomic groups compared to freshwater environments (European Centre for Ecotoxicology and Toxicology of Chemicals 2001). In order to protect the more diverse marine environment risk assessment for the latter is generally based on an additional assessment factor of 10 as compared to risk assessment for the freshwater environment.

The basis for a solid risk assessment is the availability of reliable environmental data. Historically, the marine environment had received fewer attention than freshwater environments. Yet, in the past two decades, several studies have investigated the occurrence of a multitude of chemicals in marine environments including the North Sea (Ghekiere et al. 2013; Huysman et al. 2019; Thomas and Hilton 2004; Vanryckeghem et al. 2019). Due to extensive input from some major European rivers (i.e. the Elbe, Meuse, Rhine, Scheldt and Thames), the North Sea is one of the most contaminated marine systems worldwide (Halpern et al. 2008). This input results in the presence of a variety of man-made chemicals including pesticides. Beside other commonly present pesticides like the herbicides atrazine and diuron, monitoring of the Belgian part of the North Sea resulted in the detection of various neonicotinoid insecticides (Vanryckeghem 2020; Vanryckeghem et al. 2019).

Neonicotinoid insecticides have low volatilities (Anderson et al. 2015), high water solubility (0.185 to >590 g L^{-1}) and relatively low octanol-water partitioning coefficients (-0.66 – 1.26) (Tomizawa and Casida 2005). These properties make them readily absorbed by plants but also susceptible to runoff or leaching and subsequent transfer into the aquatic environment (Raby et al. 2018b). They have widespread use because of their low toxicity to mammals and high selectivity as agonists for insect nicotinic acetylcholine receptors (nAChRs) (Tomizawa and Casida 2005) to which they bind irreversibly (Tennekes 2010; Tennekes and Sanchez-Bayo 2011) causing a constant nerve stimulation that leads to changes in behavior (Raby et al. 2018b), paralysis (Arican et al. 2017) and eventually death (Arican et al. 2017; Raby et al. 2018b). Yet, the irreversible binding of neonicotinoids to the nicotinic acetylcholine receptors has been challenged (Maus and Nauen 2011) and might depend on the individual organism of exposure due to the wide variety in binding profiles seen in various organisms (Crossthwaite et al. 2017; Taillebois et al. 2018). Neonicotinoid insecticides have been detected in a multitude of surface waters in the ng L^{-1} to low µg L^{-1} range (Morrissey et al. 2015). While they are commonly known to be widely spread in freshwater, recent monitoring studies have also confirmed their occurrence in marine waters (Hano et al. 2019) including the Belgian part of the North Sea (Vanryckeghem et al. 2019). In the latter study, the authors reported neonicotinoid concentrations in the Belgian Part of the North Sea (BPNS) to range from 0.17 – 3.1 ng L^{-1} for clothianidin (CLO), 0.3 – 10 ng L^{-1} for imidacloprid (IMI), 0.34 – 65

ng L^{-1} for thiacloprid (TCP) and 1.4 – 54 ng L^{-1} for thiamethoxam (TMX) (Vanryckeghem et al. 2019).

Yet, there is only very limited neonicotinoid toxicity data for marine species (usually *Americamysis bahia*, *Cyprinodon variegatus* and *Crassostrea virginica*) available in literature. For instance, to our knowledge, marine copepods have not yet been tested for their response to neonicotinoid exposure at all. Copepods play an important role in the BPNS where they comprise 66 % of the total zooplankton abundance (Deschutter et al. 2017) serving as prey for higher trophic levels (Gee 1989). Over the past two decades the harpacticoid copepod *N. spinipes* has become a standard test species representing coastal and estuarine organisms (Breitholtz et al. 2008; Ribbenstedt et al. 2016), resulting in the development of international acute and chronic ecotoxicity testing guidelines (International Organization for Standardization 1999; 2016). It is commonly known that planktonic crustaceans like ostracods (i.e. *Ilyocypris dentifera* and *Cyretta seurati*) and cladoceran (i.e. *Chydorus sphaericus*) can be very sensitive to neurotoxic chemicals such as neonicotinoid insecticides (Sanchez-Bayo and Goka 2006). As representative of one of the most important groups of marine crustaceans, *N. spinipes* presents itself as a relevant marine test organism. Regarding the mechanism of action on the target site, one existing theory suggests the receptor binding in invertebrates to be near irreversible, leading to accumulative effects with increasing exposure time. This may exhibit delayed toxicity due to continuous nervous system stimulation (Morrissey et al. 2015) and indicates a valid need to extend existing data for acute endpoints with chronic toxicity testing. Another theory, considers the continuous binding of neonicotinoid insecticides to the nicotinic acetylcholine receptors as reversible. This theory is supported by a study that resulted in acute (7d) and chronic (28d) L/EC_{50} values for *H. azteca* to be within a factor 1.2-6.6 for the four here investigated neonicotinoids (Bartlett et al. 2019). In this case the delayed toxicity is most likely a consequence of cell energy exhaustion or receptor dysfunction due to continuous nervous system stimulation. In order to correctly assess risks of neonicotinoids for the marine environment, several aspects have to be taken into consideration: (i) various standard test species i.e. *Daphnia magna and Oncorhynchus mykiss* are not suitable for neonicotinoid risk assessment (Beketov and Liess 2008) due to low sensitivity, and (ii) given that the mechanism of action of different neonicotinoids is the same, toxicity thresholds may act via concentration addition (i.e. toxicity can be directly estimated by adding concentrations of neonicotinoid compounds) when simultaneously present in mixtures (Morrissey et al. 2015), even though toxicokinetics and toxicodynamics may differ.

In this study, we aimed at generating ecotoxicity data for the marine copepod *N. spinipes* to fill data gaps for neonicotinoid insecticides. Further, the generated marine data together with existing ecotoxicity data was used to derive EQS for the marine environment since currently available predicted no-effect concentrations (PNECs) for

four neonicotinoid insecticides (i.e. clothianidin, imidacloprid, thiacloprid and thiamethoxam) were derived specifically for freshwater systems (European Commission 2018). Finally, we aimed to assess potential risks for the BPNS based on the presence of neonicotinoid insecticides solely and as a mixture. Therefore, in this study we report acute (mortality and immobilization) and chronic (larval development) toxicity of four neonicotinoid insecticides for *N. spinipes*. In addition, we compiled literature ecotoxicity data and derived (marine) EQS for all of these. Ultimately, we assessed potential individual and mixture risks of neonicotinoids for the BPNS.

Materials and methods

Reagents and solutions

Clothianidin (CAS 210880-92-5), imidacloprid (138261-41-3), thiacloprid (111988-49-9) and thiamethoxam (153719-23-4) were purchased from Sigma Aldrich (Overijse, Belgium) with a purity grade of analytical standards (≥99 %).

Seawater for the culturing of test organisms was collected 500 m offshore from the coast of Blankenberge, Belgium and filtered using 0.2 µm PALL Supor®-200 membrane filters. The actual medium used for culturing was prepared by diluting the collected seawater to a salinity of 7 PSU using deionized water (subsequently called test medium).

Stock solutions of the tested neonicotinoid insecticides were prepared by diluting the respective neonicotinoid directly in test medium using ultra sonication if needed.

Screening study and compound selection

Within the framework of a screening study in the BPNS (Vanryckeghem 2020; Vanryckeghem et al. 2019) we found neonicotinoid insecticides to be most acutely toxic to a marine copepod among 23 pesticides and pharmaceuticals. This inspired us to focus on neonicotinoid effects and risks in the BPNS in the present study. More details about the selection criteria for test compounds and the acute toxicity data for all non-neonicotinoids are provided in the supportive information (S1).

Test organisms

Copepods were kindly provided by the Department of Environmental Science and Analytical Chemistry (ACES) at Stockholm University, where *Nitocra spinipes* has been in continuous culture since 1975 when it was isolated from the Tvären Bay in the Baltic Sea. A culture was established in our laboratory since 2016 (Koch and De Schamphelaere 2019) according to the methods described in Breitholtz and Bengtsson (2001) (Breitholtz and Bengtsson 2001). *N. spinipes* was cultured in test medium and the culture was permanently maintained in darkness at 22 ± 1°C.

Acute copepod testing

Acute copepod tests were performed according to ISO 14669 (International Organization for Standardization 1999) and simultaneously for all four neonicotinoids. In short, 3-4 week old copepods were exposed to different concentrations of selected chemicals and mortality was monitored daily over 96h. The guidance test duration of 48h was extended to 96h because lethal effects have been found to be typically delayed (Beketov and Liess 2008). Mortality was defined as no swimming and no appendage movement during 10 seconds such as described in ISO 14669 (International Organization for Standardization 1999). In addition, immobility was recorded and defined as inhibited swimming behavior (i.e. no controlled vertical or horizontal movement) of copepods during 10 seconds. This additional endpoint was introduced since many copepods in pre-tests showed considerable immobility while appendages movements were frequently observed. Consequently, according to the definition of mortality, organisms would have been evaluated alive while there was a clear effect on mobility. During the test, organisms were permanently kept in darkness at 22 ± 1°C. For each concentration treatment (CT), 20 organisms were randomly selected regardless of gender (including egg-carrying females) and separated into 4 replicates of 5 organisms. They were exposed in 2.5 mL test medium spiked with the respective test substance in sterilized non-treated 24-well VWR® (Oud-Heverlee, Belgium) cell culture plates. These plates were deemed suitable because neonicotinoid insecticides have low log K_{OW} and there is no risk of test substance loss to the plastic vessels.

For the test, a geometric dilution series was prepared directly in the well plates. In addition, 24 wells distributed over different plates were filled with pure diluted natural seawater and 5 copepods each to serve as control treatments (total number of organisms in control treatments = 120). pH was measured in one well of the lowest and highest test concentrations of each substance as well as two control wells at test start and end. All test details including test substances and CTs, as well as the pH measurements can be found in Table SI5.

7-day larval development testing

Larval development tests were performed according to ISO 18220 (International Organization for Standardization 2016). In short, nauplii younger than 24h from *N. spinipes* were exposed to six different concentrations of the four monitored neonicotinoid insecticides. Naupliar development to the copepodite stage was recorded after 6 and 7 days of exposure It was expressed as larval development ratio (LDR) being the number of copepodites divided by the sum of nauplii plus copepodites. Nauplii were exposed to nominal CLO concentrations ranging from 0.04 – 10 µg L^{-1}. Nominal test concentrations for IMI, TCP and TMX ranged from 0.33 – 100 µg L^{-1}. At test start, 9 ± 1 nauplii were placed in eight replicate wells per concentration or control treatment for CLO, IMI, and

TMX. Because of a limited availability of nauplii at test start of TCP only 6 ± 1 nauplii were used in the replicates and controls for TCP. Seventy percent of the test medium was refreshed once on day 4 and pH and salinity were measured at test start, day 4 and day 7. Organisms were exposed in 10 mL test medium spiked with the respective test substance (or not spiked in case of control treatments) in sterilized non-treated 6-well VWR® (Oud-Heverlee, Belgium) cell culture plates. During the tests, organisms were constantly kept in darkness at 22 ± 1°C.

Derivation of Environmental Quality Standards

EQS were derived according to the technical guidance document for deriving EQS (European Commission 2011) using the assessment factor method. Toxicity data was obtained from two databases: the US Environmental Protection Agency (EPA) ECOTOX database (US Environmental Protection Agency 2019b) and the US EPA Office of Pesticide Programs (OPP) Pesticide Ecotoxicity database (US Environmental Protection Agency 2018). More details about the data origin and the applied search criteria for both databases can be found in the supportive information (S2).

Data from organisms belonging to a different crustacean order (amphipoda, cladocera, decapoda, isopoda, mysida and harpacticoida) was treated as data for separate taxa because life form and feeding strategy varied considerably between the different species. For algae and fish, no differentiation was made between orders and data for the most sensitive species was used when data for several species was available. All other taxa, i.e. insects and mollusks were treated as additional taxa.

The Technical Guidance Document (TGD) for Deriving EQS gives detailed instructions on combining freshwater and marine species data for EQS derivation (European Commission 2011). In short, all toxicity data was first logarithmically transformed. Then, log-transformed freshwater and saltwater datasets of each neonicotinoid insecticide were tested for equal or unequal variance using a F-test ($\alpha = 0.05$). This showed equal variance ($p \geq 0.3$) between freshwater and saltwater datasets for all four neonicotinoids. Next, two-tailed t-tests ($\alpha = 0.05$) were performed to test for differences between the freshwater and marine datasets. This resulted in no significant difference in sensitivity ($p \geq 0.42$) and the two datasets were therefore combined for EQS derivation for all four neonicotinoids, as per the EQS Technical Guidance Document. A detailed overview of the data used for the derivation of all EQS values and more detailed information on data reliability and methods are given in the supportive information (S3-S5).

Chemical analysis

Acute copepod testing

Samples of the test medium (control and contaminant-spiked) for analysis were taken at the beginning and end of the tests. More precisely, at test start 1 mL of each stock solution as well as the different CTs were taken for each substance. Samples from different substances were merged per CT. At test end, 250 µL were taken from each replicate well and merged in an amber glass bottle. Next, the corresponding CTs of each test substance were merged i.e. CT1 of all test substances was merged in one bottle, CT2 of all test substances in a second bottle etc. Samples from different biotests were merged in order to reduce sample handling and preparation time as well as analytical costs. Nevertheless, the analytical method applied for the analysis of the samples was initially developed for screening of environmental water samples and therefore highly suitable to deal with the simultaneous presence of various chemicals (Vanryckeghem et al. 2019).

7-day larval development testing

Known volumes (90 mL, 56 mL and 75 mL) of the test medium were taken at day 0 (test start), day 4 and day 7 of each test. Samples taken on day 4 consisted of 7 mL taken from each of the 8 replicate wells of each CT. Samples at day 0 were samples taken directly from the prepared stock solution. Samples at day 7 were a combination of the remaining test medium in all 8 replicate wells of each CT.

Sample preparation and analytical settings

Samples of all tests were first filtered using 2.7 µm glass microfiber filters (Whatman™ GF/D, GE Healthcare) and then stored in amber glass bottles in darkness at -20 °C until analysis. Before extraction, all sample volumes were adjusted to 250 mL using diluted natural seawater (7 PSU). Next, 200 mL per sample plus a diluted natural seawater blank were extracted using solid phase extraction with Oasis® HLB cartridges (6 mL, 200 mg sorbent, supplied by Waters, Brussels, Belgium), and analyzed on a reversed phase ultra-high performance liquid chromatography (UHPLC) system coupled to a hybrid quadrupole-Orbitrap high-resolution mass spectrometer (Q-Exactive™, Thermo Scientific). Details about the chemical analytical method have been published earlier (Vanryckeghem et al. 2019).

Risk characterization

Risks at various monitoring locations (see supportive information S1) in the BPNS for two independent sampling campaigns (SC) were calculated using the toxic unit (TU) approach. For each substance an individual TU was calculated as the ratio between the measured concentration of the substance and its Annual Average (AA-)EQS for saltwater

environments. For the mixture risk assessment, the individual neonicotinoid TUs were summed (∑TU) per sampling location as given in equation 1. A $TU_i > 1$ or $\sum_{i=1}^{n} TU_{i,x} > 1$ indicates a risk of the individual substance or the mixture, respectively (i.e., Risk Quotient, RQ > 1). The Risk Quotient (RQ) was calculated as follows:

$$RQ = \sum_{i=1}^{n} TU_{i,x} = \sum_{i=1}^{n} \frac{C_i}{EQS_i} \qquad \text{(equation 1)}$$

where n is the number of mixture components considered and TU_i is the toxic unit of component *i* in the mixture. The TU_i, a dimensionless parameter, is defined as the ratio between concentration of component *i* in the mixture (C_i) and its AA-EQS for saltwater environments in the mixture (EQS_i).

This research is part of the larger project called NEWSTHEPS (www.newstheps.be), which consisted of five field sampling campaigns in total. The risk assessment presented in this manuscript, was only applied in two of those campaigns, i.e. sampling campaigns 2 and 3 (SC2 and SC3). For the sake of comparability between different publications (Huysman et al. 2019; Vanryckeghem et al. 2019), related PhD theses (Huysman 2019; Moeris 2020; Vanryckeghem 2020) and the final project report (www.newstheps.be/final-report), we kept the project-related numbering of the sampling campaigns in this manuscript as well. Water grab samples were taken on 25/11/2016 and 02/02/2017 for SC2 and 13/04/2017 and 20/06/2017 for SC3, respectively. The three sampling locations were inside the harbor of Zeebrugge (HZ, 51°20'25.68"N; 3°12'12.11"E), approximately 3 km in front of Zeebrugge harbor (SZ, 51°21'37.78"N; 3°6'49.01"E), and inside the harbor of Ostend (HO, 51°13'34.68"N; 2°56'8.00"E), in the BPNS. The environmental concentration of the neonicotinoid insecticides was calculated as the average concentration during sampler deployment and retrieval. Each moment, triplicate samples were analyzed. More details are described in Vanryckeghem (2020). A summary of the measured environmental concentrations is given in the supportive information (Table SI2).

Statistics

All statistical analysis was performed in R Studio (RStudio Team 2016).

Acute copepod testing

Dose response models were generated using the "drc" package (Ritz et al. 2015) and visualizations were created with ggplot2 (Wickham 2016). For dose-response analysis, a two-parameter log-logistic model was used (fct = LL.2 (upper = 100), logDose = 10). EC_{10} and EC_{50} values with their 95 % confidence intervals were derived from the model.

7-day larval development testing

Statistical analysis was performed using non-parametric tests. Differences between specific treatments and the control were assessed using the Mann-Whitney-U test. Based on this analysis, the no-observed effect concentration (NOEC) for each substance was defined as the highest concentration showing no statistically significant (Mann-Whitney-U-Test, $\alpha = 0.05$) effect on larval development. In addition to that, the lowest-observed effect concentration (LOEC) was defined as the lowest concentration showing a statistically significant (Mann-Whitney-U-Test, $\alpha = 0.05$) effect on larval development. In a separate analysis, a concentration-response model was fitted in R Studio using the "drc" package (Ritz et al. 2015) to determine the EC_{10}. For CLO, a hormetic four-parameter model (CRS.4c) was fitted to the data (Cedergreen et al. 2005). For IMI and TCP a log-logistic two-parameter model (llogistic2) was used (Ritz et al. 2015) where the maximum LDR was set to the observed average LDR of the control treatment. For TMX, no effects were observed and thus no model fitted.

Results

Chemical analysis

Chemical analysis of the acute copepod testing resulted in constant concentrations with the concentration at test end (C_{end}) being within 94 % - 111 % of the concentration at test start (C_{start}) except for 3 treatments that showed 122 – 133 % of C_{start} at test end (for details, see supportive information Table SI3). For the 7-day larval development testing, concentrations remained constant over 7 days with C_{end} being within 81 – 120 % of C_{start} except for 4 treatments that showed 61 – 73 % of C_{start} at test end (for details see supportive information Table SI 3). A detailed overview of the chemical analytical results is given in the supportive information (S6).

Acute copepod testing

The pH across all measurements within a test varied maximally 0.3 units (7.1 - 7.4, for CLO) and on average 0.08 units. The complete pH data can be found in the supportive information (Table SI4). In addition to the standard endpoint mortality we also monitored immobilization for all four neonicotinoid insecticides. Immobilization resulted in clearly lower (2.6 – 1000 times lower) 96h-EC_{50} values as compared to mortality. Table 1 shows the EC_{50} values of the four neonicotinoid insecticides including their confidence intervals. The differences between mortality and immobilization EC_{50} values were generally larger after short exposure times (24h – 48h). Immobilization EC_{50} (96h) values were 2.6, 6.2, 847 and 1000 times lower than the mortality EC_{50} (96h) for CLO, TMX, TCP and IMI, respectively. The lowest immobilization EC_{50} (96h) were observed for CLO at 6.9 µg L^{-1} and TCP at 7.2 µg L^{-1}, whilst the observed effects for CLO and TMX

became increasingly comparable for the two endpoints (mortality and immobilization) over time. This was not the case for IMI and TCP where mortality was a clearly less sensitive endpoint than immobilization even after 96h as shown in Figure 1.

7-day larval development testing

Clothianidin

The 7-day larval development testing of CLO ended after 6 days when the control treatments reached a mean LDR of 69 %. The pH across all measurements within the test varied maximally 0.4 units (7.2 – 7.6) and salinity not more than 0.3 PS (6.9 – 7.2 PSU). The test resulted in the determination of a NOEC of 2.5 µg L^{-1} and a LOEC of 14 µg L^{-1} (Figure 2A). These results indicate a statistically significant delay in development of *N. spinipes* at a concentration of 14 µg L^{-1}. Next to this, statistical analysis identified 0.08 µg L^{-1} to cause a significantly higher LDR as compared to the control treatment, suggesting a hormetic response. The EC_{10} (6d) was defined at 2.6 µg L^{-1} (95%-CI: 0.62 – 4.5 µg L^{-1}) (Figure 2B). This EC_{10} value should be used with caution due to the fact that only one concentration treatment showed statistically significant effects on the larval development. This resulted in an uncertain model fit and the NOEC should be used preferentially.

Imidacloprid

The 7-day larval development testing of IMI ended after 7 days when the control treatments reached a mean LDR of 75 %. The pH across all measurements within the test varied maximally 0.32 units (8.00 – 8.32) and salinity not more than 0.3 PSU (7.0 – 7.3 PSU). The test resulted in the determination of a NOEC of 4.2 µg L^{-1} and a LOEC of 13 µg L^{-1} (Figure 2C). The EC_{10} (7d) was defined at 0.18 µg L^{-1} (0.01 – 2.1 µg L^{-1}) (Figure 2D). The EC_{10} was a factor of 23 lower than the NOEC and should be used with caution since it results from an extrapolation (EC_{10} is lower than the lowest concentration treatment).

Thiacloprid

The 7-day larval development testing of TCP ended after 7 days when the control treatments reached an average LDR of 60 %. The pH across all measurements within the test varied maximally 0.32 units (8.00 – 8.32) and salinity not more than 0.3 PSU (7.0 – 7.3 PSU). The test resulted in the determination of a NOEC of 2.7 µg L^{-1} and a LOEC of 8.6 µg L^{-1} (Figure 2E). These results indicate a statistically significant delay in development of *N. spinipes* as of 8.6 µg L^{-1}. The EC_{10} (7d) was defined at 1.1 µg L^{-1} (0.4 – 3.2 µg L^{-1}) where the top was set to the average LDR of the respective control treatment (Figure 2F).

Thiamethoxam

The 7-day larval development testing of TMX ended after 6 days when the control treatments reached a mean LDR of 59 %. The pH across all measurements within the test varied maximally 0.50 units (7.52 – 8.02) and salinity remained constantly at 7.1 PSU in all treatments. The test resulted in no statistically significant developmental effects of *N. spinipes* in the tested concentration range of 0.32 - 99 µg L^{-1} (Figure 2G). Therefore the NOEC is > 99 µg L^{-1}, the highest test concentration.

An overview of the endpoints and models used to evaluate the 7-day larval development testing is given in Table 2.

Deriving Environmental Quality Standards (EQS)

Conventional approach

A summary of the available toxicity data from the two databases used for the derivation of EQS values for all four neonicotinoid insecticides, including the used assessment factors (AFs), is shown in Table 3. An overview of all derived EQS values can be found in Table 4.

In the technical guidance document for the derivation of EQS, “data for additional marine taxonomic groups” has been defined as “data from studies with marine organisms other than algae, crustacean and fish, and/or having a life form or feeding strategy different from that of algae, crustaceans or fish” (European Commission 2011). This definition gives a certain degree of freedom to the risk assessor and makes an EQS derivation a somewhat subjective process that needs expert judgement and justification. Assumptions and justifications taken during this exercise are provided in the supportive information (S5).

A link to the complete archived data used for the EQS derivation of each substance can be found in the supportive information (S3). *N. spinipes* was found to be the most sensitive species for long-term exposure to CLO. Further, for the derivation of the saltwater AA-EQS (AA-EQS_{sw}) of TMX, the availability of long-term data for *N. spinipes* as an additional marine taxonomic group lead to the reduction of the AF from 100 to 50. The lowest Maximum Allowable Concentration (MAC-)EQS and AA-EQS were derived for IMI with 0.065 µg L^{-1} and 0.002 µg L^{-1}, respectively. The highest difference between MAC-EQS and AA-EQS was observed for TMX with the latter being 325 times lower.

Using acute EC_{10} values for AA-EQS derivation

Because we found that some acute EC_{10} (96h) values for adult *N. spinipes* were lower than some chronic EC_{10} (7d) values for larval *N. spinipes* (Table 2) and because one may need to protect both life stages and endpoints to protect *N. spinipes* populations, we also calculated AA-EQS for the scenario in which we considered the acute 96h-EC_{10} values for *N. spinipes*. This had an impact on the derived AA-EQS for CLO and TMX, which became a factor 8 and 2 lower, respectively, compared to the regulatory conventional method (only using chronic data), resulting in 0.0062 and 0.0086 µg L^{-1} for CLO and TMX, respectively. The AA-EQS for IMI and TCP remained unchanged (0.0048 and 0.016 µg L^{-1}, respectively).

Risk characterization

Conventional EQS derivation

Figure 3 gives an overview of the calculated toxic units for two sampling campaigns. The highest neonicotinoid concentrations were measured at the harbor of Ostend (HO) resulting in exceedance of the AA-EQS of a factor 4.0 – 5.6, 12 - 15, 3.0 – 4.0 and 8.3 - 21 for IMI, TCP, TMX and the neonicotinoid mixture. For the harbor of Zeebrugge (HZ) and the open sea location in front of this harbor (SZ) no exceedance of the AA-EQS for individual substances was observed. Yet, the AA-EQS-based ∑TU of the four neonicotinoids together slightly exceeded the trigger value of 1 at HZ for both sampling campaigns (AA-EQS-based ∑TU = 1.2 and 1.02 for SC2 and SC3, respectively). This exceedance was mainly driven by IMI (61 – 74 %) and TCP (25 – 28 %) while CLO (0.6 – 1.0 %) and TMX (7.5 -11 %) had only minor contribution. No exceedance of the MAC-EQS was observed for any sample.

Using acute EC_{10} values for EQS derivation

When basing the risk characterization on the EQS derived including acute EC_{10} values that were lower than chronic EC_{10} values for *N. spinipes*, we found an exceedance of the AA-EQS of a factor 1.2 – 1.4 for TMX during SC2 at HO in addition to the results shown for the AA-EQS in Figure 3. In addition to that, the TU_{mix} at the open sea location (SZ) was 0.88 for samples from SC2, suggesting a relatively small margin of safety for mixture risks.

Discussion

In acute toxicity testing with *N. spinipes*, neonicotinoid insecticides were found to be the most toxic among 23 personal care products, pesticides and pharmaceuticals that had been detected in a monitoring study in the BPNS. Based on the results of these tests (data for non-neonicotinoids is shown in the supportive information, S8), long-term effects of

neonicotinoid insecticides were further investigated in 7-day larval development tests with *N. spinipes*. Ultimately, EQS were derived and risks for the BPNS were assessed for four neonicotinoids individually and as a mixture. During the acute toxicity testing, we found that for neonicotinoid insecticides immobilization is a more sensitive endpoint than mortality. Finally, the risk characterization revealed exceedance of the AA-EQS_{sw} especially at HO for IMI and TCP (SC2) and IMI and TMX (SC3) as well as the mixtures of neonicotinoids. In addition to that, the ∑TU of the neonicotinoids exceeded the trigger value of 1 at HZ while individual substances indicated no immediate risk.

Acute copepod testing

Acute toxicity testing of neonicotinoid insecticides with *N. spinipes* revealed that immobilization was a more sensitive endpoint as compared to mortality. Similar effects have been observed for IMI on 3 freshwater ostracods and 2 freshwater cladoceran species (Sanchez-Bayo and Goka 2006) and for TCP on a freshwater copepod species (Arican et al. 2017). Sanchez-Bayo and Goka (2006) suggested that immobilization due to neonicotinoid exposure can seriously endanger populations of these organisms in the wild and listed the following 2 main reasons: (1) immobilization makes the zooplankton easy prey vulnerable to attacks by their numerous predators, (2) the paralysis induced by neonicotinoids is likely to cause starvation because organisms may experience difficulties in feeding. Overall, neonicotinoids elicited acute toxic responses from *N. spinipes* over a concentration range of 17-fold, with CLO being the most toxic and TMX being the least toxic indicating that toxicity among neonicotinoids can vary widely. The same has been confirmed by Raby et al. (2018) for the freshwater crustacean *Hyalella azteca* with EC_{50} values ranging 81-fold (Raby et al. 2018a).

While EC_{50} values are a commonly recognized endpoint for acute toxicity studies, barely any attention is given to acute EC_{10} values. Here, the derived immobilization EC_{10} (96h) values for *N. spinipes* (Table 1) were a factor of 7.0, 26, 3.6 and 52 lower than the respective EC_{50} (96h) values for CLO, IMI, TCP and TMX, respectively.

Our study has shown that immobilization of *N. spinipes* exposed to neonicotinoid insecticides is likely to occur at concentration levels in the low µg L^{-1}-range and such immobilization might negatively affect *N. spinipes* populations. Further, the four neonicotinoids could be ranked according to their acute toxicity as follows: CLO > TCP >> IMI >> TMX.

7-day larval development testing

While the tests with IMI and TCP ended after 7 days, the tests with CLO and TMX were finished after 6 days. This is in agreement with the test guideline stating that *"If the ratio of copepodites to the total number of surviving early-life stages (nauplii + copepodites) is*

within 60 % ± 20 % the test is terminated" (International Organization for Standardization 2016). Extending the test duration for CLO and TMX would have led to the test organisms in the lower concentration treatments to be fully developed. This would have prevented the definition of EC_{10} or NOEC values. The 7-day larval development testing of *N. spinipes* resulted in NOECs in the low µg L^{-1} range for CLO, IMI and TCP while no effects were observed for TMX up to 99 µg L^{-1}. These findings are comparable to chronic LC_{10} (7d) values for the freshwater crustacean *H. azteca* ranging from 2.8 µg L^{-1} (CLO) to 160 µg L^{-1} (TMX) (Bartlett et al. 2019). Further, the 7-day larval development results confirmed the neonicotinoid potency ranking (CLO > TCP > IMI > TMX) suggested by acute testing and in a study investigating acute (7d) and chronic (28d) effects of the same neonicotinoids on the freshwater amphipod *H azteca* (Bartlett et al. 2019). Thus, despite similar structure and the same mode of action, neonicotinoid insecticides differ in their toxicity. These differences are most likely related to variability in their toxicokinetics and/or toxicodynamics (Focks et al. 2018) determined by e.g. differences in binding sites, binding affinities and/or specificity of binding between compounds (Kayser et al. 2004; Kayser et al. 2016; Taillebois et al. 2018; Tomizawa and Casida 2005; Wellmann et al. 2004).

Next to that, NOEC and EC_{10} values were observed to be within a factor of 2.5 for CLO, TCP and TMX, while a factor of 23 was observed for IMI. For CLO and TMX this is in line with the range reported for the comparison of lethal (14d) and sublethal (40d) toxicity values for *Chironomus dilutus* (Cavallaro et al. 2017). The difference for IMI did not have any influence on the EQS-derivation, but requires careful evaluation when using these data in risk assessment. The EC_{10} for IMI was extrapolated below the lowest test concentration, which may explain the relatively high uncertainty on the EC_{10} (CI = 0.01 – 2.1 µg L^{-1}). Thus, for IMI the use of the NOEC is recommended over using the EC_{10}.

Deriving Environmental Quality Standards

When deriving EQS, one faces several challenges and needs to consider many different aspects. In the following paragraph we would like to list a few of these challenges, explain how we dealt with them and justify our decisions. In addition, information about data reliability and detailed justifications for certain choices can be consulted in the supportive information (S4 and S5).

Several authors described midges and mayflies (Anderson et al. 2015; Morrissey et al. 2015; Raby et al. 2018b) as the most sensitive aquatic organisms to neonicotinoids in both acute and chronic exposure scenarios. While literature data confirmed this generally, our experiments with *N. spinipes* resulted in a lower EC_{10} value (7-day larval development) for CLO as compared to the lowest NOEC/EC_{10} values in the used databases.

Ecotoxicity databases

EQS derivation is a hazard-based approach aiming to define thresholds with a high protection goal for the freshwater and marine environment. It is therefore crucial to reduce any uncertainty to a minimum by including as much data as possible into the decision-making process (European Commission 2011). Here, we focused on the US EPA ECOTOX and OPP databases because they complemented each other due to (mainly) different data sources, and could be regarded as to cover the majority of toxicity data directly available to us at the time of retrieval. While online databases provide an extensive amount of toxicity data for neonicotinoids, there is a clear lack of data for marine species. Next to that, data reliability is a critical point and requires thorough checking which might lead to a reduction of the already scarce data for e.g. EQS derivation.

The use of freshwater and saltwater species data

The use of both freshwater and saltwater data lead to an overall increase of data resulting in a decrease of uncertainty for the EQS derivation. Nonetheless, merging the two datasets for the EQS derivation of neonicotinoids can be questioned due to the fact that freshwater insects have been shown to be among the most sensitive species to neonicotinoids (Anderson et al. 2015; Morrissey et al. 2015). These insects usually spend their juvenile stages in freshwater habitats until maturation. Only a very limited number of insect species have shown tolerance to low salinity (Thorpe 1927). Thus, the relevance of insect data for the derivation of EQS_{sw} is questionable. On the other hand, data for marine species in risk assessment or EQS derivation is usually scarce and often basing EQS_{sw} derivation on a combination of freshwater and saltwater data is the best practice to lower the AFs in use (Gustavsson et al. 2017).
The freshwater: saltwater data ratio in our datasets was 47:12, 175:8, 73:8 and 63:7 for CLO, IMI, TCP and TXM, respectively. Thus, data for saltwater species represented only 4 – 20 % of the available data for EQS derivation. Excluding freshwater data in this case would thus have led to an increase of the AFs from 10 to 50 for IMI, TCP and TMX but no change for CLO for the MAC-EQS. For the AA-EQS it would have led to an increase from 50 to 100 for CLO, from 50 to 500 for IMI and TCP, and from 50 to 1000 for TMX. This would result in a slight increase of the MAC-EQS for CLO and IMI (to 0.32 and 0.025 µg L^{-1}, respectively) and an overall considerable decrease of the MAC-EQS for TCP and TMX (to 0.20 and 2.4 µg L^{-1}, respectively) and of the AA-EQS for CLO, TCP and TMX (to 0.025, 0.00033 and 0.0022 µg L^{-1}, respectively). Finally, the derivation of an AA-EQS for TMX would not have been possible since no endpoint would have been available.

Extrapolation using assessment factors

One important difference between freshwater and saltwater EQS derivation is the use of different AFs. AFs used for the saltwater environment are usually set a factor of 10 higher to deal with the higher biodiversity in the marine environment and the ongoing uncertainty to represent the most sensitive organisms (European Commission 2011). Next to the basic set of toxicity data (algae, crustacean and fish), the AF for saltwater EQS derivation can be further reduced when data for additional marine species is available. This includes taxa different from the basic set of algae, crustacean and fish such as e.g. mollusks or echinoderms, but also marine organisms belonging to the taxa algae, crustacean or fish with either a different life form or feeding strategy (European Commission 2011). In the present study we used toxicity data of several freshwater and saltwater algae and the aquatic plant *Lemna gibba*. If data was available for several algae, they were always considered as representatives of one taxonomic group and the lowest endpoint was considered for EQS derivation. *L. gibba* was considered as an additional freshwater species representing a separate taxon. For crustaceans, overall data was available for 8 different species representing 6 order, i.e. amphipoda, cladocera, decapoda, isopoda, mysida and harpacticoida. Short-term data for an additional marine taxonomic group was available in the form of mollusk data for all four neonicotinoids. Long-term data was available for *A. bahia* and additionally provided by our 7-day larval development tests with *N. spinipes* for all substances but TMX. Assessment factors in use ranged from 10 to 50 proving a relatively low uncertainty for the EQS derivation. This was justified by the fulfilment of the criteria described in Tables 3.3 and 3.5 in the TGD for deriving EQS (European Commission 2011).

Using acute EC_{10} values for EQS derivation

In this study, we observed a rather rare case where short-term exposure of adult organisms resulted in lower effect concentrations (mortality or immobilization 96h-EC_{10} values) as compared to long-term exposure of their early life stages (7d-NOECs). It is commonly recognized that early-life stages are usually more sensitive than adult organisms of the same species, but exceptions do exist. Holan et al. (2018) found adult individuals of the marine bivalve *Gaimardia trapesina* to be more sensitive than juveniles when exposed to copper (Holan et al. 2018). Since one of the overarching goals of the derivation of EQS is to protect species at a population level, there is no clear reason for not including acute EC_{10} values in the derivation of the AA-EQS. The consideration of EC_{10} (96h) values from *N. spinipes* short-term exposure to neonicotinoid insecticides for the AA-EQS derivation led to a more conservative AA-EQS for CLO and TMX. These findings highlight the importance of allowing some flexibility when deriving EQS. Here, we show that using short-term EC_{10} values as additional endpoints for the AA-EQS derivation may lead to a more adequate protection of *N. spinipes* populations.

Comparison of EQS and literature threshold values

Due to their extensive use and subsequent detection in the aquatic environment (Raby et al. 2018b), neonicotinoid insecticides have been found to adversely affect a wide range of non-target organisms, specifically insects (Anderson et al. 2015). Nevertheless, two (IMI, TCP) out of four neonicotinoids tested in our study currently remain approved for the European market, with CLO and TMX being banned with national exceptions for a variety of countries. Notably, the use of IMI is restricted to application in permanent greenhouses (European Commission 2019).

In a review about neonicotinoid insecticides in the Canadian aquatic environment, Anderson et al. (2015) concluded that in terms of toxicity data most studies have been performed for IMI, while data for CLO and TMX was generally scarce (Anderson et al. 2015). We do not fully agree with this statement since – based on data derived from only two of the many existing ecotoxicity databases – we found toxicity data for 16 freshwater species covering 6 different taxonomic groups and for 5 saltwater species covering 4 taxonomic groups for CLO which, combined, formed a solid basis for EQS derivation. For TCP and TMX, data for saltwater species was indeed very scarce and EQS derivation for the saltwater environment was associated with a higher degree of uncertainty.

The MAC-EQS derived in our study were a factor of 1.7 to 48 lower than threshold values reported in literature so far (supportive information S9, Table SI7). Whereas the MAC-EQS for IMI and TMX were only slightly lower (1.7 to 6 times lower), the MAC-EQS for CLO and TCP were up to a factor of 48 and 41 lower than the US-reported aquatic life benchmark (LB) for invertebrates (US Environmental Protection Agency 2019a). Nevertheless, the derivation of EQS and LB differs significantly with the latter being based on either the lowest 48h- or 96h-EC_{50} or LC_{50} of a standardized test with usually a midge, a scud or a daphnid. This EC_{50} is then reduced using a level of concern (LOC, comparable to an AF) of 0.5 for the acute value. For LBs based on the lowest no-observed adverse effect concentration (NOAEC) from a life-cycle test with usually a midge, a scud or a daphnid, a LOC of 1 is applied to the chronic endpoint. Thus, an LB only takes data from the respective taxon (in this case crustacean data) into account and is as such not really comparable to an EQS that aims to protect a whole ecosystem rather than few taxonomic groups. In a Dutch study, Smit et al. (2015) derived a MAC-EQS_{fw} of 0.065 µg L^{-1} for IMI using the AF approach, resulting in the exact same value than in our study and thereby confirming our approach (Smit et al. 2015).
The derived AA-EQS, on the other hand, were within a factor of 0.006 – 202 of threshold values reported in literature so far. There was a relatively high discrepancy between the derived AA-EQS and the US-LB (US Environmental Protection Agency 2019a) or the Canadian long-term thresholds (Health Canada Pest Management Regulatory Agency (HC-PMRA) 2018) due to very distinct derivation methods. Comparison with PNEC

values proposed by the Joint Research Center (European Commission 2018) on the other hand, resulted in the AA-EQS being a factor of 0.42 – 4.2 of those reported in literature. This is logic due to the very similar approaches for derivation of EQS and PNEC values under European legislation.

Risk Characterization

Overall, risk characterization resulted in rather comparable patterns for the two sampling campaigns. The AA-EQS-based TU for IMI and the sum of toxic units for neonicotinoids exceeded the measured seawater concentrations during both sampling campaigns at HO and the two harbors (HO and HZ), respectively. The only difference between both campaigns was the exceedance of the TU for TCP during SC2 and for TMX during SC3. Of course, two time points are not a solid basis to evaluate spatio-temporal patterns and it would be highly recommended to monitor the BPNS more regularly over an extended time period to better estimate potential chronic effects.

Neonicotinoid insecticides have been identified as contaminants of concern for aquatic ecosystems due to their frequent occurrence and relatively low effect thresholds in various organism groups (e.g. insects and crustaceans) (Morrissey et al. 2015; Sanchez-Bayo and Goka 2006). Mixtures of neonicotinoids (including the here investigated ones) have been reported to represent a significant threat to 14/19 surface waters (Morrissey et al. 2015). In addition, maximum concentrations of IMI measured in the Llobregat River (north-east Spain) have been reported to be close to the short-term threshold (0.1 - 0.07 µg L^{-1}) and exceeding the long-term threshold (0.03 µg L^{-1}) proposed in this study (Rico et al. 2018). For TMX risks due to short-term exposure are very unlikely to occur even in freshwater ecosystems due to the relatively high MAC-EQS of 5.2 µg L^{-1} which has been concluded before (Finnegan et al. 2017). The proposed AA-EQS on the other hand was found to be exceeded at HO and long-term exposure to such concentrations may pose a risk to the Belgian marine environment. Further, we found exceedance of the TU_{mix} for the four neonicotinoids at HZ where individual substances did not exceed the threshold. While risk assessment for individual neonicotinoids and their mixtures has been conducted for a variety of freshwater ecosystems, our study is to our knowledge the first to evaluate potential risks for marine ecosystems. The exceedance of the AA-EQS at the two investigated harbors should serve as an early warning for the BPNS. This is further supported by the relatively high AA-EQS-based RQ_{mix} of 0.72 and 0.22 observed for SC2 and SC3 at the SZ open sea location resulting in a relatively limited margin of safety for this sampling location. In addition, the ban of CLO and TMX in Europe and the currently restricted use of IMI might lead to an increased use of TCP as an alternative neonicotinoid insecticide, resulting in an increasing input of this substance into marine waters. This is disconcerting since TCP was among the two substances contributing the

most to the ∑TU together with IMI. Thus, a replacement of banned neonicotinoid insecticides by other neonicotinoids might turn out to be a very regrettable solution.

Conclusion

Neonicotinoid insecticides are used worldwide and have become of global concern for the aquatic environment. Harpacticoid copepods are, other than many other crustacean, very sensitive to neonicotinoids. Acute toxicity testing with *N. spinipes* revealed that immobilization is a much more sensitive endpoint than the standard endpoint mortality. Overall, the data generated for *N. spinipes* led to a refinement of the saltwater AA-EQS for CLO, and contributed considerably to the reduction of uncertainty (assessment factor) in the definition of the saltwater AA-EQS for TMX. In addition, considering short-term EC_{10} values for AA-EQS derivations resulted in a reduced AA-EQS for CLO and TMX, highlighting the importance of short-term EC_{10} values for threshold values (e.g. EQS) derived for a protection on the population level. Compared to measured concentrations in the BPNS, we found exceedance of the AA-EQS for IMI at the harbor of Ostend for both sampling campaigns, and for TCP and TMX at the harbor of Ostend during SC2 and SC3, respectively. Finally combination toxicity (RQ_{mix}) of the four neonicotinoids led to an exceedance at HO and HZ for both sampling campaigns. Further, when calculating the RQ_{mix}, we found a relatively low margin of safety for the open sea sampling location in the BPNS. Therefore, with regards to pesticide use prognostics potential risks for the BPNS in the future cannot be excluded and further monitoring is suggested.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx.

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FIGURE CAPTIONS

Figure 1. Concentration-response curves for Nitocra spinipes exposed to four neonicotinoid insecticides (clothianidin, imidacloprid, thiacloprid and thiamethoxam) measured daily for 96h. Black circles show the mean mortality of quadruplicates in percent, and blue triangles the mean immobilization of quadruplicates in percent. Lines are fitted log-logistic dose-response models.

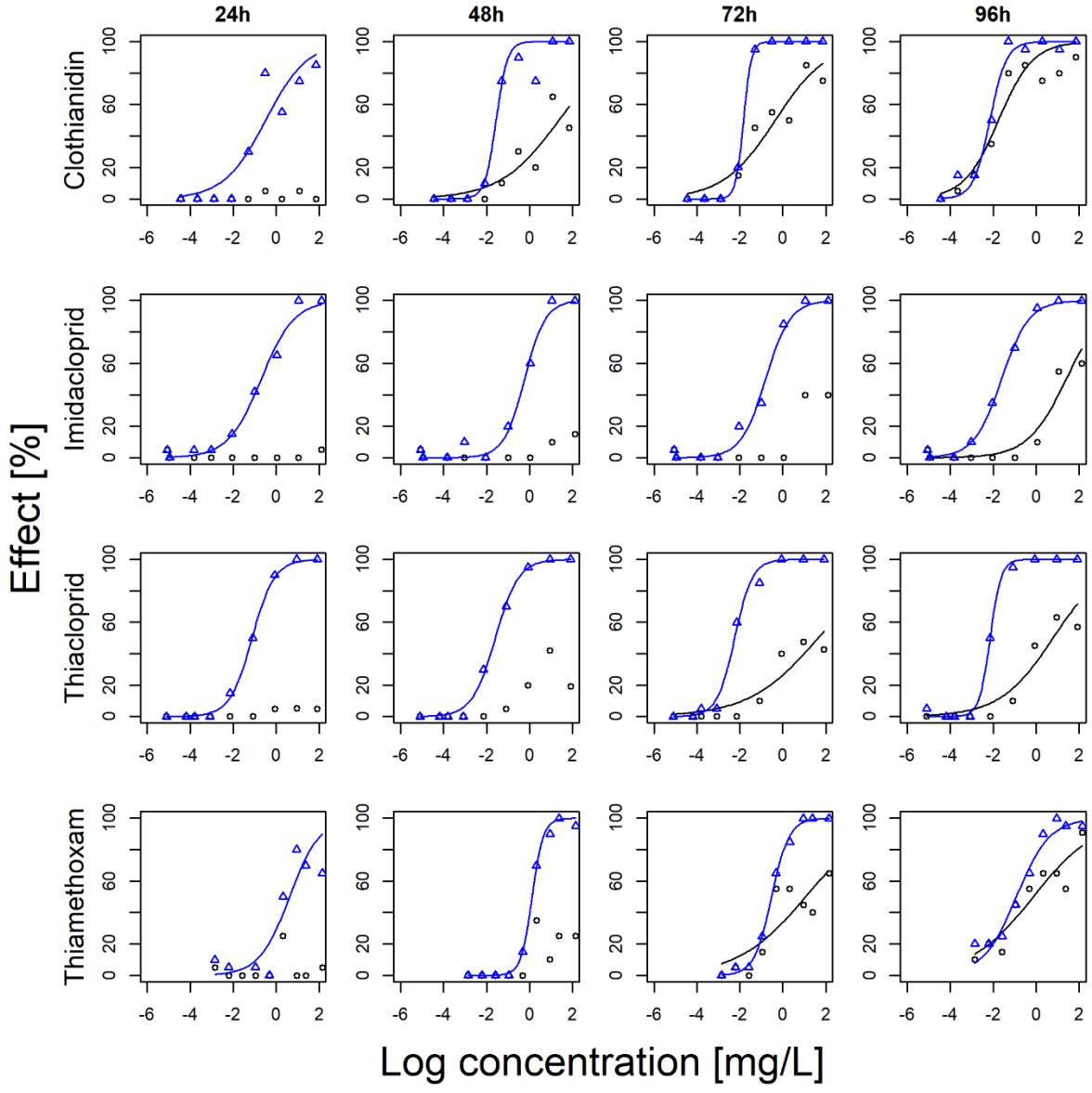

Figure 2. Results of the 7-day larval development tests with N. spinipes for clothianidin (CLO, A and B), imidacloprid (IMI, C and D), thiacloprid (TCP, E and F) and thiamethoxam (TMX, G and H). The boxplots (left column) show the results after 6 (CLO and TMX) or 7 (IMI and TCP) days of exposure for the different concentration treatments as compared to the control treatments (Control). The boxes indicate the 25th to 75th percentiles and the upper and lower limits indicate the minimum and maximum data points excluding outliers (more and less than 1.5x upper and lower quartile). The bold line shows the median larval development ratio (LDR). Treatments marked with an * indicate concentrations with a statistically significant difference (Mann-Whitney-U test, p-value < 0.05) of LDR compared to the control treatment. Blue circles in both plots represent the individual data points per replicate. The concentration response curves (right column) show the fitted dose response models for the LDR vs. the logarithmic concentration in µg L 1. No model could be fitted for TMX. The black triangles indicate the average LDR per concentration treatment and the grey zone indicates the 95 % confidence interval on responses predicted by the dose-response model.

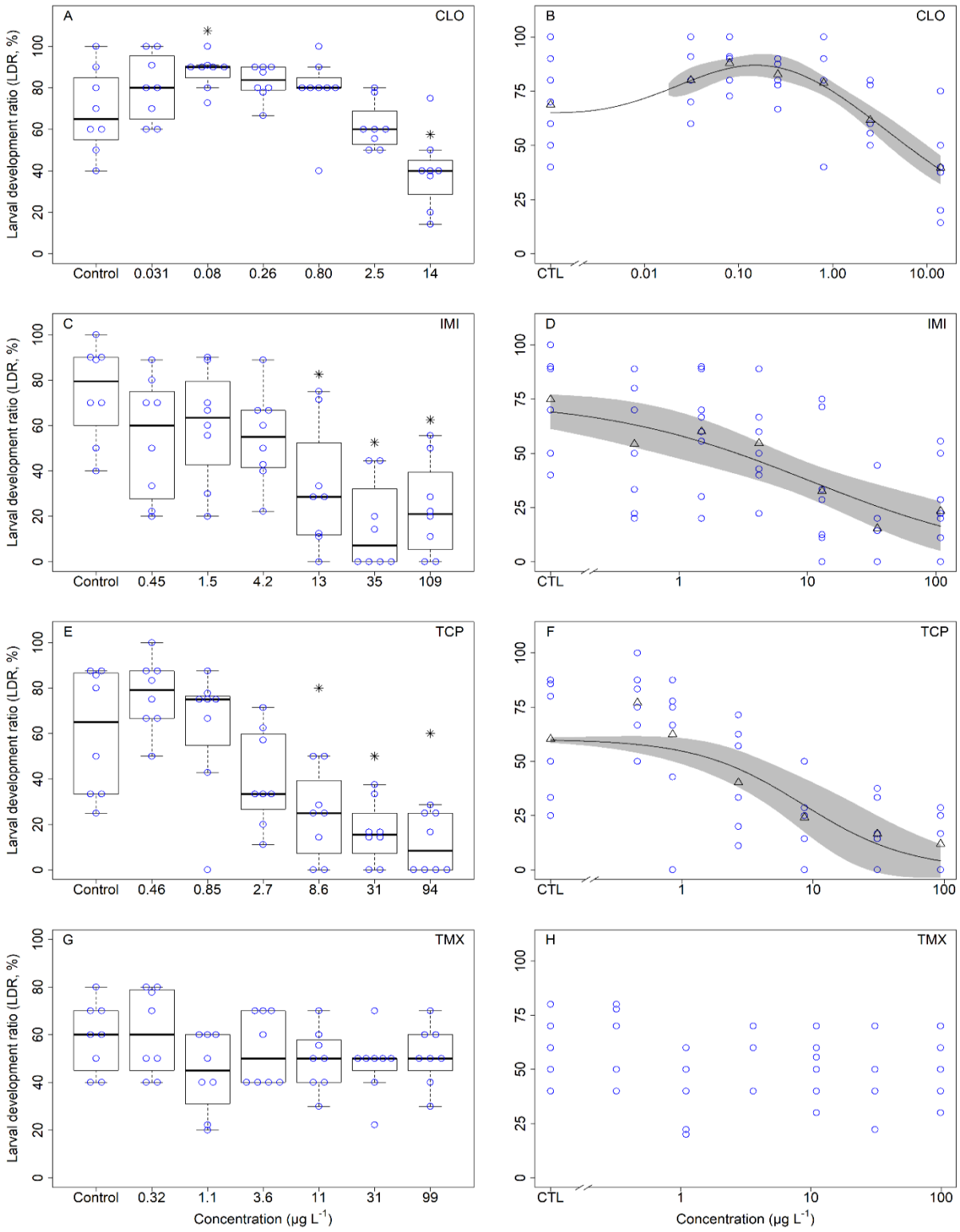

Figure 3. Risk quotients (RQ) for the three sampling locations in the Belgian Part of the North Sea based on grab sampling for sampling campaign 1 (A) and 2 (B) and derived Annual Average-Environmental Quality Standards (AA-EQS). HO, HZ and SZ describe the harbor of Ostend, the harbor of Zeebrugge and an open sea location in front of Zeebrugge, respectively. The Neonicotinoids bars indicate the sum of toxic units of the four neonicotinoid insecticides. Bars show the average of triplicates and error bars express their standard deviation.

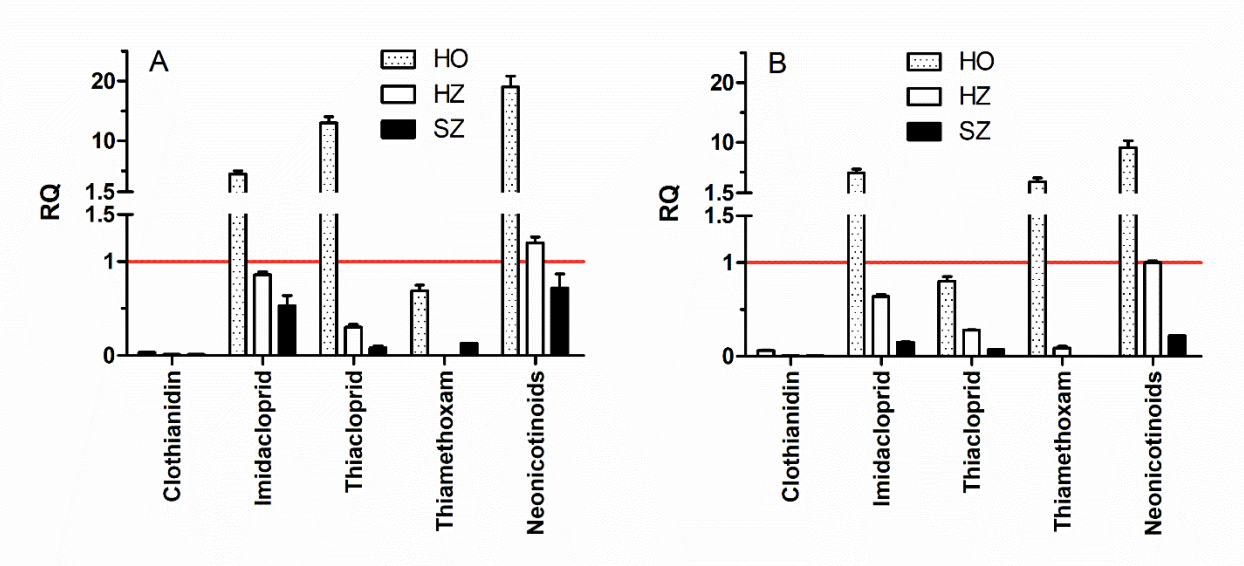

Table 1 Acute EC_{10} and EC_{50} (in µg L^{-1}) values for the four neonicotinoid insecticides and their 95% confidence intervals (in parentheses) for the two endpoints mortality and immobility.

EC_{10}	**Mortality**				**Immobility**			
	24h	**48h**	**72h**	**96h**	**24h**	**48h**	**72h**	**96h**
Clothianidin	>72,000	24	0. 94	0.31	2.4	7.5	5.9	0.99
		(16 –120)	(0.84 – 3.7)	(0.12 – 1.4)	(0.9 – 11)	(3.8 – 19)	(5.8 – 6.0)	(0.51 – 2.5)
Imidaclop rid	>132,000	>132,000	>132,000	270	4.2	51	8.8	0.96
				(31 – 840)	(2.8 – 9.7)	(44 – 108)	(5.8 – 21)	(0.43 – 1.5)

Thiacloprid	>100,000	101 (31 – 556)	13 (5.3 – 57)	12 (10 – 47)	5.5 (3.3 – 7.8)	1.7 (0.78 – 2.6)	0.72 (0.018 – 1.4)	2.0 (0.52 – 3.4)
Thiamethoxam	>142,000	>142,000	4.1 (0.16 – 25)	0.43 (0.28 – 2.0)	121 (23 – 597)	349 (215 – 482)	38 (24 – 52)	2.3 (0.81 – 6.1)

EC_{50}	Mortality				Immobility			
	24h	**48h**	**72h**	**96h**	**24h**	**48h**	**72h**	**96h**
Clothianidin	>72,000	24,000 (5,800 – 77,000)	450 (22 – 1,100)	18 (6 – 41)	330 (290 – 940)	28 (10 – 46)	15 (15 – 15)	6.9 (3.2 – 11)
Imidacloprid	>132,000	>132,000	>132,000	25,000 (20,000 – 55,000)	200 (80 – 330)	590 (290 – 890)	160 (70 – 250)	25 (18 – 31)
Thiacloprid	>100,000	>100,000	54,000 (21,000 – 180,000)	6,100 (2,500 – 16,000)	76 (62 – 90)	26 (18 – 330)	5.7 (3.4 – 81)	7.2 (6.2 – 8.2)
Thiamethoxam	>142,000	>142,000	12,000 (5,900 – 40,000)	740 (430 – 1,800)	4,200 (1,700 – 11,000)	1,300 (1,100 – 1,600)	300 (250 – 350)	120 (39 – 200)

Table 2 Endpoints of the 7-day larval development testing with *Nitocra spinipes* in µg L^{-1}. Shown are the no-observed effect concentration (NOEC), the lowest-observed effect concentration (LOEC), the effect concentration showing 10 % effect (EC_{10}) and its 95% confidence interval (95%-CI). Model indicates the model fitted to the data for the determination of the EC_{10}. Where the upper limit was fixed to the average larval development ratio of the control treatments (LDR_{CTL}).

	NOEC	**LOEC**	**EC_{10}**	**95%-CI**	**Model (as in drc package for R)**
Clothianidin	2.5	14	2.6	0.62 – 4.5	CRS.4c(names = c("b", "d", "e", "f"))
Imidacloprid	4.2	13	0.18	0.01 – 2.1	llogistic2 (fixed = c(NA,0,LDR_{CTL},NA,1))
Thiacloprid	2.7	8.6	1.1	0.4 – 3.2	llogistic2 (fixed = c(NA,0,LDR_{CTL},NA,1))
Thiamethoxam	>99	>99	>99	/	/

Table 3 Data used for saltwater (sw) Environmental Quality Standard (EQS) derivation for neonicotinoid insecticides. All effect concentrations (EC_x and NOEC) are given in µg L^{-1}.

	MAC-EQS$_{sw}$						**AA-EQS$_{sw}$**					
Substance	**Lowest EC_{50}**	**Endpoint**	**Species**	**Test duration [d]**	**Total number of species**	**AF**	**Lowest NOEC or EC_{10}**	**Endpoint**	**Species**	**Test duration [d]**	**Total number of species**	**AF**
Clothianidin	2.3	Mortality	*Chironomus dilutus*[a]	4	10	10	2.5	Larval development	*Nitocra spinipes*[b,c]	6	8	10^{f_w} 50_{sw}
Imidaclorid	0.65	Mortality	*Epeorus longimanus*[a]	4	13	10	0.1	Length	*Epeorus sp.*[a]	20	8	10^{f_w} 50_{sw}
Thiacloprid	4.6	Mortality	*Baetis rhodani*[a]	4	9	10	0.24	Mortality	*Cloeon dipterum*[a]	7	7	10^{f_w} 50_{sw}
Thiamethoxam	52	Mortality	*Cloeon dipterum*[a]	4	9	10	0.81	Mortality	*Cloeon dipterum*[a]	28	6	10^{f_w} 50_{sw}

[a]insects

[b]copepods

[c]this study

[fw/sw]freshwater/saltwater

Table 4 Derived Environmental Quality Standards (EQS) for four neonicotinoid insecticides (by dividing the lowest toxicity value by the assessment factor as reported in Table 3). Derived are the Maximum Allowable Concentration (MAC-) EQS and the Annual Average (AA-) EQS for both fresh water (fw) and salt water (sw) environments. All values are expressed in µg L^{-1}.

Substance	**MAC-EQS$_{fw}$**	**MAC-EQS$_{sw}$**	**AA-EQS$_{fw}$**	**AA-EQS$_{sw}$**
Clothianidin	0.23	0.23	0.25	0.05
Imidacloprid	0.065	0.065	0.01	0.002
Thiacloprid	0.46	0.46	0.024	0.0048
Thiamethoxam	5.2	5.2	0.081	0.016