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Acute toxicity of selected organic pollutants to saltwater (mysid *Siriella armata*) and freshwater (cladoceran *Daphnia magna*) ecotoxicological models

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Abstract The acute toxicity of three organophosphate pesticides (diazinon, chlorpyrifos, pirimiphos-methyl) two chlorinated biocides (endosulfan, pentachlorophenol) and a nonsteroidal anti-inflammatory drug (diclofenac) was tested on <24 h neonates of saltwater mysidacea (*Siriella armata*) in laboratory acute toxicity test. The 50 and 10 % lethal effective concentrations (LC_{50} and LC_{10}), NOEC and LOEC values were calculated. The three organophosphate pesticides showed an apparently biphasic dose–response profile, supporting that its mechanism of action in crustaceans differ from other organic compounds. The biphasic pattern of response was confirmed using the common aquatic ecotoxicological model *Daphnia magna*. According to the 96-h LC_{50} values for *S. armata*, the ranking of toxicity was chlorpyrifos (0.13 µg/L) < pirimiphos-methyl (1.3 µg/L) < endosulfan (3.2 µg/L) < diazinon (4.03 µg/L) < pentachlorophenol (262.2 µg/L) < diclofenac (2919 µg/L). In general, mysids resulted at least one order of magnitude more sensitive than model daphnia, which stresses the need for using marine species for the derivation of seawater quality standards.

Keywords Mysidacea · *Siriella armata* · *Daphnia magna* · Ecotoxicology · Insecticides · Pharmaceuticals

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Introduction

Synthetic organic pesticides are still intensively used in agriculture and, despite the replacement of persistent by non-persistent compounds, they continue to pose a risk to aquatic ecosystems because of their high selective toxicity to certain non-target species (e.g. Bellas et al. 2005; Palma et al. 2008; Mhadhbi and Beiras 2012). Recently, the detection of a wide variety of pharmaceutical products in measurable concentrations has also become a new environmental problem (Ferrari et al. 2003; Haap et al. 2008).

Insecticides are a large group of compounds, and the great majority of them are nerve poisons. Among them, organophosphorus (OPs) work through the inhibition of acetylcholinesterase (AChE), an enzyme essential for the correct transmission of the nerve impulse in the central nervous system. *Chlorpyrifos* (*O*,*O*-diethyl-*O*-[3,5,6,-trichloro-2-pyridyl] phosphorothioate), *pirimiphos-methyl* (*O*-[2-diethylamino-6-methylpyrimidine-4-yl] *O*,*O*-dimethyl phosphorothioate) and *diazinon* (*O*,*O*-diethyl-*O*[2-isopropyl-6-methylpyridimidinyl] phosphorothioate) are among the most common OPs. Insecticides represent 17 % of total pesticides used in the rural region of Galicia (NW Iberian Peninsula), with the OPs insecticides chlorpyrifos, pirimiphos-methyl and diazinon comprising the most use, with values around 28, 8 and 6 Tn/year respectively (AEPLA, pers. comm. 2007).

Within these compounds, pirimiphos-methyl and chlorpyrifos remain as authorized active substances by Regulation (EC) No 1107/2009, its implementation (EC) No 540/2011, and their subsequent amendments, although Directive 2013/39/EC has classified the latter as priority substance.

Cyclodiene organochlorines (OCs) are pesticides that affect synaptic transmissions by acting gamma aminobutyric acid (GABA) gated chloride channel antagonists.

Inside this group, *Endosulfan* (6,7,8,9,10,10-Hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepine-3-oxide) has been widely used in agriculture as a plant protection product. It is currently catalogued as a hazardous substance by the European Water Framework Directive and priority substance by Directive 2013/39/EC but its effects continue to raise environmental concern due to extensive use in other countries and its persistence, potential of bioaccumulation and high toxicity. The 2,3,4,5,6-pentachlorophenol (*PCP*), an herbicide, disinfectant and antimicrobial agent has been added in the last decades to adhesives, paints, paper coatings, food cans, and storage containers, and is used in leather tanning or paper production (Carrizo et al. 2008). PCP has been recognized as a persistent priority pollutant in the United States, Europe and China, classified as priority substance by Directive 2013/39/EC and carcinogen according to the International Agency for Research on Cancer (IARC) (Jin et al. 2012; Xing et al. 2012), and its use is restricted by the REACH (EC 1907/2006) at concentrations lower than 0.1 % by mass in substances or preparations placed on the market.

Among pharmaceuticals detected in the environment, nonsteroidal anti-inflammatory drugs (NSAIDs) are one of the most representative groups worldwide. Their mechanism of action is based on the inhibition of the synthesis of prostaglandin, involved in the process of inflammation, by the inhibition of cyclooxygenases. In particular, the anti-inflammatory, antipyretic, and analgesic drug *diclofenac* (2-(2-(2,6-dichlorophenylamino) phenyl)acetic acid), widely used in humans and animals to reduce inflammation and pain, was one of the most important active synthetic compounds present in the water cycle (Cleuvers 2004). Diclofenac and two other pharmaceuticals were identified by the European Parliament as new substances of environmental concern for which environmental quality standards (EQS) should be set (Decision EP-PE_TC1-COD (2011)0429, of the European Parliament), but due to unexplained reasons the ulterior revision of priority substances (Directive 2013/39/EU) did not include those pharmaceuticals.

It is well documented that organisms with a more cephalized nervous system, such as crustaceans or chordates, show higher sensitivity to neurotoxic drugs compared to other marine taxa frequently used in ecotoxicology such as bivalves or echinoderms (Roast et al. 1999; Bailey et al. 2001; Bellas et al. 2005; Montagna and Collins 2007). In that way, crustaceans are one of the taxonomic groups with more relevance among the different biological models used in ecotoxicology. *Daphnia magna i*s a freshwater cladoceran crustacean widely used as an aquatic toxicological model because of its wide distribution, ecological relevance, easy handling and rapid parthenogenetic reproduction, and its high sensitivity to organic and inorganic chemicals is well documented (Adema 1978; Villegas-Navarro et al. 1999; Sarma and Nandini 2006). Mysids are among the most used biological models for marine toxicity tests (ASTM 1998). The mysidacea *Siriella armata* is a hyperbenthic saltwater crustacean with a distribution ranging from the North Sea to the Mediterranean, which lives in shallow waters, between 0 and 30 m depth. Due to its widespread distribution, abundance, availability throughout the year, ease of handling, short life cycle and sensitivity, *S. armata* is a suitable species to be used in ecotoxicological bioassays (Pérez and Beiras 2010).

Risk assessment evaluates the probability that adverse ecological effects occur as a result of one or more harmful agents, and it has been incorporated into European environmental legislation, (Directive 2000/60/CE; REACH 1907/2006). This legislation establishes a basic level of assessment which demands the determination by acute toxicity testing of the EC_{50} in species belonging to three trophic levels. In marine ecotoxicology, standard toxicity tests with microalgae (primary producers) (EPA 2002, ISO 10253:2006; OECD 2006) and early life stages of sea urchins and bivalves (primary consumers) (ASTM 1995, 2004) are available but there is a lack of standard tests for secondary consumers representative of marine ecosystems.

The aim of this study was to evaluate the acute toxicity of diazinon, chlorpyrifos, pirimiphos-methyl, endosulfan, PCP and diclofenac on *S. armata* in order to validate the use of this species in prospective risk assessments in marine environments.

Moreover, for the two most toxic compounds in mysids, acute tests were carried out in *D. magna*, a freshwater ecotoxicological crustacean model widely used as a biological tool to assess environmental quality, evaluating the use of bioassays with marine organisms in the development of EQSs.

Materials and methods

Biological material

Swarms of *S. armata* were collected by divers using a hand net in Ría de Vigo (Galicia, NW Iberian Peninsula). Organisms were carried to *Estación de Ciencias Mariñas de Toralla* (ECIMAT) and maintained in 100 L polyvinyl chloride (PVC) tanks with circulating sand-filtered seawater, with 100 % volume exchange every 50 min. Every day nauplii or metanauplii of *Artemia salina* were added and mysids fed ad libitum. Daily, temperature (ranged between 17 and 18 °C) salinity (from 34.4 to 35.9 %) and oxygen (>6 mg/L) were checked daily.

Daphnids are cultured in ECIMAT since 2007 for their use as bioassay organisms using standard methods (UNE-EN ISO 6341:1996; USEPA 2002). A 20 °C isothermal room with 16 h light: 8 h dark photoperiod was used, where 50 daphnids per 4 L plastic jar with reconstituted hard water (RHW; hardness between 160 and 180 mg $CaCO_3$/L) were fed 3×10^6 cells of *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*) every second day, after water renewal.

Experimental solutions

The acute toxicity of three organophosphate pesticides (diazinon, chlorpyrifos, pirimiphos-methyl) two chlorinated pesticides (endosulfan, PCP) and one nonsteroidal anti-inflammatory drug (diclofenac) were tested. Stock solutions were prepared in analytical grade using dimethyl sulfoxide (DMSO) as a carrier. Selected experimental concentrations were prepared in *S. armata* tests by addition of adequate volumes of the stock solution to 0.22 µm filtered seawater (FSW) of oceanic characteristics, and to RHW prepared with Milli-Q water in case of daphnids. During this dilution, equal amounts of DMSO (maximum of 400 µL/L in both cases), found not to be toxic in preliminary testing, were added for each concentration. All testing materials were rinsed with acetone, washed up to 24 h in acid–water (HNO_3 7 %) and cleaned with distilled water before their use.

Experimental concentrations were chosen after previous range-finding trials, and taking also into account the solubility limits, in order to avoid working above those limits. Mysids were tested from 0.7 to 400 µg/L for diazinon, 0.01 to 40 µg/L for pirimiphos-methyl, 2.05 to 1500 ng/L for chlorpyrifos, 0.01 to 100 µg/L for endosulfan, 12.5 to 1600 µg/L for PCP and 0.25 to 20 mg/L for diclofenac. For daphnids, concentrations from 0.01 to 8000 µg/L for pirimiphos-methyl and 0.001 to 3600 µg/L for chlorpyrifos were tested.

In all the bioassays, at least six concentrations plus controls were used. Both FSW (or RHW in case of *D. magna*) and the highest carrier solvent concentration used to prepare the experimental concentrations were used as controls.

Experimental procedure

Following the method proposed by Pérez and Beiras (2010), *S. armata* mature females bearing embryos in the last post-nauploid phase (Cuzin-Roudy and Tchernigovtzeef 1985) were separated 1 day before the start of the test in order to obtain neonates <24 h old the next day. We used 0.45 dm^3 individual PVC chambers to avoid cannibalism and allow females to release new-born mysids. The chambers were closed at the bottom with 150 µm mesh, half-submerged into the main tank and well-aerated. After 24 h neonates were collected and the test was initiated. Experimental solutions were distributed in 20 mL glass vials, using a total of 20 replicates in each concentration, and one neonate per vial was added. When the number of organisms was limited, less replicates were used per concentration, always ensuring a minimum of 15.

Using standard methods for acute toxicity bioassays (UNE-EN ISO 6341:1996; USEPA 2002) *D. magna* mature females were separated 1 day before the start of the test, to collect neonates after 24 h. Experimental solutions were distributed in 25 mL glass vials, and ten <24 h daphnids per replicate and four replicates per treatment were used. In both bioassays, vials were closed by a top of polytetrafluoroethylene (Teflon) and they were incubated in an isothermal room at 20 °C and 16 h light: 8 h dark photoperiod.

Oxygen concentration, pH, salinity (in *S. armata* tests) and water hardness (in *D. magna* tests) were determined at the beginning of each test.

Daphnids mortality was recorded after 48 h, and animals were not fed during the test. For mysids, a 96 h exposure time was selected according to Pérez and Beiras (2010), although they were counted after 24, 48, 72 and 96 h in order to observe variations in the effects with time. The 96-h acute test seems to be the best predictor of demographic response for the mysid population (Kuhn et al. 2000). Mysids were fed 10–15 nauplii of 24–48 h post-hatch *A. salina* daily.

Mathematical models

The modified Weibull model was used as the dose–response function (Rial et al. 2010):

$$R = K\left\{1 - \exp\left[-\ln 2\left(\frac{D}{m}\right)^{\alpha}\right]\right\} \quad (1)$$

where R is the response (with K as the maximum value), m is the dose corresponding to the semi-maximum response (i.e., the LC_{50} when $K = 1$) and is a shape parameter related to the maximum slope of the response.

To obtain directly the confidence intervals (CI) of doses with responses differing from the semi-maximum response, Eq. 1 was reparametrized to make explicit the corresponding dose:

$$R = K\left\{1 - \exp\left[\ln\left(1 - \frac{X}{K}\right)\left(\frac{D}{LC_x}\right)^{a}\right]\right\} \quad (2)$$

where X is the level of response, in our case 0.1 for the LC_{10} and 0.5 for the LC_{50}. In those cases where the dose:response curve showed a biphasic profile it was fitted

to a model consisting of the sum of two sigmoid equations (Murado et al. 2011):

$$R = K_1\left\{1 - \exp\left[-\ln 2\left(\frac{D}{m_1}\right)^{a_1}\right]\right\} + K_2\left\{1 - \exp\left[-\ln 2\left(\frac{D}{m_2}\right)^{a_2}\right]\right\} \quad (3)$$

This allows the separate estimation of the m values for each of the effectors 1 and 2 (m_1 and m_2) as well as the LC_{10} and LC_{50} values.

Statistical analyses

Both LC_{50} and LC_{10} values (estimated concentrations that cause 50 and 10 % mortality of the population) were calculated by minimization of the sum of quadratic differences between experimental and model-predicted values using the nonlinear least-squares method provided by the macro Solver of the Microsoft Excel XP spreadsheet. Values were confirmed and 95 % CI calculated using DataFit v 9.0 (Oakdale Engineering, 1995–2008, http://www.curvefitting.com/).

No observed effect concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) values were calculated with the Kruskal–Wallis test and the Mann–Whitney U-non-parametric test ($p < 0.05$) using SPSS version 15.0 for Windows software (SPSS, Inc., Chicago, Illinois, www.spss.com), because data did not satisfy parametric criteria.

The extra sum-of-squares F test and the Akaike's information criterion (AIC) were used for comparing models (Motulsky and Christopoulos 2003). The F test is based on statistical hypothesis testing and is only appropriate for nested models, while AIC is based on entropy concept and is valid for any kind of model comparison. In both criteria the value of F or AIC statistic summarize goodness-of-fit, as residual sum of squares (RSS), against the number of parameters (p) for the same data set (n), and their aim is to avoid overfitting and select a excessively complex model.

The F statistic is given by:

$$F = \frac{(RSS_a - RSS_b)/RSS_b}{(df_a - df_b)/df_b} \quad (4)$$

where RSS_a and RSS_b are the residual sum of squares of model a (simpler) and b respectively, and df_a and df_b their degrees of freedom. The probability of the F distribution can be calculated taking the value of the F-Statistic, $df_a - df_b$ as degrees of freedom for the numerator and df_b for the denominator. If the probability is lower than 0.05 it is accepted that the more complex model describes better the data.

AIC corrected can be defined as (Motulsky and Christopoulos 2003):

$$AIC = n\ln\left(\frac{RSS}{n}\right) + 2(p+1) + \left[\frac{2(p+1)(p+2)}{n-p-2}\right] \quad (5)$$

The model with the lowest AIC is the one with the highest likelihood of being correct. The relative probability (Pr) of the chosen model being correct between two equations a and b can be calculated as indicated now:

$$Pr = \frac{\exp(-0.5\Delta AIC_{b-a})}{1 + \exp(-0.5\Delta AIC_{b-a})} \quad (6)$$

Results

Water quality parameters were similar and acceptable in all the tests; oxygen concentration was always >6 mg/L, pH ranged between 7.8–8.2 in mysids and 7.6–8.0 in daphnids, salinity in saltwater tests ranged between 34 and 35 %, and hardness in freshwater tests was 160–180 mg $CaCO_3$/L. At the end of the bioassays, all daphnid tests showed a control survival of 100 % and all mysid tests had >85 % control survival.

The NOEC, LOEC, LC_{10} and LC_{50} values with their 95 % CI are summarized in Table 1. Values of 96-h LC_{50} for *S. armata* in increasing order of toxicity were 0.13 µg/L (chlorpyrifos), 1.3 µg/L (pirimiphos-methyl), 3.2 µg/L (endosulfan), 4.03 µg/L (diazinon), 262.2 µg/L (PCP) and 2919 µg/L (diclofenac). Chlorpyrifos and pirimiphos-methyl, which showed the higher toxicity in mysids, were also tested on daphnids, obtaining a 48-h LC_{50} of 14.5 µg/L for chlorpyrifos and 103.5 µg/L for pirimiphos-methyl.

Among the six compounds tested on *S. armata*, the three organophosphate pesticides showed an apparently biphasic dose–response profile (Fig. 1), a feature that was even more remarkable for *D. magna* (Fig. 2). The comparisons using the F test between the models 1 and 3 indicated that the more complex biphasic model showed significantly higher likelihood in all cases, and the AIC test confirmed this finding (Table 2). The calculation of LC_{10} by the use of the model 3 or model 1 produced statistically significant values in two of the five cases with an apparent biphasic profile (Tables 1, 2). The LC_{50} calculated by model 3 were statistically significant in only two of the five cases, which contrasts with the statistical significance of the LC_{50} calculated with model 1 (Tables 1, 2). LC_{50}s values obtained using model 3 for *D. magna* proved to be 2.0–3.4 times lower than those calculated by the model 1. In contrast, for *S. armata* LC_{50}s calculated by model 1 are 1.2–2 times lower than those obtained by model 3. Regardless of the fitting model used, 96 h-LC_{50} values for the marine species

Table 1 Parametric estimates corresponding to a single-effector dose:response model (see Eq. 1 in the text) fitted to toxicity data obtained for daphnids at 48 h and mysids at 96 h: LC_{50}, LC_{10}, NOEC, LOEC (±95 % CI)

	Siriella armata						*Daphnia magna*	
	Chlorpyrifos	Pirimiphos-methyl	Endosulfan	Diazinon	PCP	Diclofenac	Chlorpyrifos	Pirimiphos-methyl
LC_{50}/48 h (μg/L)	0.269 ± 0.002	2.271 ± 0.030	4.8 ± 0.0	8.325 ± 0.557	673.1 ± 38.1	10.436 ± 53	14.5 ± 12.3	103.5 ± 56.8
LC_{50}/96 h (μg/L)	0.128 ± 0.036	1.331 ± 0.187	3.2 ± 0.3	4.026 ± 2.006	262.2 ± 39.4	2919 ± 1921	–	–
LC_{10}/48 h (μg/L)	0.185 ± 0.005	1.962 ± 0.003	4.2 ± 0.1	2.476 ± 0.441	457.2 ± 70.1	9995 ± 1	0.2 ns	0.2 ns
LC_{10}/96 h (μg/L)	0.023 ± 0.015	0.481 ± 0.176	1.6 ± 0.3	0.347 ns	90.7 ± 31.5	238 ns	–	–
NOEC/48 h (μg/L)	0.167	2	2.5	1.6	400	8000	3	0.1
NOEC/96 h (μg/L)	0.0062	0.1	1	–	50	250	–	–
LOEC/48 h (μg/L)	0.278	5	5	4	800	10,000	5	1
LOEC/96 h (μg/L)	0.0185	0.5	2.5	–	100	500	–	–
RSS	0.0216	0.0142	0.0048	0.0377	0.0107	0.0980	0.1276	0.0608
adj. R^2	0.9843	0.9908	0.9961	0.9610	0.9904	0.8922	0.9361	0.9568

For mysids toxicity parameters were also calculated at 48 h

RSS Residual sum of squares, *adj.* R^2 adjusted coefficient of multiple determination, *ns* not statistically significant at $\alpha = 0.05$ confidence level

S. armata are 20 to 70-fold lower than values for the classical ecotoxicological model *D. magna*.

Discussion

The organophosphate compounds chlorpyrifos and pirimiphos-methyl were the most toxic compounds tested, whereas the lowest toxicity was obtained for diclofenac (96 h-LC_{50} 2919 μg/L). For the pesticides tested with both model organisms, the toxicity was 1 or 2 orders of magnitude higher for mysids (LC_{50}/96 h of 0.128 and 1.331 μg/L for chlorpyrifos and pirimiphos-methyl respectively) than for daphnids (LC_{50}/48 h of 14.5 and 103.5 μg/L for chlorpyrifos and pirimiphos-methyl). Literature data of acute effects of diazinon, endosulfan, PCP and diclofenac in daphnids, discussed below, also supported the same conclusion. Although the period of exposure for *D. magna* (48 h) is shorter than for *S. armata* (96 h), and this could partly explains the lower LC_{50} values of the mysids, if 48 h-LC_{50} values for both species are compared (Table 1) *S. armata* still shows higher sensitivity than *D. magna*.

Chlorpyrifos was the compound with the highest acute toxicity for both mysids (96-h LC_{50} of 0.128 μg/L) and daphnids (48-h LC_{50} of 14.5 μg/L). *Neomysis integer* showed a very similar 96 h-LC_{50} of 0.14 μg/L (Roast et al. 1999), and *Americamysis bahia* a LC_{50} of 0.04 μg/L (Schimmel et al. 1983). Palma et al. (2008) obtained a 48 h EC_{50} of 0.74 μg/L for *D. magna*; this low value could be explained as the endpoint was immobility, not mortality. 48 h EC_{50} was also reported by Liu et al. (2012) found a value of 7.1 μg/L. Montagna and Collins (2007) obtained a chlorpyrifos 96 h-LC_{50} of 0.49 μg/L for the freshwater prawn *Palaemonetes argentinus* showing higher toxicity than the other OPs tested (endosulfan, 96 h-LC_{50} of 6.28 μg/L). Guzzella et al. (1997) tested eleven OPs in both *Artemia* sp. and the rotifer *Brachionus plicatilis* and concluded that chlorpyrifos was the most toxic OP insecticide to both species (24 h-EC_{50} of 2 and 1.7 mg/L respectively). Mhadhbi and Beiras (2012), working with larvae of the fish *Psetta maxima*, also found that chlorpyrifos was the most toxic pesticide of the seven tested in that study (96 h-LC_{50} of 94.65 μg/L).

As opposed to the abundant information available about the toxicity of chlorpyrifos, no data of acute toxicity was found for *pirimiphos-methyl* in crustaceans, although this study demonstrates high toxicity on both the marine and the freshwater test species. In other aquatic organisms little data are also available. Brown et al. (1998) found a 96 h-

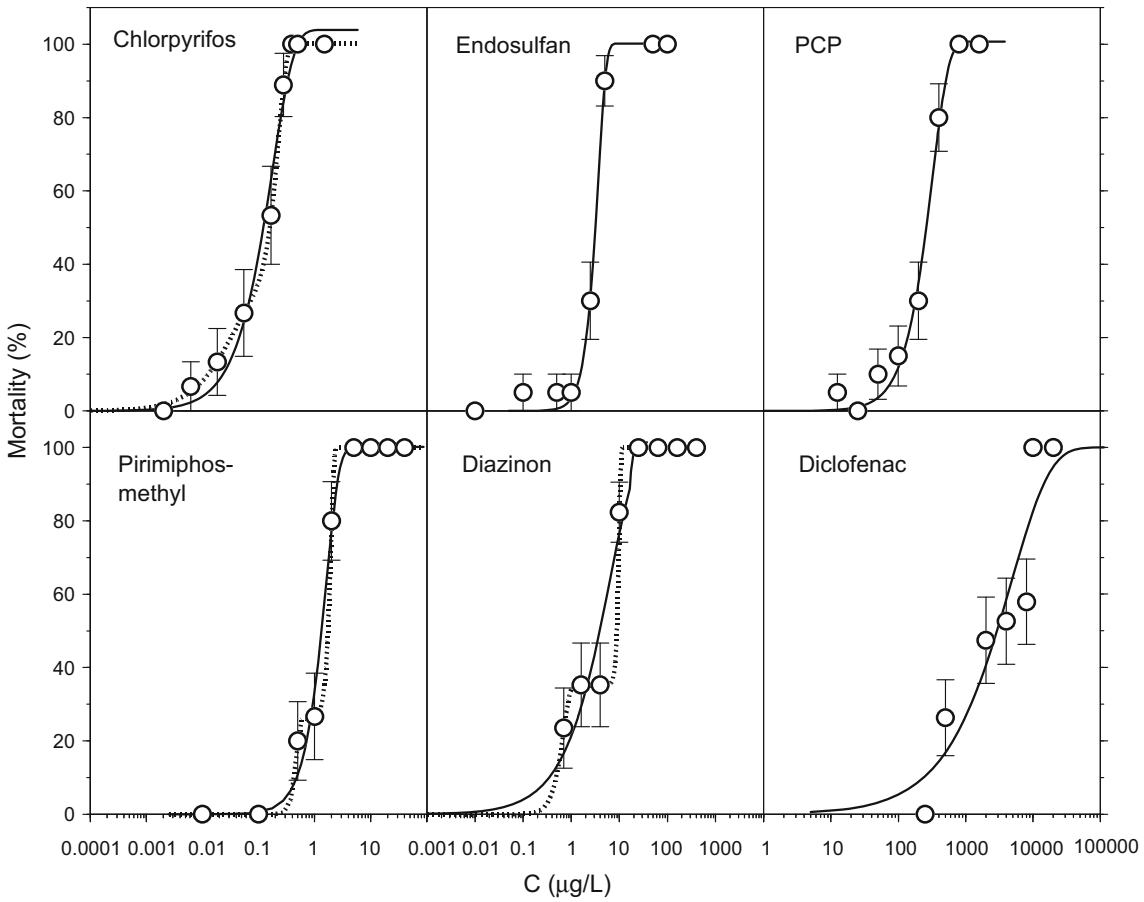

Fig. 1 Mortality (%) of *Siriella armata* neonates after 96 h exposure to (*white circle*) chlorpyrifos, pirimiphos-methyl, endosulfan, diazinon, PCP and diclofenac. Concentration in µg/L. Continuous line correspond to a single effector dose:response model and dotted line to a two-effectors dose:response model (see text)

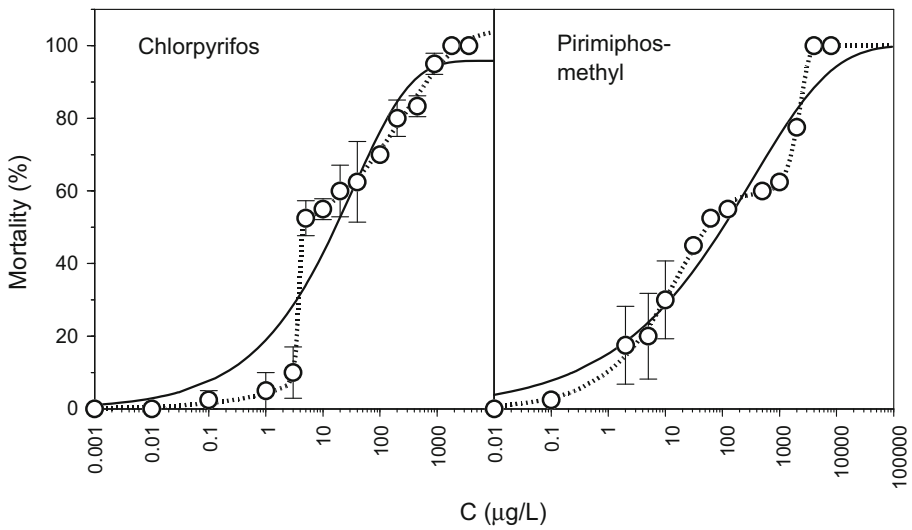

Fig. 2 Mortality (%) of *Daphnia magna* neonates after 48 h (*white circle*) exposure to chlorpyrifos and pirimiphos-methyl. Concentration in µg/L. Continuous line correspond to a single effector dose:response model and dotted line to a two-effectors dose:response model (see text)

LC_{50} of 91 µg/L for the Australian blue eye *Pseudomugil signifer*. European Commision (2006) reported a 96 h-LC_{50} values of 0.404 and 0.20 mg/L for the rainbow trout *Oncorhynchus mykiss*. Mhadhbi and Beiras (2012) also showed a 96 h-LC_{50} of 560 and 452 µg/L for embryos and larvae stage of *P. maxima*.

Table 2 Parametric estimates corresponding to a two-effectors dose:response model (see Eq. 3 in the text): LC_{50}, LC_{10} (±95 % CI)

	Siriella armata			*Daphnia magna*	
	Chlorpyrifos	Pirimiphos-methyl	Diazinon	Chlorpyrifos	Pirimiphos-methyl
K_1	0.307 ± 0.280	0.263 ns	0.353 ± 0.165	0.433 ± 0.099	0.592 ± 0.039
m_1 (µg/L)	0.022 ns	0.454 ns	0.605 ns	3.7 ns	9.4 ± 2.4
a_1	1.033 ± 0.698	7.487 ns	3.147 ns	10.584 ns	0.572 ± 0.090
K_2	0.694 ± 0.283	0.737 ns	0.647 ± 0.165	0.607 ± 0.132	0.410 ± 0.056
m_2 (µg/L)	0.201 ± 0.045	1.843 ns	9.509 ns	128.1 ± 81.7	2111.0 ± 242.4
a_2	3.004 ± 1.499	7.753 ns	12.532 ns	0.460 ± 0.134	2.881 ± 2.062
LC_{50} (µg/L)	0.157 ± 0.079	1.713 ns	8.913 ns	4.3 ns	53.0 ± 12.1
LC_{10} (µg/L)	0.013 ± 0.004	0.432 ns	0.468 ns	3.0 ns	0.9 ± 0.4
RSS	0.0005	0.0000	0.0000	0.0039	0.0020
adj. R^2	0.9993	1.0000	1.0000	0.9981	0.9979
F test Prob	0.001*	0.000*	0.000*	0.000*	0.000*
AIC Prob.	0.000	1.000*	1.000*	1.000*	1.000*

p value from Fisher's *F* test and probability based on AIC scores of accepting model 3 as more likely to be correct than model 1

RSS Residual sum of squares, *adj.* R^2 adjusted coefficient of multiple determination, *ns* not statistically significant

An asterisk indicates a higher probability of model 3 to be correct

In case of *diazinon*, the values here obtained with *S. armata* are also in line with those reported for other mysids. Nimmo (1981) found for *A. bahia* a 96-h LC_{50} of 4.82 µg/L, and Cripe (1994) obtained a 96-h LC_{50} of 8.5 µg/L for the same organism. In case of *D. magna*, Burkepile et al. (2000) and Jeon et al. (2010) previously reported a 48 h-LC_{50} of 2.39 and 1.07 µg/L respectively. For other crustaceans, Cripe (1994) found a 96-h LC_{50} of 21 µg/L for the prawn *Penaeus duorarum*, and Guzzella et al. (1997) showed a 24 h-EC_{50} of 19 and 28 mg/L to *Artemia* sp. and the rotifer *B. plicatilis* respectively. In fish, Mhadhbi and Beiras (2012) obtained a 96 h-LC_{50} of 1837 µg/L for embryos and 1230 µg/L for the larval stage of *P. maxima*, and cited 96 h-LC_{50} values of 1170 µg/L for *Oncorhynchus mykiss*, 1500 µg/L for *Cyprinus carpio*, or 7830 µg/L for *Oreochromis niloticus*. In all cases fish had lower toxicity than crustaceans.

Toxicity data for the two chlorinated biocides are scarce, but in both cases are in concordance with the present study. In case of *endosulfan*, *A. bahia* showed a 96 h-LC_{50} of 0.84 µg/L whereas a 48 h-LC_{50} for *D. magna* was 328 µg/L (USEPA2002). Values of Montagna and Collins (2007) showed a 96 h-LC_{50} of 6.28 µg/L for *P. argentinus*. In fishes, 96 h-LC_{50} were 2.03, 1.15 and 2.41 µg/L for *P. promelas*, *O. mykiss* and *Cyprinodon variegatus* respectively (USEPA 2002). Jin et al. (2012) studied the toxicity of *PCP* and reported an 96 h-LC_{50} of 140 µg/L for the freshwater shrimp *Macrobrachium superbum*, and values of 90 and 130 µg/L for the fishes *Plagiognathops microlepis* and *Erythroculter ilishaeformis*.

Diclofenac was not tested with daphnids in this study, but for *D. magna* Haap et al. (2008), Cleuvers (2004) and Ferrari et al. (2003) obtained a 48-hEC_{50} of 39.9, 68 and 22.4 mg/L respectively, again one order of magnitude higher than the value found here for *S. armata* (2.9 mg/L), even though the endpoint of daphnids in all of them was immobility, not death. This suggests that again mysids result more sensitive than cladocerans.

In contrast to other compounds tested previously (Pérez and Beiras 2010) the behaviour of daphnids and mysids at the end of bioassays for the three organophosphates compounds exposed in this study exhibited a different pattern. Even though the endpoint in all our test was the death, in case of OPs we observed that living organisms (especially in case of daphnids) showed sublethal effects at low concentrations like deformation of carapace, swimming difficulties or low mobility. This suggests that the use of a sublethal endpoints would further decrease the toxicity parameters (ECx, LOEC, NOEC) with OPs. For diazinon, chlorpyrifos and pirimiphos-methyl a clearly biphasic pattern of response emerges from inspection of Figs. 1 and 2, and this is backed by the statistical analysis. The fact that this was not an isolated observation, but repeated in the three organophosphate insecticides tested suggests that the mechanism underneath the biphasic response may differ from other organic biocides.

Biphasic profiles obtained for organophosphate pesticides could be explained mainly by three different hypotheses: (a) existence of two subpopulations with different levels of resistance, (b) variation of the mode of action

depending on the concentration of exposure, (c) exposition to more than one agent due to the appearance of breakdown metabolites. According to our results, the first hypothesis seems to be the weakest one, as biphasic profiles obtained for wild mysids are less pronounced than for daphnids, a very homogeneous biological material where intraclonal genetic variability can occur only by mutations or sexual reproduction (Baird et al. 1989). Regarding the second hypothesis, Printes and Callaghan (2004) studied the AChE inhibition by four organophosphates in *D. magna* and its relation with mobility inhibition, and found that binding sites other than AChE were involved in the toxic mechanism. It is well known that other enzymes inhibited by organophosphate pesticides are butyrylcholinesterases and carboxylesterases (Stenersen 2004). The interaction of a pesticide with secondary targets generally occur at higher concentrations than corresponding to its primary mechanism of action (Krieger 2001). However, Barata et al. (2004) found that carboxylesterase of *D. magna* showed more activity and equivalent or higher sensitivity than AChE and pointed out that this could be a mechanism of detoxification. Regarding the third hypothesis, it may be explained by the oxidation of the parent compound catalyzed by CYP enzymes that generate the oxon. The kinetics of accumulation of the parental compound, genesis and elimination of the oxon have been described in vertebrates, where biotransformation within the organisms undergoes oxidate desulfuration to convert the relatively inactive compounds (parental OPs, containing P=S moiety), to a more potent and reactive metabolites (oxons, P=O). The oxons are: truly responsible of their high neurotoxicity as cholinesterase inhibitors (which caused a disrupting of a normal nervous system function), usually more persistent and soluble than their parent compounds (Krieger 2001; Xuereb et al. 2007). Those reactions are likely to be similar in crustaceans, but with different enzymes involved in the reactions. Although the biphasic dose–response pattern found for the organophosphate pesticides is conspicuous (see Figs. 1 and 2), the experimental design of the present study does not allow to distinguish among the hypotheses above mentioned. That should be the subject of future investigation.

Current European guidelines for the derivation of water and sediment EQS (European Commission 2011) rely on laboratory testing with model species representative of different trophic levels of the aquatic ecosystems for the calculation of toxicity parameters, such as LC_{50}. The EQS value is then estimated as the toxicity parameter divided by an assessment factor that accounts for experimental uncertainty. We have shown here how LC_{10} and LC_{50} values calculated from the same data sets can vary up to two orders of magnitude depending on the mathematical model chosen for the fitting (see Tables 1 and 2). In the case of modelling the toxicity of organophosphate insecticides to *Daphnia*, the LC_{50} values are much lower when a two-effector model, more complex but with a better fitting, is used. This is very relevant, since many organics readily undergo biological or chemical degradation once in solution, but the breakdown products may be equally or even more toxic than the parental compound. A two-effector model allows fitting toxicity data for chemicals showing biphasic behaviour. For the case of some organophosphate insecticides here tested with *Daphnia*, applying the biphasic model for the LC_{50} calculation would yield much lower EQS values.

In those regulations freshwater organisms are most commonly used to develop EQS than marine organisms, even when EQS related to the marine environment are established. However, based on our results and relying on literature (Crane et al. 2002), the sensitivity of marine organisms could be even higher, and it should be considered, especially when it needs to establish EQS in marine environment.

Currently, the EU legislation establishes toxicity categories for aquatic organisms. On the basis of this scheme, the Directive 93/67/ECC classifies all the compounds with an $EC_{50} < 1$ mg/L as "very toxic to aquatic organisms"; those between 1 and 10 mg/L as "toxic to aquatic organisms" and between 11 and 100 mg/L as "harmful to aquatic organisms". According to our study, all of the compounds tested would be classified as very toxic to aquatic organisms (diazinon, chlorpyrifos, pirimiphos-methyl, endosulfan and PCP) except for diclofenac, which would be considered as toxic. Comparing our results with the European EQS for priority substances (Directive 2013/39/EC) we found that the EQS expressed as maximum allowable concentration (MAC-EQS) for chlorpyrifos (0.1 μg/L) is higher than the acute toxicity value (NOEC) calculated for *S. armata* (0.0062 μg/L). In view of the results obtained in this study it seems clear that the MAC-EQS for this organophosphate insecticide given in the current legislation is too permissive, and may not protect aquatic ecosystems.

To summarize, results achieved in this study for *S. armata* allow to demonstrate (a) the importance of using an adequate mathematical model to establish toxicity, which results in more realistic values of EQS; (b) the use of marine ecotoxicological models as the most appropriate tools to establish marine EQS; and additionally (c) it is expected that these results, combined with literature data of acute toxicity values for other marine organisms, could be useful in the establishment of the concentration of each toxicant that causes no adverse effect to the environment [Predicted no effect concentration (PNEC)]. By determining the ratio between the concentration expected to find in the environment [Predicted environmental concentration

(PEC)] and the PNEC, it can be estimated a priori the environmental risk assessment to predict the probability of future adverse effects.

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Conflict of interest The authors declare that they have no conflict of interest.

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