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Determination and validation of an aquatic Maximum Acceptable Concentration-Environmental Quality Standard (MAC-EQS) value for the agricultural fungicide azoxystrobin☆

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ABSTRACT

The main goal of the present study was to determine and validate an aquatic Maximum Acceptable Concentration-Environmental Quality Standard (MAC-EQS) value for the agricultural fungicide azoxystrobin (AZX). Assessment factors were applied to short-term toxicity data using the lowest EC_{50} and after the Species Sensitivity Distribution (SSD) method. Both ways of EQS generation were applied to a freshwater toxicity dataset for AZX based on available data, and to marine toxicity datasets for AZX and Ortiva® (a commercial formulation of AZX) obtained by the present study. A high interspecific variability in AZX sensitivity was observed in all datasets, being the copepoda *Eudiaptomus graciloides* ($LC_{50,48h}$ = 38 μg L^{-1}) and the gastropod *Gibbula umbilicalis* ($LC_{50,96h}$ = 13 μg L^{-1}) the most sensitive freshwater and marine species, respectively. MAC-EQS values derived using the lowest EC_{50} (≤0.38 μg L^{-1}) were more protective than those derived using the SSD method (≤3.2 μg L^{-1}). After comparing the MAC-EQS values estimated in the present study to the smallest AA-EQS available, which protect against the occurrence of prolonged exposure of AZX, the MAC-EQS values derived using the lowest EC_{50} were considered overprotective and a MAC-EQS of 1.8 μg L^{-1} was validated and recommended for AZX for the water column. This value was derived from marine toxicity data, which highlights the importance of testing marine organisms. Moreover, Ortiva affects the most sensitive marine species to a greater extent than AZX, and marine species are more sensitive than freshwater species to AZX. A risk characterization ratio higher than one allowed to conclude that AZX might pose a high risk to the aquatic environment. Also, in a wider conclusion, before new pesticides are approved, we suggest to improve the Tier 1 prospective Ecological Risk Assessment by increasing the number of short-term data, and apply the SSD approach, in order to ensure the safety of aquatic organisms.

1. Introduction

For retrospective aquatic risk assessment, two types of information are required: exposure levels and toxic effects on non-target organisms, and the risk is expressed as the ratio between exposure concentrations and critical effect concentrations. The latter could be set by an Environmental Quality Standard (EQS) value, which may be generated by applying assessment factors to ecotoxicity data (European Commission, 2011). If a large dataset for different taxonomic groups is available, a probabilistic methodology based on statistical extrapolation techniques such as the Species Sensitivity Distribution (SSD) method might be applied, and therefore lower assessment factors can be used. The SSD approach assembles single-species toxicity data in order to predict hazardous concentrations (HC_x) affecting a certain percentage (x) of species in a community. The most conservative form of this approach uses the lower 95% tolerance limit of the estimated percentage to ensure that the specified level of protection is achieved. Hose and Van den Brink (2004) confirmed this concept of species protection by comparing laboratory-based SSD curves with both local mesocosm

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experiments and field monitoring data. SSD curves are constructed by fitting a cumulative distribution function to a plot of species toxicity data against rank-assigned percentiles (Wheeler et al., 2002). The greater the number of species tested, the lower the uncertainty of the risk assessment attributable to interspecies differences in sensitivity. In addition, this approach may reduce the uncertainty resulting from differences in the sensitivity of standard test species and those expected to be exposed in nature by also using non-standard test species data. According to Newman et al. (2000), sample size producing HC_5 (hazardous concentration for 5% of species) estimates with minimal variance should range from 15 to 55.

According to international authorities, azoxystrobin (AZX, CAS No. 131860-33-8), the world's No. 1 agricultural fungicide (PAN UK, 2015; Royal Society of Chemistry, 2016; Van Alfen, 2014), is considered to be of low acute and chronic toxicity to mammals, birds and bees (EFSA, 2010; US-EPA, 1997). However, despite the absence of critical areas of concern related to non-target species, an exception was made for aquatic organisms, since a toxicity data gap was identified after the peer-review of the AZX risk assessment of EFSA (2010). In addition, studies on AZX toxic effects on marine organisms are considered scarce by Rodrigues et al. (2013). Therefore, a comprehensive study was designed in order to contribute and timely respond to this critical area of concern, and the median effective concentration for growth rates (EC_{50}) and mortality (LC_{50}) were determined for species representative of several functional and trophic levels of marine ecosystems.

Pesticides are rarely used individually, and additives such as stabilizers, carrying solvents or emulsifiers are added to the final-product (Walker et al., 2001). Accordingly, it has already been shown that commercial formulations of pesticides can be more toxic than their active ingredients (e.g., Mesnage et al., 2014; Puglis and Boone, 2011). The AZX active ingredient is presently registered under different trade names, such as Abound®, Amistar®, Ortiva®, among others. The latter is a mixture of declared hazardous components which are reported in its Safety Data Sheet: 22.9% weight/weight of AZX and 10–20% weight/weight of propane-1,2-diol (Syngenta, 2010). Since sensitivities may be compared by means of the SSD concept (Leung et al., 2001), both Ortiva and AZX SSD curves were plotted to find whether Ortiva is more toxic to marine communities than its active ingredient.

A general strategy to assess the risk of pesticides for marine environments consists of applying safety factors to the risk level calculated based on freshwater toxicity data (ECHA, 2015). Since the available ecotoxicological data on AZX derive mostly from assays with freshwater species (Rodrigues et al., 2013), an SSD curve could also be generated for freshwater species so as to compare sensitivities of both marine and freshwater species by means of the SSD concept (Leung et al., 2001).

The main goal of the present study was to determine and validate a water column Maximum Acceptable Concentration (MAC)-EQS value in line with the European Commission (2011) for AZX. To attain this main goal, three specific objectives were delineated:

1) Determining whether the commercial formulation Ortiva is more toxic than its active ingredient AZX.
2) Comparing the sensitivity of marine species to AZX with that of freshwater species.
3) Determining if MAC-EQS values generated using SSD curves are more protective and conservative than those derived using the lowest EC_{50}.

Since the statistical extrapolation SSD approach for aquatic regulatory purposes is still under debate (Del Signore et al., 2016), this comprehensive study may provide important insights on this subject. In addition, the present study contributes to the establishment of EQSs in the field of water policy under the Water Framework Directive, and allows AZX regulatory risk characterization.

2. Material and methods

2.1. Ethical statement

All animal experiments were conducted in accordance with the ethical guidelines of the European Union Council (Directive 2010/63/EU) and the Portuguese Agricultural Ministry (Decreto-Lei 113/2013) for the protection of animals used for experimental and other scientific purposes. The person in charge of experimental procedures with live animals has accreditation for the use of live animals for scientific purposes (category C) according to the Federation of European Laboratory Animal Science Associations (FELASA) education and training guidelines, granted by the Portuguese General Directorate of Veterinary.

2.2. Marine experimental design

Short-term toxicity assays using both the AZX analytical standard and the commercial formulation Ortiva fully complied with internationally recognized guidelines and protocols (Table 1). The selected species include both standard and non-standard test species, such as non-pathogenic bacteria (*Vibrio fischeri*), microalgae, rotifers (*Brachionus plicatilis*), macrocrustaceans (*Artemia franciscana*), gastropod molluscs (*Rissoa parva* and *Gibbula umbilicalis*) and fish (*Solea senegalensis*). In order to have phytoplankton representativeness, microalgae were chosen from among four phylogenetic groups: Bacillariophyceae (the pennate diatom *Phaeodactylum tricornutum* and the centric diatom *Thalassiosira weissflogii*), Cryptophyceae (*Rhodomonas lens*), Eustigmatophyceae (*Nannochloropsis gaditana*) and Haptophyceae (*Isochrysis galbana*). With a single exception, the *R. parva* assay, all lethal assays were performed using early life stages, larvae or juveniles, as they generally tend to be more sensitive to pollutants than later life stages (Buchwalter et al., 2004; Mohammed, 2013).

2.3. Analytical standard and Ortiva solutions

Azoxystrobin PESTANAL analytical standard (99.9% purity) was purchased from Sigma-Aldrich (31697). Stock standard solutions were prepared in *pro analysis* grade acetone and stored at −18 °C. The fungicide Ortiva was kindly provided by the tree nursery Almeida Rodrigues Viveiros Agrícolas Lda (Coimbra, Portugal). Ortiva intermediate solutions and both AZX and Ortiva exposure media were freshly prepared on the day of use in reconstituted marine water (tropic marin salt, Tropical Marine Centre) using ultra-pure water purified with a Milli-Q Biocel System (Millipore) at salinities presented in Table 1. In the case of the *V. fischeri* assay, the exposure medium was prepared in diluent supplied by Microtox (Modern Water), whereas for the *B. plicatilis* and *A. franciscana* assays, the exposure media were prepared using reagent grade chemicals supplied by MicroBioTest kits: Rotoxkit M and Artoxkit M, respectively. Nominal concentrations were confirmed using a validated chemical method according to section 2.4: the solutions used to start the serial dilutions in the bacteria and microalgae assays, and the exposure solutions collected at the end of the lethal assays. The concentrations used in the statistical analysis were attained by calculating the geometric mean of nominal and measured concentrations, as recommended by Traas (2001).

Table 1
Bioassays experimental design and conditions.

Species	T (°C)	Photoperiod	Salinity	Exposure conditions				No. replicates	No. organisms/ replicate	Guideline
				AZX Nominal concentration range	Ortiva Nominal concentration range	Dilution factor	Exposure time			
V. fischeri	4	–	–	0.26–16 mg L^{-1}	0.13–4.1 g L^{-1}	2	5 min	1	–	WCMUC, 1994
P. tricornutum	20	24 h L	33	32-4096 μg L^{-1}	32-4096 μg L^{-1}	2	72 h	3	10^3 cells mL^{-1}	ISO 10253, 2006
T. weissfolgii	20	24 h L	33	50-6400 μg L^{-1}	50-6400 μg L^{-1}	2	72 h	3	10^4 cells mL^{-1}	ISO 10253, 2006
R. lens	20	24 h L	33	50-6400 μg L^{-1}	50-6400 μg L^{-1}	2	72 h	3	10^4 cells mL^{-1}	ISO 10253, 2006
N. gaditana	20	24 h L	33	13-6400 μg L^{-1}	13-6400 μg L^{-1}	2	72 h	3	10^4 cells mL^{-1}	ISO 10253, 2006
I. galbana	20	24 h L	33	5.0–160 μg L^{-1}	5.0–160 μg L^{-1}	2	72 h	3	10^5 cells mL^{-1}	ISO 10253, 2006
B. plicatilis	25	24 h D	15	0.60–6.8 mg L^{-1} (a)	1.0–6.2 mg L^{-1} (a)	1.2	24 h	6	5	ASTM E 1440, 1991
A. franciscana	25	24 h D	35	150-774 μg L^{-1}	500-2580 μg L^{-1}	1.2	24 h	3	10	ASTM E 1440, 1991
R. parva	15	16/8 h L/D	34	101-513 μg L^{-1}	–	1.5	96 h	4	5	ASTM E 729, 2002
G. umbilicalis	15	16/8 h L/D	34	8.2–63 μg L^{-1} (b)	8.2–63 μg L^{-1} (b)	1.5	96 h	4	5	ASTM E 729, 2002
S. senegalensis	20	12/12 h L/D	35	75-1282 μg L^{-1} (c)	75-1282 μg L^{-1} (c)	1.5	48 h	4	5	ASTM E 729, 2002

T: temperature; L: light; D: dark.
a)No confirmatory chemical analysis were performed since no mortality was observed.
b)No confirmatory chemical analysis were performed since some nominal concentrations were below the method's limit of quantification.
c)No confirmatory chemical analysis were performed since there was no sufficient volume at the end of the assay.

2.4. Chemical analytical methodology

Azoxystrobin analyses in water samples were carried out in the laboratory of Instituto Superior Técnico (University of Lisbon, Portugal). Extraction was performed using 10 mL of each sample in 0.5 mL of dichloromethane by the liquid-liquid methodology. The separation and quantification of AZX was done by GC-MS. A Restek TG-5MS column, 30 m × 0.25 mm, 0.25 μm (Supelco) was employed using helium as a carrier gas at a 1.0 mL min^{-1} flow rate. The temperature of the injector was kept at 250 °C. The oven temperature was as follows: 230 °C at 20 °C min^{-1} held for 1 min, then 310 °C at 25 °C min^{-1} held for 6 min. The mass detector conditions were: 310 °C as the transfer line temperature and 250 °C as the ion source temperature. Selected Ion Monitoring (SIM) mode was chosen and several specific ions were selected: 329, **344**, 345, 372, 388, 403 (in bold, ion used for quantification). The limit of the AZX quantification method was 13 μg L^{-1}.

2.5. Marine single-species short-term toxicity assays

Since AZX is considered stable to hydrolysis (US-EPA, 1997), static non-renewal tests were performed in the conditions established in Table 1. A negative and a solvent control, the latter containing the highest concentration of the solvent used, were considered in each AZX bioassay (APHA, 1989), whereas for Ortiva bioassays, due to its water solubility, only negative controls were required. Concentration replicates were also considered, as reported in Table 1. Physico-chemical conditions such as salinity, pH and dissolved oxygen were measured in the media at the beginning of each bioassay using the multi-parameter Hach HQ30d.

The sensitivity of the bacterium *V. fischeri* was assessed using a standardized bioluminescent assay developed by MICROTOX® Bioassay Testing System, which was coupled with the Microtox Omni Windows software. The Microtox 81.9% Basic Test was used. In the AZX assay, the solvent control was tested as 50 μL of acetone in 10 mL of the Microtox diluent, and no bacteria luminescence inhibition was observed. Therefore, to compute the median effective concentration, only negative control data was considered.

Microalgae cultures were supplied by AQUALGAE (Spain). Stock culture maintenance and bioassays were both performed according to ISO 10253 (2006). Briefly, stock cultures were maintained axenically in 100 mL-Erlenmeyers with 50 mL of growth medium prepared with reconstituted water supplemented with 10 mL L^{-1} of "optimedium" (AQUALGAE). An extra supplement of sodium silicate (45 μg L^{-1}) was added to the *T. weissflogii* culture medium. Cultures were placed in a 20 °C constant-temperature cabinet (Binder KBW_{400}) with illumination programmed to continuous wide-spectrum light from cool daylight lumilux lamps (Osram L18W/865). Light intensity at the surface of the culture vessels was about 3300 lux (Delta OHM HD9221). An orbital shaker (Heidolph rotamax 120) was used to ensure adequate culture homogenization, and the environment inside the cabinet was enriched with air filtered through a 0.22 μm syringe filter (Minisart, Sartorius Stedim Biotech). Since microalgae growth rates are highly influenced by environmental factors such as light, salinity and nutrient availability, growth curves were generated from daily cell counts. Cell doubling time and daily growth rates were determined using the Doubling Time 1.0.10 software (http://www.doubling-time.com). Hence, to start the bioassays, algae were inoculated from exponential growth-phase stock cultures and placed in glass test tubes of 4.0 mL and 10 mm ∅ covered with parafilm, using 1.0 mL of exposure medium prepared by serial dilutions. Acetone concentration in solvent controls was 640.4 μL L^{-1} for all microalgae assays, except for the *I. galbana*, which was 256 μL L^{-1}. The bioassays took place in the same conditions used for maintaining the stock cultures, and the test tubes were vortexed and repositioned daily. At the end of the bioassays, and as recommended by Marie et al. (2014), samples were preserved with glutaraldehyde (Fluka 49632) 0.25% (final concentration) and then deep-frozen (−80 °C, Haier DW-86L628) for subsequent counting by flow cytometry. No preservation was performed in *N. gaditana* and *I. galbana* samples. Flow cytometry analyses were, therefore, made with live cells. All the counts were achieved using the True Volumetric Absolute Counting technique, which is available in the Partec CyFlow Space flow cytometer used. The data acquisition FloMax software was optimized by using both the scatter (forward and side scatter) and the auto-fluorescence properties of the cells. To eliminate the large amount of debris signal, the auto-fluorescence signal of the cells was used to gate the particles in the forward *versus* side scatter (both in logarithmic scale). In the cytogram, a region was defined

around the cloud of cells for each microalgae species, which was kept constant throughout the analyses. Due to the size of *T. weissflogii* cells, counts were performed using a Neubauer chamber.

The bioassays using rotifers *B. plicatilis* were conducted according the Rotoxkit M protocol. The PVC multi-well plates supplied with the kit were 24 h-conditioned with exposure media. Concerning the bioassays using artemia *A. franciscana*, the multi-well test plates supplied by the kit were replaced by 4.0 mL and 10 mm ∅ glass tubes with 1.0 mL of exposure media. Acetone concentration in solvent controls was 182.4 μL L^{-1}.

Gastropods were collected in April (*R. parva*) and May (*G. umbilicalis*) of 2015, during low tide, in an intertidal rocky shore of the Portuguese Atlantic coast (40°10′16.5″N, 8°53′33.6″W). The water temperature at the time of collection was 15 °C. According to MarMAT, a multimetric method to classify the ecological quality status of coastal areas based on marine macroalgae, the selected site was considered of good/high quality (Neto et al., 2012). The stocks of gastropods were maintained in a 15 °C constant-temperature cabinet with illumination programmed to 16-h light (~1500 lux)/8-h dark periods. The stocks were maintained in aerated glass tanks with 2.0 L of reconstituted water for at least 20 days (acclimation period). Air filtration was done through a 0.22 μm syringe filter and the media were replaced in whole every three days. The organisms were supplied *ad libitum* with fresh *Ulva*, although they were starved in the 48 h prior to the assays, thus ensuring that all animals were at a similar starting point. During the starvation period, 150 mg L^{-1} of sodium hydrogen carbonate ($NaHCO_3$, Sigma S5761) was added to the reconstituted water since the carbonate is used by gastropods for their skeletons (shells). Calibrated snails, *R. parva* (2.4–3.8 mm length) and *G. umbilicalis* (6.5–8.1 mm), were randomly introduced in 50 mL- and 250 mL-Erlenmeyer flasks, respectively, containing exposure media (40 mL for *R. parva* and 150 mL for *G. umbilicalis*) prepared in $NaHCO_3$-supplemented reconstituted water. The length of *R. parva* was measured using a microscope Leica M-80 with a calibrated ocular micrometer, and *G. umbilicalis* was measured using an electronic digital caliper (VMR 1819-0012). To start the assay, *R. parva* were observed under a binocular microscope to perceive mobility and those carrying egg masses were discarded. Concerning *G. umbilicalis*, mobility was observed with the naked eye. Acetone concentration in solvent controls was 2.6 mL L^{-1} and 312.5 μL L^{-1} for *R. parva* and *G. umbilicalis* assays, respectively. During the assays, each test vessel was covered with a watch glass and checked twice a day, and emerged snails were gently submerged. At the end of the assays, the criterion used to determine mortality was failure to respond to gentle physical stimulation observed under a binocular microscope.

Senegal sole pelagic larvae (newly hatched) *S. senegalensis* were kindly provided by the marine fish farm A. Coelho & Castro (Estela, Portugal). On arrival to the laboratory, larvae were immediately placed in a 20 °C constant-temperature cabinet for three hours. Then, they were randomly introduced in each replicate test vessel (4.0 mL and 10 mm ∅ glass tubes with 1.0 mL of exposure media). Acetone concentration in solvent controls was 128.2 μL L^{-1}. The assays took place in the above mentioned constant-temperature cabinet with illumination programmed to 12-h light (850 lux)/12-h dark periods. The 48-h exposure period covered the yolk-sac stage, thus making feeding unnecessary during the assay.

2.6. Statistical analysis

The EC_{50s} and 95% Confidence Intervals (CI) for *V. fischeri* data were determined using the Microtox Omni Windows software by graphing the log of the sample concentration *versus* the percentage of light decrease. The EC_{50s} (95% CI) for microalgae growth inhibition data were determined using the standard method as described by the ISO 10253 (2006) and the STATISTICA 7.0 software. This software was also used to test, through a *t*-test, the statistical significance of the difference between negative and solvent controls in the microalgae AZX assays. A value equal or inferior to 0.05 was considered statistically significant. The LC_{50s} (95% CI) from mortality records were determined by probit analysis (Probit 1.63 software).

The SSD curves and HC_{5s} were generated by the E_TX 2.1 software (Van Vlaardingen et al., 2004). This software computes hazardous concentrations assuming a lognormal distribution of the toxicity data using the methodology described by Aldenberg and Jaworska (2000). Lognormality of data was verified by the Anderson-Darling test included in the E_TX software package. Associated with hazardous concentrations, 95% and 50% CIs were also derived by setting the lower limit HC_5 ($LLHC_5$) and the median HC_5, respectively.

2.7. Freshwater toxicity data collection

Data on the toxicity of AZX to freshwater organisms were compiled from two main sources: scientific literature and the ECOTOX (http://cfpub.epa.gov/ecotox/) database. The ECOTOX database is an internationally recognized database regarded as one of the most reliable toxicity databases available (Cronin and Schulz, 2003). All gathered data are reported in Table 2, being the concentration range of values between 38 and 13,900 μg L^{-1}. The invertebrate copepoda *Eudiaptomus graciloides* and the planktonic species *Anabaena flosaquae* were the most and the least sensitive freshwater species to AZX, respectively. In order to avoid toxicity data overrepresentation of one particular species, and as recommended by Newman et al. (2000), the geometric mean was determined for the two *A. flosaquae* growth inhibition test results available. Hence, an $EC_{50,120h}$ = 13,443 μg L^{-1} was then considered for the SSD curve. Also, for *Navicula pelliculosa*, an $EC_{50,120h}$ of 85 μg L^{-1} was considered. Concerning the macrophyte *Lemna gibba*, the $EC_{50,14d}$ outcome is 3299 μg L^{-1}, and for the five *Dapnhia magna* $LC_{50,48h}$ available results, a $LC_{50,48h}$ of 176 μg L^{-1} was determined. Therefore, a total of 19 freshwater species: 1 fungi, 5 microalgae, 1 macrophyte, 7 invertebrates (cladocera, copepods and amphipods) and 5 fish, were used to generate the SSD curve.

2.8. Estimation of MAC-EQS values

Marine and freshwater MAC-EQS values for AZX and a marine MAC-EQS value for Ortiva were calculated in compliance with the European Commission (2011). MAC-EQS values for the freshwater pelagic community were calculated by dividing the lowest value of the toxicity dataset by 100, and from the median HC_5 value, which was divided by an uncertainty factor of 10. Concerning the marine pelagic community, since toxicity data of two additional specific taxonomic groups (bacteria and gastropoda) and more than two additional specific taxonomic groups (bacteria, bivalvia and gastropoda) are available for Ortiva and AZX datasets, respectively, an uncertainty factor of 100 was applied to the lowest value of each toxicity dataset. Moreover, for the MAC-EQS values which derived from median HC_5 values, an uncertainty factor of 10 was applied.

3. Results

3.1. Marine single-species short-term toxicity results

Results of the microalgae laboratory toxicity tests showed that, with the exception of *N. gaditana*, all the microalgae species tested

Table 2
Freshwater short-term toxicity data (μg L^{-1}) reported in literature for azoxystrobin.

Species	Group	Endpoint (exposure time)	EC_{50} and LC_{50} (95% CI)	Data source
Saprolegnia sp. strain JL	fungi, oomycota	growth inhibition (48 h)	212 (97–992)	Hu et al., 2013
Anabaena flosaquae	microalgae, blue-green	growth inhibition (120 h)	13,000 (12,000–14,000)	US-EPA, 1992
A. flosaquae			13,900	European Commission, 2009
Navicula pelliculosa	microalgae, bacillariophyceae		49 (43–58)	US-EPA, 1992
N. pelliculosa			146	European Commission, 2009
Pseudokirchneriella subcapitata	microalgae, chlorophyta		106 (92–121)	US-EPA, 1992
Selenastrum capricornutum		growth inhibition (96 h)	360	European Commission, 2009
Chlorella vulgaris			510 (440–600)	Liu et al., 2015
Lemna gibba	macrophyte	no. of fronds (14 d)	3400 (3000–3900)	Smyth et al., 1993
L. gibba			3200	European Commission, 2009
Daphnia galetea	invertebrate, cladocera	immobilization (48 h)	95	Lauridsen, 2003
D. magna, neonates			259 (126–644)	US-EPA, 1992
D. magna, neonates			340 (320–360)	Ochoa-Acuña et al., 2009
D. magna, clone Gammelmosen, neonates			71 (34–126)	Warming et al., 2009
D. magna, clone Herlev Gadekær, neonates			98 (66–139)	Warming et al., 2009
D. magna, clone Langedam, neonates			277 (145–427)	Warming et al., 2009
D. pulex			200	European Commission, 2009
Eudiaptomus graciloides	invertebrate, copepoda		38	Lauridsen, 2003
Macrocyclops fuscus			130	European Commission, 1998
Gammarus fossarum, adult males	invertebrate, amphipoda	mortality (7 d)	148 (128–169)	Zubrod et al., 2014
G. pulex, adults		mortality (96 h)	270 (170–450)	Beketov and Liess, 2008
Carassius auratus	fish, cyprinidae	mortality (48 h)	2712 (2314–3039)	Hu et al., 2013
Ctenopharyngodon idella, juveniles			549 (419–771)	Liu et al., 2013
Cyprinus carpio		mortality (96 h)	1600	European Commission, 2009
Oncorhynchus mykiss	fish, salmonidae		470 (400–580)	US-EPA, 1992
Lepomis macrochirus	fish, centrarchidae		1100 (900-1700)	US-EPA, 1992

CI: confidence interval.

showed no significant differences between negative and solvent control responses, with $P \geq 0.162$. Concerning *N. gaditana*, there were significant differences between negative and solvent control treatments ($P = 0.028$), but the percentage of difference was lower than 10% (8.6%) and was thus considered negligible. Therefore, the EC_{50} value for *N. gaditana* was considered in the AZX SSD curve (Table 3). The diatom *P. tricornutum* presented low sensitivity to AZX, showing no growth inhibition up to 5.9 mg L^{-1}, which is approximately the value of the AZX maximum solubility in water (6.7 mg L^{-1}, European Commission, 1998). Similarly, data from *T. weissflogii* and *R. lens* only allowed EC_{20} calculations. Therefore, for those species, in order to determine if Ortiva is more toxic than its active ingredient, the EC_{20} were calculated also for Ortiva. Thus, for *T. weissflogii*, the EC_{20} (95% CI) were 5.0 (3.9–6.0) mg L^{-1} and 2.6 (1.9–3.4) mg L^{-1} for AZX and Ortiva, respectively; while for *R. lens*, the EC_{20} (95% CI) were 4.7 (3.7–5.7) mg L^{-1} and 2.3 (2.2–2.4) mg L^{-1}, respectively. Table 3 comprises the EC_{50} values of the species which allowed data analysis.

In what concerns lethal tests, mortality was observed in the control of the *A. franciscana* assay and in the solvent control of the *S. senegalensis* assay. However, mortality was below the acceptance criterion of 10% in both assays. The rotifer *B. plicatilis* presented low sensitivity to both AZX and Ortiva, showing no mortality up to nominal concentrations of 6.8 mg L^{-1} and 6.2 mg L^{-1} for AZX and Ortiva, respectively. Therefore, no LC_{50s} were determined for this species. For all the other assays, time-dependent LC_{50} values are presented in Table 3. This table also includes AZX short-term toxicity data regarding marine species collected in literature. The

Table 3
Marine short-term toxicity data (μg L^{-1}) assessed in the present study, as well as data reported in literature, for azoxystrobin and Ortiva.

Species	Group	Endpoint (exposure time)	EC_{50} and LC_{50} (95% CI)		Data source
			AZX	Ortiva	
V. fischeri	bacteria	luminescence inhibition (5 min)	6961 (5856–8176)	868,681 (665,989–1,129,285)	present study
P. tricornutum	microalgae, bacillariophyceae	growth inhibition (72 h)	>5900	2997 (2757–3237)	present study
T. weissflogii			>5400	4309 (3333–5063)	present study
Skeletonema costatum			300	–	EFSA, 2010
R. lens	microalgae, cryptophyceae		>5600	2406 (2310–2502)	present study
N. gaditana	microalgae, eustigmatophyceae		298 (193–403)	243 (121–364)	present study
I. galbana	microalgae, haptophyceae		31 (24–38)	29 (24–33)	present study
B. plicatilis	invertebrate, rotifer	mortality (24 h)	>6800	>6200	present study
Chydorus sphaericus	invertebrate, cladocera	mortality (48 h)	370	–	Lauridsen, 2003
A. franciscana, larvae	invertebrate, artemiidae		345 (284–434)	1256 (1070–1496)	present study
Americamysis bahia, juveniles	invertebrate, mysidae	mortality (96 h)	56 (35–110)	–	Kent et al., 1993
Crassostrea gigas	invertebrate, bivalvia	mortality (48 h)	1300 (1100–1400)	–	US-EPA, 1992
R. parva, adults	invertebrate, gastropoda	mortality (96 h)	118 (100–140)	–	present study
G. umbilicalis, juveniles			13 (10–16)	17 (13–22)	present study
S. senegalensis, larvae	fish, soleidae	mortality (48 h)	698 (576–855)	1271 (1226–1318)	present study
Cyprinodon variegatus	fish, cyprinodontidae	mortality (96 h)	671 (560–800)	–	US-EPA, 1992
Sparus aurata, juveniles	fish, sparidae		729 (585–944)	–	Rodrigues et al., 2015

CI: confidence interval.

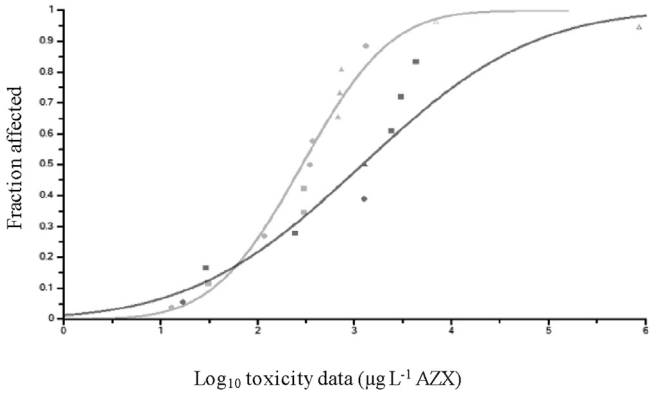

Fig. 1. Comparison of SSD curves for azoxystrobin active ingredient (light grey) and Ortiva commercial formulation (dark grey) for marine species (△ bacterium, ■ microalgae, ● invertebrates, ▲ fish).

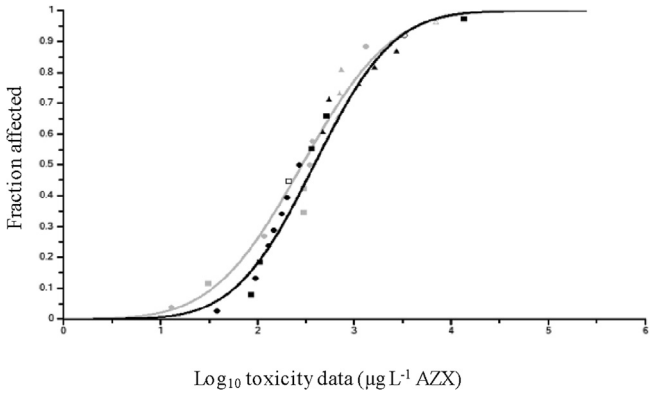

Fig. 2. Comparison of SSD curves for marine (light grey) and freshwater (black) species for azoxystrobin active ingredient (△ bacterium, □ fungi, ■ microalgae, ○ aquatic plant, ● invertebrates, ▲ fish).

range of values for the AZX toxicity dataset varies from 13 to 6961 µg L^{-1}, whereas the range of values is from 17 to 868,681 µg L^{-1} for the Ortiva toxicity dataset. In general, among the studied species, toxicity results showed several orders of magnitude for both AZX and Ortiva datasets. The marine invertebrate gastropod *G. umbilicalis* was the most sensitive species to both AZX and Ortiva. Conversely, the marine bacterium *V. fischeri* was the least sensitive species to both AZX and Ortiva (Table 3).

3.2. SSD results

As to cumulative frequency distributions for marine species, a total of 13 species (seven from this study and six gained from literature): 1 bacterium, 3 microalgae, 6 invertebrates (crustacea, bivalvia and gastropoda) and 3 fish, were used to generate the SSD curve for AZX, whereas a total of 9 species (all from this study): 1 bacterium, 5 microalgae, 2 invertebrates (crustacea and gastropoda) and 1 fish, were used to determine the curve for Ortiva. Even though parity was assured for bacteria, microalgae, crustaceans, gastropods and fish, as they were present in both AZX and Ortiva datasets, the same did not happen with bivalves, which were absent from the Ortiva dataset. Also, the representativeness of microalgae is greater in the Ortiva dataset, whereas invertebrate and fish representativeness is greater in the AZX dataset. Although some uncertainties remain over representativeness, a comparison of marine organisms' sensitivity to Ortiva and AZX was attempted by plotting both SSD curves in the same graph (Fig. 1). Results show that the response distributions cross each other, revealing that Ortiva affects the most sensitive species to a greater extent than AZX.

Regarding the comparison of sensitivity of marine and freshwater organisms to AZX, some lack of parity between both datasets should be highlighted, since an aquatic plant (*L. gibba*) is present in the freshwater dataset which do not exist in their counterpart. Nevertheless, the representativeness of microalgae, invertebrates and fish is similar. Hence, the marine and freshwater SSD curves for AZX are presented in Fig. 2 and show a systematic shift of both datasets with similar slopes. Results suggest that marine species are generally more sensitive to AZX than freshwater species.

3.3. HC_5 and MAC-EQS values

An overview of freshwater (AZX) and marine (AZX and Ortiva) lower limit and median HC_5 values derived from the SSD curves, as well as MAC-EQS values, are presented in Table 4.

4. Discussion

4.1. Determining whether the commercial formulation Ortiva is more toxic than AZX

The short-term effects exerted by AZX and Ortiva were studied in order to determine if Ortiva is more toxic to marine communities than its active ingredient. Concerning the effects of AZX on both diatoms *P. tricornutum* and *T. weissflogii*, and the cryptophyceae *R. lens*, as well as on the rotifer *B. plicatilis*, experimental testing concentrations as high as those required to obtain EC_{50s} or LC_{50s} were impractical due to the low sensitivity of these species. Nevertheless, such high concentrations are of limited ecological relevance since the maximum water concentration of AZX found in natural environments is 11 µg L^{-1} (Table 5). In order to compare the toxic effects of AZX and Ortiva, data evaluation from nine species of four different trophic groups (decomposers, primary producers and consumers, and secondary consumers) suggested that AZX and Ortiva provoke different levels of toxicity to marine species, depending on the species (high interspecific variability in sensitivity). For instance, results showed similar toxicities of AZX and Ortiva in two microalgae species (*N. gaditana* and *I. galbana*) and in the gastropod *G. umbilicalis*. However, in the cases of bacteria (*V. fischeri*), crustaceans (*A. franciscana*) and fish (*S. senegalensis*), toxicity was primarily due to the active ingredient. Conversely, for the generality of phytoplankton taxa (*P. tricornutum*, *T. weissflogii* and *R. lens*), Ortiva presented higher toxicity than AZX. Similar sensitivities to AZX and its commercial formulation Quilt® were also observed for *Bufo cognatus* tadpoles (Hooser et al., 2012). Nevertheless, the results of the present study suggest that the so-called 'inert' ingredients or the declared chemical propane-1,2-diol may have an influence on the toxicity of Ortiva within the microalgae group. In addition, regulatory attention should be drawn to the lower tails of the distributions presented in Fig. 1, where Ortiva is perceived as the one which affects the most sensitive species to a greater extent than AZX. The most sensitive species to both AZX and Ortiva was the gastropod *G. umbilicalis*, with LC_{50s} of 13 and 17 µg L^{-1}, respectively.

Using the SSD key tool to assess the ecotoxicological threat of AZX and Ortiva to marine biodiversity, the data gathered by the present study concluded that, in order to protect 95% of the species, the water concentration of AZX cannot exceed 3.5 µg L^{-1} ($LLHC_5$). This value should ensure low risk to marine organisms. However, regarding the Ortiva toxicity dataset, a much more protective concentration value was attained for marine environments

Table 4
Lower limit and median HC_5 values (μg L^{-1}) derived from the SSD curves, as well as water column MAC-EQS values (μg L^{-1}) calculated from both the lowest EC_{50} and median HC_{5s} values, for azoxystrobin. N, number of toxicity data points used to determine the lower limit and median HC_5 values.

		Freshwater AZX (N = 19)	Marine	
			AZX (N = 13)	Ortiva (N = 9)
Lower limit HC_5		11	3.5	0.078
Median HC_5		32	18	5.2
MAC-EQS	Lowest EC_{50}/100	0.38	0.13	0.17
	Median HC_5/10	3.2	1.8	0.52

Table 5
Azoxystrobin maximum concentration (μg L^{-1}) in natural water samples. Maximum concentration measured is highlighted.

Country	Location	Aquatic system	No. samples	Collecting period	Detection frequency (%)	Maximum concentration	Data source
US	13 States	Streams (29)	103	2005/2006	45	1.13	Battaglin et al., 2011
	Maine, Idaho, Wisconsin	Streams, ponds (12)	60	2009	58	0.06	Reilly et al., 2012
	Nebraska	Streams, ditches	92	July 2014	38	2.47	Mimbs et al., 2016
	Maine, Idaho, Wisconsin	Groundwater (12)	12	2009	17	0.0009[a]	Reilly et al., 2012
	Colorado, Montana,Wyoming	Lakes, creeks (15)	26	summer 2009	3.8	0.06	Keteles, 2011
	7 States	Amphibian habitat ponds	54	2009/2010	9.3	0.16	Smalling et al., 2012
Brazil	Neópolis, Sergipe	Surface, groundwater	26	October 2009	11.5	0.19	Filho et al., 2010
Norway	Agricultural areas	Surface water	–	July 2012	–	0.045	Petersen et al., 2015
Denmark	Experimental field sites	Surface water	450	2004–2009	24.4	1.4	Jørgensen et al., 2012
	Experimental field sites	Groundwater	1173	2004–2009	<1.0	0.01	Jørgensen et al., 2012
	Island of Funen	Streams (14)	–	April–August 2009	43	0.51	Rasmussen et al., 2012
Germany	**Braunschweig, Lower Saxony**	**Streams (20)**	–	**April, May, June 1998–2000**	–	**11**	**Liess and von der Ohe, 2005**
France	Lyon, Morcille catchment	Streams (1)	–	March 2007–March 2008	–	0.54	Rabiet et al., 2010
Portugal	Mondego estuary	Surface water (7 sites)	42	January 2010–January 2011	57	0.09	Cruzeiro et al., 2016a
	Tagus estuary	Surface water (7 sites)	–	April 2010–February 2011	90	0.02	Cruzeiro et al., 2016b
	Ria Formosa Lagoon	Surface water	–	2012/2013	100	0.16	Cruzeiro et al., 2015
Vietnam	Lower Mekong river delta	Surface water	11	March 2012–January 2013	66.3	2.41	Chau et al., 2015
Australia	Melbourne	Urban and peri-urban wetlands (24)	24	April 2010	8	0.178	Allinson et al., 2015

[a] Concentraion below the detection limit and estimated by authors.

($LLHC_5$ = 0.078 μg L^{-1}).

4.2. Comparing the sensitivity of marine and freshwater species to AZX

The AZX concentration settled by the freshwater SSD curve as the negligible risk level to organisms is 11 μg L^{-1} ($LLHC_5$). This value is about three times less protective than the one established, and above mentioned, for marine environments using the AZX $LLHC_5$, and much less protective (~140 ×) when considering the Ortiva $LLHC_5$. Also, despite some minor reservations with regard to parity, when comparing marine and freshwater species, the greater sensitivity of marine species to AZX was also highlighted by visually comparing both SSD curves (Fig. 2). The same conclusion was also highlighted by Del Signore et al. (2016), in their critical review of the SSD approach.

4.3. MAC-EQS values generated using SSD curves vs using the lowest EC_{50}

The MAC-EQS values determined by the present study allow us to conclude that EQSs obtained using different methodologies may vary and the ones based on the lowest EC_{50} were lower than those obtained through the SSD method for AZX (Table 4). Several studies corroborate this conclusion, as Jin et al. (2012) for 2,4,6-trichlorophenol and Nam et al. (2014) for gold (III) ion. Moreover, the MAC-EQS values derived from active ingredient and commercial formulation toxicity data, or from marine and freshwater toxicity data, may also vary. In general, the following ranking of environmental protection for AZX was attained: marine Ortiva > marine AZX > freshwater AZX. In the case of the MAC-EQS values extrapolated using the SSD approach, the difference between active ingredient and commercial formulation derivations is of about three times, and the difference between marine and freshwater derivations is of about two times for AZX and six times for Ortiva data. It should be noteworthy that these factors are not in line with the guidance document of the European Chemicals Agency for the derivation of marine no-effect levels based on freshwater data, which recommends a safety factor of 10 (ECHA, 2008, 2015).

4.4. Validation of a MAC-EQS value for AZX

Starting from the MAC-EQS values derived by the more scientifically robust SSD approach, the lowest EQS value obtained in the present study (0.52 μg L^{-1} derived from marine Ortiva toxicity data) should be the one considered in risk calculations for AZX. Therefore, a validation of this value by comparing it with annual average concentration (AA-EQS) values, which protect against the occurrence of a prolonged AZX exposure, was carried out. Ten long-term

NOEC values derived from freshwater species, covering from aquatic fungi to fish, were reported by Rodrigues et al. (2013), and the data ranged from 14 to >5000 μg L^{-1}. These data also have an associated uncertainty. Therefore, the lowest NOEC value was divided by an assessment factor of 10, according to the European Commission (2011), thus providing an AA-EQS of 1.4 μg L^{-1}. An AA-EQS for AZX of 0.95 μg L^{-1} was also reported by the Norwegian Institute for Water Research (Petersen et al., 2015). Accordingly, the MAC-QS generated by the Ortiva dataset (0.52 μg L^{-1}) is lower than 0.95 μg L^{-1} (the smallest AA-EQS), which makes little ecological sense. This is possible due to the low number of toxicity data points in the Ortiva dataset (N = 9) and/or due to the influence of the extremely insensitive *V. fisheri*. On the other hand, the second smallest MAC-QS value (Table 4), which was derived from marine AZX toxicity data (1.8 μg L^{-1}), is higher than the above mentioned AA-EQS. Thus, an aquatic MAC-EQS of 1.8 μg L^{-1} is validated and recommended for AZX. Moreover, in the present study, all the MAC-EQS values derived from the lowest EC_{50s} (EQSs ≤0.38 μg L^{-1}) are lower than the AA-EQS considered for validation (0.95 μg L^{-1}), thus being acknowledged as overprotective. Therefore, the SSD method was considered more suitable than the lowest EC_{50} to assess an aquatic MAC-EQS for AZX.

4.5. Linking aquatic effects and exposure

The retrospective aquatic risk characterization of AZX could be determined as the Risk Quotient (RQ), the ratio of measured environmental concentrations and its MAC-EQS value. Therefore, since the maximum concentration of AZX reported in natural waters is 11 μg L^{-1} (Table 5), it is possible to conclude that this pesticide currently poses potential high risk to aquatic organisms (RQ > 1).

5. Conclusion

The results of the present study contribute to the hazardous assessment of AZX by setting a MAC-EQS of 1.8 μg L^{-1} as a protective concentration value to protect against possible effects from short-term concentration peaks. Since this target value was derived from short-term marine AZX toxicity data, results highlight the importance of testing marine species. Gathered results also allow concluding that Ortiva affects the most sensitive marine species to a greater extent than AZX, and marine species are more sensitive than freshwater species to AZX.

Almost 15 years later (AZX was firstly presented at the Brighton Conference in November 1992), the retrospective aquatic risk characterization of AZX indicates that this pesticide currently poses potential high risk to aquatic organisms, thus allowing us to point out that regulatory prospective aquatic ecotoxicological studies might have to be improved before new pesticides are approved. Therefore, we suggest increasing the number of short-term data, and applying the SSD approach to the Tier 1 prospective Ecological Risk Assessment of pesticides in the aquatic environmental compartment.

Conflict of interest

There are no conflicts of interest to declare.

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