Ecotoxicology and Environmental Safety 149 (2018) 72–79

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

The ecotoxicity of binary mixtures of Imazamox and ionized ammonia on freshwater copepods: Implications for environmental risk assessment in groundwater bodies

Walter D. Di Marzio[a,*], Marco Cifoni[b,c], María E. Sáenz[a], Diana M.P. Galassi[b], Tiziana Di Lorenzo[c]

[a] Programa de Investigación en Ecotoxicología, Departamento de Ciencias Básicas, Universidad Nacional de Luján - Comisión Nacional de Investigaciones Científicas y Técnicas CONICET, Argentina
[b] Department of Life, Health and Environmental Sciences, University of L'Aquila, Via Vetoio 1, Coppito 67100, L'Aquila, Italy
[c] Institute of Ecosystem Study - CNR - National Research Council of Italy, Via Madonna del Piano 10, 50019 Sesto Fiorentino, Florence, Italy

ARTICLE INFO

Keywords:
Imazamox
Ammonia
Mixture
Copepods
Groundwater
Stygofauna

ABSTRACT

Groundwater bodies are impacted by substances such as pesticides and N-fertilizers, which usually occur in the environment as complex mixtures rather than isolated pollutants. The threat that these mixtures pose to groundwater-dwelling organisms is still poorly understood. The aims of the present study were to test the acute effect of a binary mixture of a herbicide (Imazamox) and NH_4^+ on epigean (*Eucyclops serrulatus*) and hypogean (*Diacyclops belgicus*) freshwater copepod species. In addition, to evaluate if the effect of the mixture can be explained by referencing non-interaction models or by more complex interaction models; and the implications for groundwater risk assessment. Compared with the action of the compounds evaluated separately, the effects of Imazamox and NH_4^+ in the binary mixture were more than additive or synergistic for both species. MixTox models evidenced a dose ratio and dose level deviations from concentration addition and independent action traditional models. The hypogean species was three times more sensitive to NH_4^+ that the epigean species when assayed as a single chemical. However, *D. belgicus* was only 1.13 times more sensitive than *E. serrulatus* when NH_4^+ was assayed in the mixture. The use of an integrated approach for substances that are known to interact in groundwater, should include copepods species as test organisms.

1. Introduction

Chemical pollution represents a threat to aquatic ecosystems, which poses a risk to aquatic organisms and human health (Malaj et al., 2014). According to WATERBASE, the European Environmental Agency database of EU fresh- and marine water bodies (www-eea.europa.eu/data-and-maps/data/waterbase-transitional-coastal-and-marine-waters-8), 34% of EU groundwater monitoring sites demonstrated ionized ammonia (NH_4^+) concentrations exceeding quality standards in the period 2000–2011 (EPA, 2010). Although the NH_4^+ concentration has been decreasing in EU surface water bodies in the last decades, it still remains higher than the natural level in several groundwater bodies as a result of agricultural treatments, such as crop fertilization by ammonia-N (NH_4^+ + NH_3) compounds (EEA, 2010, 2015). Pesticides, in association with N-fertilizers, have also been widely detected in surface and groundwater bodies at concentrations exceeding the current EU quality standard (0.1 μg/L; EEA, 2015). The Water Framework Directive (WFD, 2000/60/EC) requires setting environmental quality standards (EQSs) for substances of EU-wide concern (such as pesticides) and threshold values (TVs) for substances of national or local concern (such as NH_4^+). TVs are set by each Member State (EC, 2014a). According to Annex V, point 1.4.3 of the WFD, a waterbody is in good chemical status when the concentrations of pollutants, both as individual compounds and as compounds in mixtures, comply with the relative EQSs and TVs (EC, 2014b).

The Guidance Document n. 27 (TGD) is the official EU technical document for deriving both EQSs and TVs (EC, 2011). TGD highlights the importance of ecotoxicological data in this process (EC, 2011). The base set of taxa that TGD suggests using consists of algae and/or macrophytes, the cladoceran *Daphnia* and fish (EC, 2011). However, none of these taxa dwell in groundwater habitats (Gibert et al., 1994). For many years, several researchers have warned about using surface water species to infer the sensitivity of groundwater taxa due to relevant differences in their metabolism (Hose, 2007; Avramov et al., 2013; Di Lorenzo et al., 2014, 2015c). TGD also highlights the importance of deriving EQSs and TVs for mixtures of substances (EC, 2011). In

* Corresponding author.
E-mail addresses: ecotoxicologia@aae.org.ar, wdimarzio@conicet.gov.ar (W.D. Di Marzio).

https://doi.org/10.1016/j.ecoenv.2017.11.031
Received 7 May 2017; Received in revised form 2 November 2017; Accepted 10 November 2017

general, contaminants do not occur as isolated substances in groundwater but rather as complex mixtures. As scientific evidence indicates that the toxicity of a mixture of chemicals can be different from that of the individual compounds as a result of antagonistic or synergistic effects, EQSs and TVs for mixtures are necessary.

The objectives of the present study were: (i) to test the acute effect of a binary mixture (MIX) of a herbicide (Imazamox) and NH_4^+ on epigean and hypogean freshwater copepod species belonging to the same family (i.e., with a relatively close phylogenetic relationship); (ii) to determine if the effect detected in the MIX bioassay can be explained by reference non-interaction models, such as a concentration addition (CA) or an independent action (IA) model, or by more complex interaction models, such as those in which the two chemicals have synergistic or antagonistic effects; (iii) to discuss the implications for groundwater risk assessment.

We selected the copepod taxon because, with more than 1100 species living in groundwater, it is by far the most abundant and species-rich group in groundwater and associated ecosystems (Galassi, 2001). For the purposes of this study, we selected two Cyclopoida, Cyclopidae species, the hypogean *Diacyclops belgicus* and the epigean *Eucyclops serrulatus*.

Finally, we investigated the effect of the binary MIX of NH_4^+, dosed by ammonium nitrate, and the herbicide Imazamox, for the following reasons: 1) EU intensive cereal agriculture is known to be associated with a high use of N-fertilizers in the forms of ammonium nitrate and urea (Erisman et al., 2007), as well as herbicides such as Imazamox (ARPAP, 2014); 2) albeit NH_4^+ is in equilibrium with NH_3 in water depending on pH and temperature, NH_4^+ only is listed in the Annex II Part B of the Groundwater Daughter Directive – GDD − 2006/118/EC as a pollutant for which the Member States have to consider establishing TVs in accordance with the Article 3 of the GDD; 3) Imazamox application to wheat requires the addition of N-fertilizers, such as urea and ammonium nitrate (Geier and Stahlman, 2009); also the increased use of Clearfield® technology, which is based on the use of both the herbicide imazamox and resistant (IMI-R) sunflower, wheat, oilseed rape and rice hybrids, resulting in an increased IMA application (http://agronotizie.imagelinenetwork.com/materiali/Varie/File/syngenta2013/syngenta-girasole2-013.pdf) 4) information from environmental fate studies indicates that Imazamox does not persist in shallow surface waters, but the groundwater ubiquity score (GUS) index for imazamox suggests it poses a leaching risk (Milan et al., 2017) and could persist in environment with low oxygen concentrations (EFSA, 2016); and 5) gaps in the ecotoxicological data concerning the risk posed to aquatic organisms by Imazamox have been recently identified (EFSA, 2016). For these reasons, the environmental fate of imazamox deserves investigation, especially given that its use is likely to keep growing into the future (Milan et al., 2017).

2. Materials and methods

2.1. Test chemicals, test organisms and culture conditions

The experiments were carried out with a binary MIX of NH_4^+, dosed by ammonium nitrate (pure crystalline solid, CAS No. 6484-52-2) and the herbicide Imazamox (commercial formulation 3.7%, Beyond, BASF, Italy, CAS No. 114311-32-9). NH_4^+ concentrations were measured at the beginning and the end of the tests by Hach method #8038, adapted from Standard Methods for the Examination of Water and Wastewater 4500-NH3 B-C, using a DR3900 Hach spectrophotometer. The limit of detection was 20 μg/L. Imazamox is highly stable in water, at least within the assayed time period (USEPA, 2008). However, the nominal concentrations were confirmed at the end of each test by HPLC-UV according to Mastan et al. (2016). The system was equipped with a reversed phase C18 analytical column of 250 mm × 4.6 mm and a particle size of 5 μm (Phenomenex Luna-C18). The injected sample volume was 10 μL. The mobile phases were acetonitrile and 0.1% ortho phosphoric acid (30:70 v/v). The flow rate used was 1.2 mL/min. The detector wavelength was 254 nm. The limit of detection was 0.5 mg/L. pH was measured daily in the test chambers by Oakton 1100 pHmeter.

Test specimens were collected from two different shallow boreholes (B1 and B2) used for gardening on the campus of Consiglio Nazionale delle Ricerche - CNR in Florence (Italy), 300 m apart from each other, in April 2014. Water samples from both bores were tested for 32 chemicals to ascertain the requisite assurance that the test organisms were obtained directly from wild populations in relatively unpolluted areas. Concentrations of NH_4^+, nitrites, nitrates, heavy metals, inorganic pollutants, polycyclic aromatic hydrocarbons, pesticides, and volatile organic compounds were lower than the Italian legislative quality standards. In particular, NH_4^+ concentrations were < 0.01 mg/L in both bores, and the Imazamox concentration was below the limit of detection. The bores were open at the top (70 cm in diameter), allowing the mixing of rainwater with shallow groundwater. Dissolved organic carbon (DOC) was 1.1 and 1.0 mg/L in B1 and B2, respectively. Bacteria were present in approximately 10^6 prokaryotic cells/mL in both bores. Both bores (depth < 10 m) were situated in a shallow quaternary porous aquifer. A phreatobiological net sampler (mesh size 60 μm) was used to collect copepods from the bottom and the water column of the wells. The specimens were transported to the laboratory with the bore water in a cooling box within 10 min after collection. The copepods were sorted using a micropipette under a stereomicroscope at 12× magnification and separated into different groups according to macroscopic differences in morphology. Specimens of each group were then identified at the species level under an optical microscope at 100× magnification using the taxonomic key of Alekseev et al. (2006). Two different species were identified, namely *E. serrulatus* (epigean) and *D. belgicus* (hypogean). Groups of 25 individuals of each species were reared in 25-mL glass beakers in a standard water (Millipore® Milli-Q® deionized water re-mineralized with chemical grade: pH 7.4, hardness 80 mg/L, alkalinity 30 mg/L). They were kept in permanent darkness in a laboratory thermostatic cabinet (Pol-Eko-Aparatura Mod. ST 3) at 15 °C, corresponding to the mean annual temperature of the bore water, which was measured monthly in both bores using a multiparametric probe (ECM MultiTM; Dr. Lange GmbH, Düsseldorf, Germany).

Individuals of *E. serrulatus* can be easily maintained with a standardized algal diet in the laboratory, where they complete their full-life cycle (egg to adult to egg) in approximately 70 days at 15 °C, producing approximately 16 eggs per clutch per female and surviving up to 80 days. In contrast, individuals of *D. belgicus* must be maintained in the laboratory in glass beakers filled with bore water, allowing them to feed on a prokaryotic diet. The development rate of this species is unknown; however, the individuals collected in April 2014 remained alive for approximately one year in the laboratory. Only one female out of 16 produced egg-sacs (6 eggs per sac) within this period and no hatched nauplii were observed. Further information about the ecological features of stygobiotic copepods can be found in Galassi (2001). Due to these differences in the life cycles of the two species, after collecting and sorting, we acclimated 250 adult individuals of each species for the tests, in two different glass beakers (500 mL) with the standard water used for the stock cultures for 3 days prior to each ecotoxicological test. To cope with unexpected events, the number of acclimated organisms was 20% more than that required for the tests (200 organisms for each species). At the end of the tests, the unused organisms were maintained in laboratory stock cultures. Copepod fecal pellets are nutrient-enriched microenvironments that act as hotspots for microbial colonization and consume oxygen in the test vials (Di Lorenzo et al., 2015a). Accordingly, acute tests with copepods are usually performed without food (Di Lorenzo et al., 2014, 2015b, 2015c). However, starvation was required also during the 3-day acclimation in our trials because both *D. belgicus* and *E. serrulatus* produce fecal pellets for three days after stopping eating. For this reason, both species were deprived of food during acclimation to allow the guts to empty completely. The digestive tract was clearly visible at 60 × magnification under a Leica Microsystems M80

stereomicroscope. After this procedure, only actively swimming copepods were picked up by a glass pipette and selected for each bioassay.

2.2. Ecotoxicological testing

Prior to mixture trials, four single-chemical trials were carried out, two with *E. serrulatus* and two with *D. belgicus*, with either NH_4^+ or Imazamox, at 15 °C. Test conditions for the trials with single chemicals are fully explained in Di Lorenzo et al., (2014, 2015b). In brief, each trial consisted of four replicates. For each replicate, 5 nominal concentrations of the respective chemical were prepared by diluting the appropriate volumes of stock solutions (see Paragraph "Test chemicals, test organisms and culture conditions"). An appropriate control (no toxicant), was prepared for each replicate. The assays were carried out in 5-cm diameter sterile glass Petri dishes, each containing 15 mL of the appropriate solution and 5 individuals, for a total of 30 individuals per concentration. Organisms were maintained (temperature: 15 °C ± 0.3) for 96 h without mechanical aeration and food and in the darkness. A plastic cover was placed on the Petri dishes. Every 24 h, each replicate was checked for the presence of dead individuals (no movement after gentle stimulation by a sorting needle). Spasms were counted as deaths. The exposure solutions were not renewed during the test. At the end of the trials, the mortality responses in each replicate were cumulated per each concentration and used to determine the LC50 values at 96 h.

Two further trials (one with *E. serrulatus* and another one with *D. belgicus*) were carried out with mixtures of the two chemicals. For the mathematical formulation of the CA/IA models, a dimensionless concept called the toxic unit (TU) is usually used, which is defined as the ratio of the actual concentration (C) of a substance to the concentration that is needed to cause a certain effect, ECx (Backhaus et al., 2004). Usually, TU = C/LC. The final TUs to be assayed in this study were chosen to start from the LC50 values at 96 h of NH_4^+ and Imazamox at 15 °C that were previously reported for *E. serrulatus* and *D. belgicus* by Di Lorenzo et al., (2014, 2015b) and from the results of the single-chemical trials of this study (full data are reported as Supplementary File A). Due to the limitation of the number of groundwater organisms to assay, we tested nine concentrations within a TU range of 0.05– 3 (Jonker et al., 2011) in each replicate. Fully measured concentration values in mg/L and the equivalent TUs can be found as Supplementary File A online. Four replicates were performed for controls and the assayed concentrations. The assays were carried out in 5-cm diameter sterile Petri dishes, each containing 5 individuals. Organisms were maintained and checked as in the single-chemical trials. At the end of the trials, the mortality responses in each replicate were cumulated per each TU mix and used for data analysis.

3. Data analysis

3.1. Concentration–response curves (CRCs)

Concentration response functions which give the intensity of an effect as a function of a substance concentration, were determined by applying the best fit method described in Scholze et al. (2001). Five different non-linear regression models were fitted to each data set (Logistic, Weibull, Logit, Generalized Logit I and II), and then the best fitting one was selected by a robust goodness of fit criterion (sum of absolute errors). Finally, CRCs of the individual compounds and the corresponding MIX were fitted using the logistic model:

$$E = f(x) = \frac{a}{(1 + (x/b)^c)}, \tag{1}$$

where E is the effect or response, x the concentration of a toxicant, a is the upper response of the control when x is zero, b the EC50 value, and c is the slope parameter or Hill's slope. Data were fitted using the Newton algorithm to find the minimum of the sum of squares.

The inverse of Eq. (1) was used in the MIX data analysis:

$$x = b^*((a - E)/E)^{1/c}. \tag{2}$$

3.2. Calculation of mixture toxicity

The assessment of the MIX toxicity with non-interaction models was accomplished using concentration addition (CA) and independent action (IA) models.

The mathematical equation for the CA model is expressed according to Berenbaum (1985):

$$ECXmix = \left(\sum_{i=1}^{n} \frac{pi}{ECXi}\right)^{-1}, \tag{3}$$

where ECXmix is the effect of the MIX concentration eliciting X% toxic effect; ECXi denotes the effect of the concentration of the *i*th component of the *n*-compound MIX when acting individually; and p*i* is the molar concentration ratio of the *i*th component in the MIX.

The alternative model is IA, introduced by Bliss (1939), which assumes that the MIX components act dissimilarly. It is also known as response addition and can be formulated as

$$ECmix = 1 - \prod_{i=1}^{n} (1 - Eci), \tag{4}$$

where ECmix is the overall effect expressed as fractions of a maximum possible effect of a mixture composed of n chemicals, c*i* is the concentration of the *i*th compound in the MIX, and E*ci* describes the effect of the *i*-chemical if applied singly in a concentration c that corresponds to the concentration of that component in the MIX. For a binary MIX, Eq. (4) is equivalent to:

$$E_{A+B} = E_A + E_B - E_A{}^*E_B, \tag{5}$$

where E_A and E_B represent the fractional effects (ranging 0–1) caused by the individual toxicants A and B and E_{A+B} is the total effect of the MIX.

The choice of using the CA and/or IA models depends on the knowledge of the mode of action of the stressors. The CA model is based on the assumption that each component of the mixture possesses a similar mode of action, acting on the same biological pathways and the same molecular target. Conversely, the IA model assumes dissimilar actions of the mixture components, with interaction with different target molecule sites. If the mode of action is unknown or ambiguous, both models should be applied to predict the expected mixture effect (Ferreira et al., 2008). The physiological modes of action of NH_4^+ and Imazamox are different. However, since it is not clear how Imazamox exerts toxicity on copepods, data from the joint acute exposure to copepods were fitted to both the reference models (CA and IA) and to conceptual models according to Jonker et al. (2005). In real scenarios, MIX may occur in a variety of concentrations, so more complex response patterns need to be addressed, such as those producing more severe (synergism: S), or less severe (antagonism: A) effects, or those dependent on "dose level" (DL, different deviations at high and low concentrations) or "dose ratio" (DR, deviations differ from MIX composition) to assure the correct assumption of MIX effects (Jonker et al., 2005). These patterns can be characterized by quantifying how the observed data deviate from either reference model. We used a statistical approach that is fully explained in Jonker et al. (2005). Briefly, deviations from the reference CA and IA models, such as S/A, DL and DR, were obtained by the addition of two deviation parameters (a and b), forming a nested framework (MixTox models), as proposed by Jonker et al. (2005). Then, the data were fitted to the conceptual models or the deviations were compared using the maximum likelihood ratio (MLR), and the best fit was chosen. When a significant deviation was identified, the pattern of the effects could be deduced directly from the parameter values, and the maximum deviation could be calculated in terms of effect concentration (CA) or effect level (IA). The analyses of the

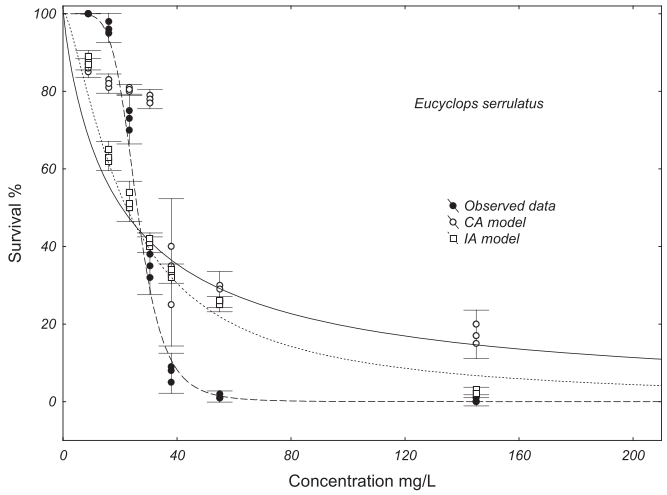

Fig. 1. The effects of the NH_4^+:IMA mixture on the survival of *Eucyclops serrulatus*. Observed data, modeled values and their 95% confidence intervals calculated using MixTox for the CA and IA models.

MixTox models were carried out using specially designed Solver spreadsheets in Microsoft Excel 2013 according Jonker et al. (2005). SigmaPlot version 13 was used to make 3D mesh and isobologram graphs. Nonlinear regressions were compared with a lack-of-fit F-test (Seefeldt et al., 1995).

4. Results

LC50–96h values for both species and chemicals, fell within LC50 ± 2 standard deviation compared with the original values presented by Di Lorenzo et al., (2014, 2015b).

The pH values during the mixture exposures ranged from 7.05 to 7.22 with an average of 7.15; and 7.11–7.32 average 7.19 for *E. serrulatus* and *D. belgicus*, respectively.

Survival data for single substances and measured concentrations for total ammonia and IMA are presented in Supplementary File A. The effects of the NH_4^+:IMA mixture on the survival of *E. serrulatus* and *D. belgicus* are shown in Figs. 1 and 2. The corresponding F test demonstrated fit for only the observed values and for both species and for the IA model with *E. serrulatus*. In addition, the MIX effects were characterized by quantifying how observed data deviated from either reference model. Additional parameters, which define the functional form

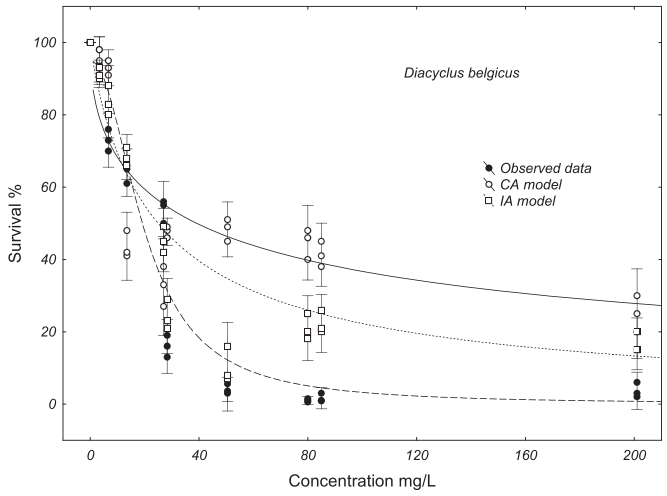

Fig. 2. The effects of the NH_4^+:IMA mixture on the survival of *Diacyclops belgicus*. Observed data, modeled values and their 95% confidence intervals calculated using MixTox for the CA and IA models.

of the deviation patterns, were substituted into the CA and IA reference models, as showed in Tables 1, 2. Only the data for *E. serrulatus* fit to the IA reference model, showing a lower sum of squared residuals (SS) with respect to the deviation patterns. With regards to *E. serrulatus*, after adding the *a* and *b* parameters, the SS value was almost the same for DR and DL deviation patterns from the independent action (IA). However, S/A deviation had a higher SS with respect to the IA reference model (Table 1 IA). Alternatively, considering the CA approach, a synergistic and a DR-dependent deviation were shown by MLR, with $p(\chi^2)$ of 0.00067 and 0.0076, respectively (Table 1 and Fig. 3). In this regard, the isobologram and 3D plots of Fig. 3A,B demonstrates a clear synergistic effect as a function of the DR between NH_4^+ and Imazamox. This DR behavior indicates an antagonistic effect when higher IMA concentrations (> 50 mg/L) are present, neutralizing the elevated NH_4^+ concentrations at levels higher than 30 mg/L. Under 50 mg of IMA/L, this effect is reduced, and a synergistic pattern appears, even at lower NH_4^+ concentrations (< 5 mg/L). In the case of *D. belgicus*, neither the CA nor the IA reference models fit at lower SS values. In contrast, a significant $p(\chi^2)$ value for the MLR test was registered (Table 2) under the S/A deviation model, indicating a synergistic effect when data were subject to the CA model. It is clearly observed in the isobologram and 3D plot of Fig. 3C,D. For analysis with the IA model, S/A and the DR deviation patterns were observed, showing decreased SS values and significant $p(\chi^2)$ for MLR tests (Table 2). The last point is not as evident or pronounced as for *E. serrulatus* when comparing the isobolograms and 3D plots from Figs. 3A,B and 3C,D.

To compare acute toxicity, specifically for NH_4^+ when it is mixed with IMA, we constructed concentration-response curves, taking into account the survival values obtained from the mixture assays and plotted them against the known separate concentrations. The LC50 values of the MIX and NH_4^+ for both species are shown in Figs. 4A and 4B. In the case of *E. serrulatus* the LC50-96h for NH_4^+ was 54.97 mg/L as single chemical and 3.06 mg/L in the NH_4^+-IMA mixture (Fig. 4A). Similarly, for *D. belgicus* (Fig. 4B), we observed that the LC50-96h for NH_4^+ was 16.19 mg/L as a single chemical and 2.71 mg/L when NH_4^+ was part of the mixture. This could indicate that the binary mixture induces a more-than-additive effect on the survival of freshwater copepods, as was highlighted under MixTox analysis. It can also be observed that the increase of toxicity in the MIX is close to one order of magnitude for both NH_4^+ and Imazamox on both species.

5. Discussion

The predicted values of mixture toxicity on the basis of CA and IA differed significantly from the observed values. The two reference models did not predict toxicity equally well, depending on the composition of the mixture and the concentrations of the compounds. For chemicals with different mechanisms of action and/or independent modes of action, as we assumed for NH_4^+ and IMA at the beginning of our experiments, IA models may be able to accurately predict the mixture toxicity. However, this was true just for *E. serrulatus* which showed a better correlation at lower concentration ratios. Alternatively, it is possible that the modes of action of the toxicity of IMA and NH_4^+ are not fully independent and that their interaction is pH-dependent, as discussed below. However, DL and DR also deviated from the IA model, demonstrating significant improvement of survival data (Table 1). In this regard, MixTox models are a useful tool to predict the toxicity of a mixture of two chemicals that do not have a specific mode of toxic action or whenever it is difficult to classify them as having a similar or independent mode of action. Our results would indicate that NH_4^+ and IMA have a more than additive behavior and it would be related with their dose ratio and dose level.

Imazamox belongs to the imidazolinone class of chemicals (EFSA, 2016). The herbicidal activity of the imidazolinones is through the inhibition of acetohydroxy acid synthase (AHAS), an enzyme only found in plants. AHAS is part of the biosynthetic pathway leading to the

Table 1
Summary of the analysis of the effect of NH_4^+ and Imazamox on the survival of *Eucyclops serrulatus*. μmax is the control response; β is the slope of the individual concentration–response curve; EC50 (mg/L) is the effective concentration at 96 hs; a, b_{dl}, and b_{NH4+} are parameters in the deviation functions; SS is the objective function; $\chi 2$ is the test statistic; df is the degrees of freedom; and $p(\chi 2)$ indicates the outcome of the likelihood ratio test. S/A is synergism/antagonism, DL is dose level–dependent deviation from the reference, and DR is dose ratio–dependent deviation from the reference. NC: not calculated.

	Concentration addition (CA)				Independent action (IA)			
	Reference	S/A	DL	DR	Reference	S/A	DL	DR
μ_{max} (%)	98.26	98.52	100.16	99.99	97.94	92.00	99.86	100.88
β_{NH4+}	0.63	1.66	4.56	5.80	1.29	16.71	1.06	2.67
β_{IMA}	2.97	3.30	3.76	3.80	3.08	7.02	3.03	2.94
$EC50_{NH4+}$	39.18	53.89	52.63	52.48	56.65	56.61	52.04	53.91
$EC50_{IMA}$	274.48	275.85	239.82	239.79	276.17	286.78	270.96	262.33
$SS \times 10^3$	3.25	1.13	1.85	2.09	0.29	4.15	0.23	0.38
a		−7.39	−9.44	−5.60		−22.25	20.68	−19.07
b_{NH4+}				−3.53				15.27
b_{dl}			−2.66				27.95	
χ^2		11.58	2.26	9.76		NC	11.53	9.65
Df		1	2	2		1	2	2
$p(\chi^2)$		**< 0.001**	0.32	**< 0.001**		NC	**< 0.001**	**< 0.001**

Table 2
Summary of the analysis of the effect of NH_4^+ and Imazamox on survival of *Diacyclops belgicus*. μmax is control response; β is the slope of the individual concentration–response curve; EC50 (in mg/L) is the median effect concentration; a, b_{dl}, and b_{NH4+} are parameters in the deviation functions; SS is the objective function; $\chi 2$ is the test statistic; df is the degrees of freedom; and $p(\chi 2)$ indicates the outcome of the likelihood ratio test. S/A is synergism/antagonism, DL is dose level–dependent deviation from the reference, and DR is dose ratio–-dependent deviation from the reference.

	Concentration addition (CA)				Independent action (IA)			
	Reference	S/A	DL	DR	Reference	S/A	DL	DR
μ_{max} (%)	97.12	100	104.24	104.18	114,20	95.36	101.38	95.24
β_{NH4+}	2.56	0.733	0.99	1.37	0,79	6.25	1.22	5.88
β_{IMA}	2.22	2.00	0.85	1.71	2,25	3.43	3.46	3.32
$EC50_{NH4+}$	15.75	21.08	31.27	28.69	3,85	17.99	11.17	17.93
$EC50_{IMA}$	181.83	180.23	180.34	180.47	154,65	194.38	185.92	193.57
$SS \times 10^3$	9.87	6.14	13.23	6.75	9.41	0.695	13.52	0.660
a		−6.71	−7.36	−3.98		−15.71	−0.046	−21.05
b_{NH4+}				−3.66				−8.70
b_{dl}			6.21				638.14	
χ^2		9.03	5.57	1.52		49.21	1.72	50.19
Df		1	2	2		1	2	2
$p(\chi^2)$		**< 0.001**	0.061	0.47		**< 0.001**	0.37	**< 0.001**

formation of branched-chain amino acids (Wersal and Madsen, 2007). Animals lack AHAS and this biosynthetic pathway. The lack of AHAS contributes to the low toxicity of Imazamox in vertebrates and invertebrates (EFSA, 2016). However, herbicides are known to affect non-target species, such as fish and crustaceans, due to alteration of metabolic, hematological and oxidative parameters. A modification of escape behavior has been observed in some copepod species exposed to herbicides (Gutierrez et al., 2010).

Because of the low toxicity of Imazamox and its metabolites, there would be no concern regarding the potential for cumulative effects of Imazamox and its degradation products with other substances with similar modes of action. As the LC50-96h values were higher than 100 mg/L for both *E. serrulatus* and *D. belgicus*, Imazamox is considered "practically non-toxic" to these two freshwater copepod species, according to the USEPA (2012). However, this toxic scenario changed when Imazamox was considered in the binary mixture with NH_4^+ due to synergistic effects. In the mixture with NH_4^+, Imazamox is considered "slightly toxic" to freshwater copepods, according to the USEPA (2012). The same logic can be applied to NH_4^+, which was "slightly toxic" as an individual compound and "moderately toxic" in the mixture with Imazamox. In attempting to explain or understand why the Imazamox-NH_4^+ mixture is more toxic than the two compounds taken singularly, we must first consider the LC50 values in the mixture, expressed as molar concentrations: 1.5/0.06 and 1.7/0.08 mM (NH_4^+/Imazamox) for *D. belgicus* and *E. serrulatus*, respectively. These values mean that there is an excess of NH_4^+ with respect to Imazamox in the mixture. The mobility of Imazamox across the cellular membrane is regulated by pH. The dissociation constant (pKa) reported for Imazamox reflects the ionization potential under typical environmental conditions. When the pH of a solution is equal to the pKa of a chemical, the chemical will be dispersed equally between the ionic and the non-ionic states. In general, the ionized forms of chemicals represent lower ecological risks because of limited penetration of cell membranes due to low lipid solubility. For weak acids, such as Imazamox, as the pH is elevated above the pKa, the proportion of the compound in an ionic state will change (Environ, 2012). In our assays, the pH was > 7. Therefore, Imazamox was present mainly as carboxylate and amide anions. These ions were likely neutralized in the mixture due to the excess of NH_4^+ cations facilitating the cellular uptake of both chemicals.

From an environmental risk assessment perspective, a main observation regarding the toxicity of the binary mixture of Imazamox and NH_4^+ resulted from our study. The differential sensitivity to NH_4^+ of the two species was evident when we compared the LC50 values for NH_4^+ assayed as single chemical (*D. belgicus* was three times more sensitive than *E. serrulatus*). The toxic effects of both NH_3 and NH_4^+ on aquatic crustaceans is exerted on a wide range of physiological processes such as osmoregulation, immunology, acid/base balance and gas exchange, the induction of oxidative stress, pathogenic susceptibility and histopathological damage (Romano and Zeng, 2013). From measured concentrations for total ammonia in mixtures exposures, we obtained a highest fraction of non-ionized form equivalent to 0.3 and

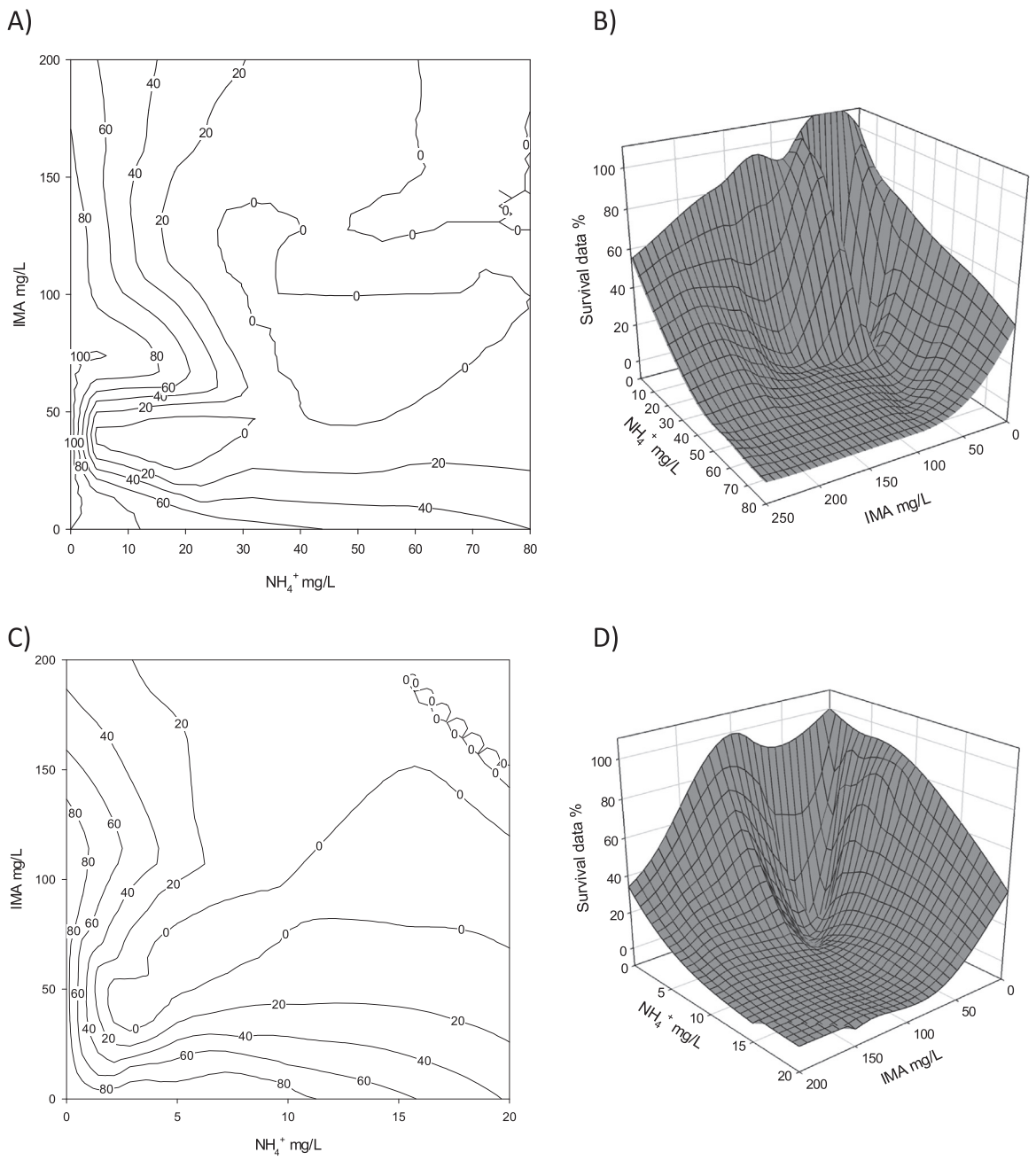

Fig. 3. The concentration–response relationship for the binary mixture of NH_4^+–IMA, showing a synergistic relationship, and the DR deviations from the CA model for the survival of *Eucyclops serrulatus*: (A) 2D isobolic surfaces and (B) 3D mesh and synergistic deviation from the CA/IA models for the survival of *Diacyclops belgicus*: (C) 2D isobolic surfaces and (D) 3D mesh. Concentrations are reported as nominal values; IMA: Imazamox.

0.08 mg/L for *E. serrulatus* and *D. belgicus*, respectively. The ammonia speciation varies markedly with T°C, pH and ionic strength, but this is more important in marine environments. In freshwater, this effect is much smaller than the effects of pH and temperature (Soderberg and Meade, 1991) and is sufficiently small compared to the typical uncertainty in LC50s and not affecting ammonia toxicity. It is well known that NH_3 is more toxic than NH_4^+, it was recognized when was observed that increased pH caused total ammonia to appear to be much more toxic. NH_3 is a neutral molecule which is able to diffuse across the epithelial membranes of aquatic organisms more readily than NH_4^+. High external concentrations of NH_3 reduce the diffusive gradients of internal excretion and cause the buildup of NH_3 in gill tissue and blood/hemolymph. Because of the importance of NH_3, it became a convention to express ammonia toxicity in terms of un-ionized form. However, ammonium ion can contribute significantly to ammonia toxicity under some conditions (USEPA, 1998). Observations that ammonia toxicity is relatively constant when expressed in terms of un-ionized ammonia come mainly from toxicity tests conducted at pH > 7.5. At lower pH, toxicity varies considerably when expressed in terms of un-ionized ammonia and under some conditions is relatively constant in terms of ammonium ion (Erickson, 1985). Also, studies have established that mechanisms exist for the transport of ammonium ion across gill epithelia (Wood, 1993), so this ion might contribute significantly to ammonia exchange across gills and affect the buildup of ammonia in tissues if its external concentration is sufficiently high. Thus, the very same arguments employed for the importance of unionized ammonia can also be applied in some degree to ammonium ion. This is not to say that ammonium ion is as toxic as unionized ammonia, but rather that, regardless of its lower toxicity, it can still be important because it is generally present in much greater concentrations than un-ionized

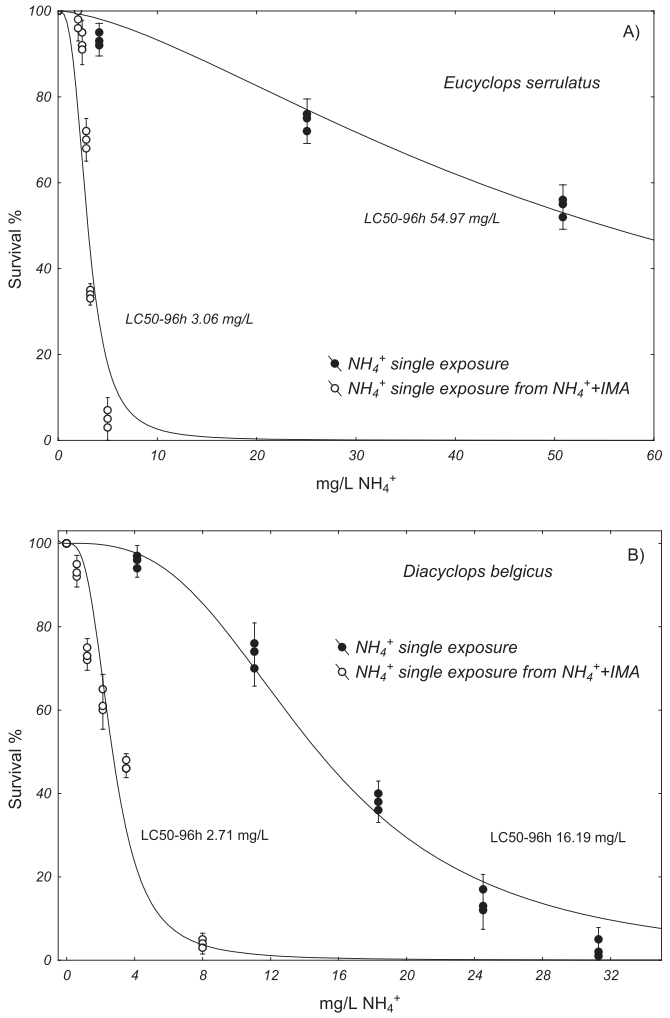

Fig. 4. LC50-96h for exposure to NH4$^+$ alone and in the binary NH_4^+-IMA mixture for *Eucyclops serrulatus* (A) and *Diacyclops belgicus* (B).

ammonia (USEPA, 1998). Also, when expressed in terms of un-ionized ammonia, ammonia toxicity is usually not constant with temperature, on average being about four-fold greater at 5 °C than at 25 °C for fish (Erickson, 1985). Because the relative amount of ammonium ion is also higher at low temperatures, this raises the possibility that ammonium ion might be in part responsible for this temperature dependence. Taking into account, the highest values measured for NH_3 in our experiences, related to the pH / T°C values, we could consider that the toxicity of total ammonia was a consequence of the highest proportion of the ionized form NH_4^+.

In crustaceans, NH_4^+ changes the hemolymph pH and affects the excretion rates (Romano and Zeng, 2013). The NH_4^+ form penetrates the cell membrane less rapidly than NH_3, which rapidly diffuses across the cell membrane via the lipid pathway (Wright, 1995). However, as NH_3 diffuses across the lipid bilayers of the cells, it is protonated to NH_4^+ (Romano and Zeng, 2013). NH_4^+ interferes with the activity of the Na^+/K^+-ATPase pump, which has a major role in NH_4^+ excretion in aquatic invertebrates. It was observed that NH_4^+ substitutes for K^+ in the activation of the ouabain-sensitive branchial Na^+/K^+-ATPase of the freshwater shrimp *Macrobrachium olfersii* (Wright, 1995). This substitution, hindered by high external NH_4^+ concentrations, alters the excretion rates in aquatic crustaceans, intoxicating the organisms. Di Marzio et al. (2009) concluded that chronic exposure to ammonium alters the post-naupliar development of freshwater copepods.

The sensitivities to NH_4^+ of the two species were similar when NH_4^+ was included in the mixture (*D. belgicus* was 1.13 times more sensitive than *E. serrulatus*). This result could be due to the different metabolic rates of the two species. One of the most striking adaptations of groundwater taxa is low metabolism, which makes hypogean species more sensitive than their epigean relatives to chemical stressors under acute exposure (Di Marzio et al., 2009; Reboleira et al., 2013; Di Lorenzo et al., 2014). The respiratory rates of *E. serrulatus* are five times higher than those of *D. belgicus* (Di Lorenzo et al., 2015c). According to other researchers (Avramov et al., 2013), the lower metabolism of hypogean species might delay the onset of defense mechanisms against a toxin and can be suggested as the reason for the higher sensitivity of *D. belgicus* to NH_4^+ tested alone (Avramov et al., 2013; Di Lorenzo et al., 2014). However, the difference in sensitivity between the two species was similar when they were exposed to the mixture. In this case, the lower metabolism of *D. belgicus* might have protected this species against the increased cellular uptake of both chemicals due to the neutralization of the ionic state of Imazamox. Even though it has not been documented yet, the line of argument suggests that metabolism should be considered in comparative ecotoxicological studies with ground- and surface water species. It is also important to identify whether such sensitivity patterns are also observed more generally across freshwater copepod species belonging to the two different ecological categories.

The relationship of the sensitivities between the two copepods species to the toxic substances observed in this study suggests that epigean copepod species can be included in the environmental risk assessment of groundwater bodies, at least when mixtures are concerned, or they can even be used in place of close hypogean relatives. This approach would provide several advantages, such as 1) reducing the impact of the collection required for toxicity testing on populations of groundwater copepod species, which are often strictly endemic and rare, as well as very old from a phylogenetic point of view (Galassi, 2001, and 2) using species that are more suitable to toxicity testing than the hypogean species due to short life cycles, high reproduction rates, suitability for handling in the laboratory and the ability to be collected in significant numbers throughout the year (Di Marzio et al., 2009).

Other consideration concerning environmental risk assessment, is how are calculated the threshold values for NH_4^+ in groundwater. The procedure adopted by the EU is clearly tailored for surface water bodies because it includes species that do not dwell in groundwater. In contrast, the procedure used in this study has the advantage of being based on copepods (one of which is a groundwater-dweller), which are the dominant taxa in groundwater ecosystems. Our results demonstrated that the environmental risk for NH_4^+ is increased when it is mixed with Imazamox. This indicates that the TV of NH_4^+ significantly underestimates the environmental risk to aquatic species of invertebrates, especially for the hypogean species. The approach used in this study matters if we consider the TVs of NH_4^+ that have been set up by the 28 EU Member States (MS): 7 MS have not yet provided TVs; Ireland has the most stringent value (0.083 mg/L); 4 MS have indicated TVs < 0.25 mg/L; 11 MS have provided a TV = 0.5 mg/L; and the remaining 6 MS have indicated TVs > > 0.5, with 4 of which being > 1.26 mg/L. This indicates that at least in 4 MS, the current TVs for NH_4^+ in groundwater are not adequate to protect groundwater fauna from acute exposure to this toxicant. The latter observation highlights the importance of including copepods in the definition of threshold values for groundwater pollutants.

6. Conclusions

For both the epigean and the hypogean copepod species, the effects of Imazamox and NH_4^+ in the binary mixture were more than additive or synergistic compared to the actions of these compounds separately, and it would be related with their dose ratios and dose levels. However, these effects were at IMA concentrations greater than those observed in the environment at the present. Both assayed species were more sensitive to NH_4^+ when it was mixed to the herbicide Imazamox. The

results from this study highlight that the EU approach for assessing quality standards for groundwater ecosystems need to be improved. The current EU TVs of NH_4^+ fails to protect groundwater copepods from acute exposure in at least four EU Member States. Based on our results, we suggest the use of an integrated approach, based on mixtures of pollutants, for substances that are known to interact in groundwater and to include copepod species as test organisms. Finally, chronic exposure to sublethal concentrations of the binary mixture of NH_4^+ and Imazamox needs to be investigated.

Acknowledgements

The project was funded by the European Community (LIFE12 BIO/IT/000231 AQUALIFE). We are indebted to two anonymous reviewers who provided comments that improved the MS.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2017.11.031.

References

Alekseev, V., Dumont, H.J., Pensaert, J., Baribwegure, D., Vanfleteren, J.R., 2006. A redescription of *Eucyclops serrulatus* (Fischer, 1851) (Crustacea: copepoda: Cyclopoida) and some related taxa, with a phylogeny of the *E. serrulatus*-group. Zoo Scripta 35. pp. 123–147.

ARPAP, 2014. Relazione monitoraggio anno 2013: attività ARPA nella gestione delle reti di monitoraggio regionale delle acque superficiali - corsi d'acqua e laghi. Agenzia Regionale per la Protezione dell'Ambiente, Piemonte, Italia.

Avramov, M., Schmidt, S.I., Griebler, C., 2013. A new bioassay for the ecotoxicological testing of VOCs on groundwater invertebrates and the effects of toluene on *Niphargus inopinatus*. Aquat. Toxicol. 130, 1–8.

Backhaus, T., Arrhenius, A., Blanck, H., 2004. Toxicity of a mixture of dissimilarly acting substances to natural algal communities: predictive power and limitations of independent action and concentration addition. Environ. Sci. Technol. 38, 6363–6370.

Berenbaum, M., 1985. The expected effect of a combination of agents: the general solution. J. Theor. Biol. 114, 413–431.

Bliss, C.I., 1939. The toxicity of poisons applied jointly. Ann. Appl. Biol. 26, 585–615.

Di Lorenzo, T., Di Marzio, W.D., Sáenz, M.E., Baratti, M., Dedonno, A.A., Iannucci, A., Cannicci, S., Messana, G., Galassi, D.M.P., 2014. Sensitivity of hypogean and epigean freshwater copepods to agricultural pollutants. Environ. Sci. Pollut. Res. 21, 4643–4655.

Di Lorenzo, T., Cifoni, M., Lombardo, P., Fiasca, B., Galassi, D.M.P., 2015a. Ammonium threshold values for groundwater quality in the EU may not protect groundwater fauna: evidence from an alluvial aquifer in Italy. Hydrobiologia 743, 139–150.

Di Lorenzo, T., Di Marzio, W.D., Cifoni, M., Fiasca, B., Baratti, M., Sáenz, M.E., Galassi, D.M.P., 2015b. Temperature effect on the sensitivity of the copepod *Eucyclops serrulatus* (Crustacea, Copepoda, Cyclopoida) to agricultural pollutants in the hyporheic zone. Curr. Zool. 61, 629–640.

Di Lorenzo, T., Di Marzio, W.D., Spigoli, D., Baratti, M., Messana, G., Cannicci, S., Galassi, D.M.P., 2015c. Metabolic rates of a hypogean and an epigean species of copepod in an alluvial aquifer. Freshw. Biol. 60, 426–435.

Di Marzio, W.D., Castaldo, D., Pantani, C., Di Cioccio, A., Di Lorenzo, T., Sáenz, M.E., Galassi, D.M.P., 2009. Relative sensitivity of hyporheic copepods to chemicals. Bull. Environ. Contam. Toxicol. 82, 488–491.

EC, Technical Guidance for deriving Environmental Quality Standards. Guidance Document No. 27, 2011 Common Implementation Strategy for the Water Framework Directive (2000/60/EC). Technical Report 2011–2055. European Commission, Brussels, Belgium.

EC, 2014a. Environment. Water. Strategies against chemical pollution of surface waters. European Commission, Brussels, Belgium (cited 2015 January 4). (Available from). ⟨http://ec.europa.eu/environment/water/water-dangersub/index.htm⟩.

EC, 2014b. Environment. Water. Priority substances under the Water Framework Directive. Ecological Effects Characterization. European Commissionm Brussels, Belgium (cited 2012 May 9). (Available from). ⟨http://ec.europa.eu/environment/water/water-dangersub/⟩.

EEA, 2010. The European Environment-State and Outlook 2010. Synthesis Report. European Environmental Agency. Publications Office of the European Union, Copenhagen, Luxembourg.

EEA, 2015b. The European Environment-State and Outlook 2015. Synthesis Report. European Environment Agency. Publications Office of the European Union, Copenhagen. Luxembourg.

EFSA, 2016. Technical report. peer review of the pesticide risk assessment of the active substance imazamox. EFSA J. 14 (4), 4432. http://dx.doi.org/10.2903/j.efsa.2016.4432.

Environ,. Screening-Level Ecological Risk Assessment of the Proposed Use of the Herbicide Imazamox to Control Invasive Japanese Eelgrass (*Zostera japonica*) in Willapa Bay, Washington State. Technical Report. ENVIRON International Corporation Seattle, 2012, Washington.

EPA, 2010. Parameters of water quality. Interpretation and standards. Technical Report. Environmental Protection Agency, Ireland. [cited2015 January 24]. Available from: ⟨https://www.epa.ie/pubs/advice/water/quality/Water_Quality.pdf⟩.

Erickson, R.J., 1985. An evaluation of mathematical models for the effects of pH and temperature on ammonia toxicity to aquatic organisms. Water Res. 19, 1047–1058.

Erisman, J.W., Bleeker, A., Galloway, J., Sutton, M.S., 2007. Reduced nitrogen in ecology and the environment. Environ. Pollut. 150, 140–149.

Ferreira, A.L.G., Loureiro, S., Soares, A.M.V.M., 2008. Toxicity prediction of binary combinations of cadmium, carbendazim and low dissolved oxygen on *Daphnia magna*. Aquat. Toxicol. 89, 28–39.

Galassi, D.M.P., 2001. Groundwater copepods (Crustacea: Copepoda): diversity patterns over ecological and evolutionary scales. Hydrobiologia 453, 227–253.

Geier, W., Stahlman, P.W., 2009. Nitrogen concentration and application timing affect Imazamox efficacy in winter wheat. *Crop Prot.* 28 (7):573-576.22 MDA-MDE. 2014. Imazamox. Technical report. Massachusetts Department of Agriculture Division of Crop and Pest Services and Massachusetts Department of Environmental Protection Office of Research and Standards, Massachussets.

Gibert, J., Danielopol, D.L., Stanford, J.A., 1994. Groundwater Ecology. Academic Press, San Diego, pp. 571.

Gutierrez, F.M., Gagneten, A.M., Paggi, J.C., 2010. Copper and chromium alter life cycle variables and the equiproportional development of the freshwater copepod *Notodiaptomus conifer* (SARS). Water Air Soil Poll. 23, 275–286.

Hose, C.G., 2007. Response to comments on assessing the need for groundwater quality guidelines for pesticides using the species sensitivity distribution approach. Hum. Ecol. Risk Assess. 13, 241–246.

Jonker, M.J., Gerhardt, A., Backhaus, T., van Gestel, C.A.M., 2011. Test design, mixture characterization, and data evaluation. In: van Gestel, C.A.M., Jonker, M.K., Kammenga, J.E., Laskowski, R., Svendsen, C. (Eds.), Mixture Toxicity. CRC Press, Pensacola.

Jonker, M.J., Svendsen, C., Bedaux, J.J., Bongers, M., Kammenga, J.E., 2005. Significance testing of synergistic/antagonistic, dose level-dependent, or dose ratio dependent effects in mixture dose-response analysis. Environ. Toxicol. Chem. 24, 2701–2713.

Malaj, E., von der Ohe, P.C., Grote, M., Kühne, R., Mondy, C.P., Usseglio-Polatera, P., Brack, W., Ralf, B., Schäfer, R.B., 2014. Organic chemicals jeopardize the health of freshwater ecosystems on the continental scale. PNAS 111 (26), 9549–9554.

Mastan, J., Srinivas, B.N., Nageswara, R.T., Apparao, K., 2016. Determination of Imazamox and Imazethapyr Herbicide residues in soybean oil. Der Pharma Chem. 8, 102–107.

Milan, M., Ferrero, A., Fogliatto, S., De Palo, F., Vidotto, F., 2017. Imazamox dissipation in two rice management systems. J. Agric. Sci. 155, 431–443.

Reboleira, A.S., Abrantes, N., Oromì, P., Gonçalves, F., 2013. Acute toxicity of copper sulfate and potassium dichromate on stygobiont Proasellus: general aspects of groundwater ecotoxicological and future perspective. Water Air Soil Pollut. 224, 15–50.

Romano, N., Zeng, C., 2013. Toxic effects of ammonia, nitrite, and nitrate to decapod crustaceans: a review on factors influencing their toxicity, physiological consequences, and coping mechanisms. Rev. Fish. Sci. 21, 1–21.

Seefeldt, S.S., Jensen, J.E., Fuerst, E., 1995. Log-logistic analysis of herbicide dose–response relationships. Weed Technol. 9, 218–227.

Scholze, M., Boedeker, W., Faust, M., Backhaus, T., Altenburger, R., Grimme, L.H., 2001. A general best-fit method for concentration-response curves and the estimation of low-effect concentrations. Environ. Toxicol. Chem. 20, 448–457.

Soderberg, R.W., Meade, J.W., 1991. The Effects of Ionic Strength on Un-ionized Ammonia Concentration. Prog. Fish.-Cult. 53, 118–120.

USEPA, 1998. Update of Ambient Water Quality Criteria for Ammonia. U.S. Environmental Protection Agency, Office of Water, NW, Washington DC.

USEPA, 2008. Environmental Fate and Effects Science Chapter: environmental Fate and Ecological Risk Assessment: registration of New Use Imazamox for the proposed new use for the control of weed in Clearfield rice (imidazolinone-tolerant rice). U.S. Environmental Protection Agency, Washington, DC.

USEPA, 2012. Technical Overview of Ecological Risk Assessment - Analysis Phase: Ecological Effects Characterization. Environmental Protection Agency. Office of Pesticide Programs, Washington DC (Available from). ⟨http://www.epa.gov/pesticide-science-and-assessing-pesticide-risks/technical-overview-ecological-risk-assessment-0#main-content⟩.

Wersal, R.M., Madsen, J.D., 2007. Comparison of Imazapyr and Imazamox for control of parrotfeather (*Myriophyllum aquaticum* (Vell.) Verdc.). J. Aquat. Plant Manag. 45, 132–136.

Wright, P.A., 1995. Nitrogen excretion: three end products, many physiological roles. J. Exp. Biol. 273, 273–281.

Wood, C.M., 1993. Ammonia and urea metabolism and excretion. In: Evans, D.H. (Ed.), The Physiology of Fishes. CRC Press, Boca Raton, FL., pp. 379–425.