

# Comparison of specific versus literature species sensitivity distributions for herbicides risk assessment

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**Abstract** Species sensitivity distributions (SSDs) are an important predictive tool in risk assessment. Usually, literature data are used to build SSDs that are mostly based on planktonic species. But, to get adequate protective thresholds for environmental communities, one could argue that SSD should be built on ecotoxicological data obtained from species found in the ecosystem that should be protected. This is particularly true when benthic algae are of concern. Due to the lack of literature data, building SSD on benthic microalgae is difficult. This paper aims in comparing SSDs, and thus protective thresholds (hazardous concentration that affects 5 % of the species of a community, HC<sub>5</sub>), built on ecotoxicological data obtained (1) from literature and (2) with specific bioassays on benthic diatoms from a lake. Thresholds were derived for protection against four herbicides separately and for a mixture of them. Sensitivity data obtained from literature were statistically lower than the specific data for all herbicides: Species tested in the literature were usually more sensitive (mainly chlorophytes), leading to more protective lower HC<sub>5</sub>. The HC<sub>5</sub> thresholds (literature or specific) derived for protection against the mixture were also compared to the observed

sensitivity of an assemblage of benthic diatom species exposed to an increasing range of herbicide mixture concentrations. We observed that one species within the assemblage (*Fragilaria rumpens*) was affected at a concentration below both the literature and the specific HC<sub>5</sub> thresholds.

**Keywords** Species sensitivity distribution · Ecotoxicological data · Benthic diatoms · Chlorophytes · Herbicides · Mixture · Assemblage

## Introduction

The mixture of chemicals found in watershed runoff and urban discharges can cause pollution of aquatic ecosystems (Loos et al. 2009; Dorigo et al. 2010; Montuelle et al. 2010). Herbicides, and more specifically photosynthesis inhibitors such as atrazine, terbutryn (s-triazines), diuron, and isoproturon (phenylureas), are among the most commonly detected compounds in aquatic ecosystems (Loos et al. 2009; Dubois and Lacouture 2011). These herbicides are regularly found in the middle of Lake Geneva (Ortelli et al. 2013) as well as at the littoral zone (diuron, 0.004–0.0257 nM; isoproturon, 0.009 nM; atrazine, 0.005–0.0278 nM; terbutryn, 0.004–0.008 nM) (INRA, unpublished data). Photosystem II (PSII) inhibitors are phytotoxic, and their environmental residues have a deleterious effect on diatoms, even at low concentrations (Tang et al. 1997; Lockert et al. 2006; Roubeix et al. 2011; Larras et al. 2012). In shallow water zones, benthic diatoms are crucial primary producers which account for a considerable proportion of the fixed biomass and carry out some of the main biochemical processes. In addition, these sensitive organisms are widely used as indicators of ecosystem water quality, especially for trophic level and physical disturbances (Rumeau and Coste 1988; Van Dam et al.

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1994). Because benthic diatoms are ecologically significant and sensitive to herbicides, they can be relevant organisms for evaluating the environmental risk of herbicides.

Predictive models have been developed for risk assessment of single substances and complex mixture at the community level (Traas et al. 2002; de Zwart and Posthuma 2005; Van den Brink et al. 2006; Gregorio et al. 2012). These models are based on the species sensitivity distribution (SSD) as proposed by Posthuma et al. (2002). The distribution represents the variation of sensitivity between species within a community and allows threshold derivation defined as the hazardous concentrations (HCs). In risk assessment, the  $HC_5$  (the concentration that affects 5 % of the species of a community) is most commonly used to derive risk quotients. SSDs are mostly built on no-observed-effect concentrations (NOEC) but can also be calculated on effect concentrations 50 % ( $EC_{50}$ ). SSDs are based on several assumptions, such as that interaction between species does not influence the sensitivity distribution and that species represent a random sample of species sensitivity found in natural community (Forbes and Calow 2002). To increase the ecological relevance of SSDs and the reliability of derived HC thresholds, selected incoming species have to be (1) sensitive to the specific mode of action of the chemical and (2) representative of the targeted ecosystem (Posthuma et al. 2002; Forbes and Calow 2002; Van den Brink et al. 2006). Several authors have raised concerns about uncertainties introduced when using species not representative of the targeted community for assessing the risk of many substances such as heavy metals and pesticides (Bossuyt et al. 2005; Maltby et al. 2005; Kwok et al. 2008). This point is difficult to respect since data available in literature are often too poor to build one reliable SSD for one substance and for one environmental community. Moreover, literature data are recognized to suffer from important heterogeneity in terms of exposure time, growth conditions, and sensitivity endpoints. With regard to PSII inhibitor herbicides, most of the sensitivity data available in the literature and from online databases concern a few planktonic microalgae species, mainly cyanobacteria and chlorophytes, which are poorly representative in terms of taxonomical diversity, or biological and ecological traits, to the natural communities. Sensitivity data for benthic microalgae (mainly diatoms) are very scarce in these sources. This makes it difficult to assess specific herbicide risks for benthic diatom communities even though these species, which make up the major part of the attached biomass in shallow water habitats, are currently exposed to these mixtures. One alternative is to perform ecotoxicological tests on chosen species, representative of the environmental community to be protected, and leading to a more robust and ecologically relevant dataset.

The first aim of this study was therefore to compare whether  $HC_5$  thresholds differ when derived from literature SSD (mainly organisms developing in the water column such as

planktonic algae and macrophytes) and specific SSD (mainly organisms developing in the biofilm matrix, such as benthic diatoms) for four single herbicides (atrazine, terbutryn, diuron, and isoproturon). The data for building the specific SSD were generated in a previous study on benthic microalgae from Lake Geneva (Larras et al. 2013). Furthermore, aquatic organisms are typically exposed to a mixture of compounds (Faust et al. 2001). Previous studies have examined methods to reliably predict the toxicity of a mixture for PSII inhibitors from dose–response curves of single species and communities (Faust et al. 2001; Backhaus et al. 2004). The concentration addition (CA) model applied on SSDs is widely used to predict the effects of mixtures when constituents of a mixture share the same toxic mechanism of action. De Zwart and Posthuma (2005) found that building a single SSD for a mixture with a CA model is reliable when the species included in the SSD have the receptor site of the chemical.

The second aim of this study was to verify which curve provides the best prediction of species sensitivity when exposed together to the herbicide mixture and protect most of them. That allowed us to define the importance of the ecological relevance of data to be used for building SSD and deriving protection thresholds. This work was performed in the framework of the protection of natural diatom communities of the benthic littoral zone of Lake Geneva.

## Materials and methods

### Sensitivity datasets

The study focused on a set of four PSII inhibitors—atrazine, terbutryn, diuron, and isoproturon. Sensitivity data ( $EC_{50}$ ) to these four herbicides were collected from experimental and literature sources to build SSDs. Specific SSDs (S-SSDs) were built on data from Larras et al. (2013) for 11 benthic diatom species found in biofilm of the Lake Geneva littoral zone. In this dataset, as in natural biofilms, there was a higher proportion of pennate diatoms (10) than centric diatoms (1) among tested species. Specific benthic species were *Fragilaria capucina* var. *vaucheriae* (FCVA), *Fragilaria rumpens* (FRUM), *Ulnaria ulna* (UULN), *Craticula accomoda* (CRAC), *Mayamaea fossalis* (MAFO), *Eolimna minima* (EOMI), *Nitzschia palea* (NPAL), *Achnantheidium minutissimum* (ADMI), *Cyclotella meneghiniana* (CMEN), *Encyonema silesiacum* (ELSE), and *Gomphonema parvulum* (GPAR). All strains were obtained from the Thonon Culture Collection (Thonon-Les-Bains, France, <http://www.inra.fr/cartel-collection>).

Literature SSDs (L-SSDs) were built using a dataset obtained from Chèvre et al. (2006), which collected  $EC_{50}$  data in literature for organisms developing in the water column such as planktonic algae (chlorophytes, diatoms, cyanobacteria,

rhodophytes) and macrophytes. The amount of sensitivity data available in the literature varied greatly between diuron ( $n=23$ ), isoproturon ( $n=20$ ), atrazine ( $n=29$ ), and terbutryn ( $n=7$ ). Only freshwater species were selected. A geometric mean was used when several sensitivity data were available for the same species.

### Assemblage microcosm experiment

In a microcosm experiment, we exposed a benthic diatom artificial assemblage to the equitoxic mixture of the atrazine, terbutryn, diuron, and isoproturon. The mixture was defined based on the molar ratios of  $HC_{50}$  of the S-SSDs (0.63 of atrazine, 0.025 of terbutryn, 0.072 of diuron, and 0.27 of isoproturon) using the CA model according to Faust et al. (2001). The  $HC_{50}$  ratio was selected because the middle of the SSD curve is the most robust part of the curve. We investigated the dose–effect relationship of this mixture using an increasing gradient of eight concentrations (nominal concentrations of 61, 176, 381, 953, 2381, 5953, 14,882, and 37,206 nM). The same ratio was kept along the gradient. Herbicides were obtained from Sigma-Aldrich (St. Louis, MO 63103, USA), and stock solutions were prepared in DV culture media ([http://www6.inra.fr/cartel-collection\\_eng/Culture-media/Composition-of-the-culture-media](http://www6.inra.fr/cartel-collection_eng/Culture-media/Composition-of-the-culture-media)) (Table 1). Atrazine and diuron were dissolved in 0.05 % dimethyl sulfoxide (DMSO) and sonicated for 30 min before adding the DV media because of the high concentration of stock solution and their low solubility in water. The non-toxicity of 0.05 % DMSO in DV media toward the diatoms was controlled and confirmed prior to the bioassay implementation.

The microcosm experiment was carried out using an artificial assemblage of the 11 specific benthic diatom species used to build the S-SSD (see above). Cultures of the 11 diatom species were introduced together in three large incubation aquaria. Glass disks of 2.54 cm<sup>2</sup> were positioned at the bottom of the incubation aquaria, as a substrate, to support benthic growth. The assemblage was grown for a period of 12 days to stabilize the community. Before contamination, five glass disks were removed from each of the three incubation aquaria: two for diatom identification and three for cell density assessment. The disks were gently shaken to dislodge any settled particles and to ensure that only diatom cells actually present in the biofilm were sampled. The experiment was carried out

in 27 aquaria measuring 15\*15\*15 cm. Eight glass disks from the same incubator aquarium were placed in all aquariums (i.e., we used the same incubator aquarium for the same replicate). The aquaria were split into one control (DV culture media and 0.05 % DMSO) and eight mixture concentrations (DV culture media and the mixture solution), and each treatment was replicated three times. We exposed the community to the eight nominal concentrations. After 4 days of exposure, we removed from each aquarium two glass disks for diatom identification and three glass disks for cell density assessment. Incubation and exposition in microcosms were performed at 21±2 °C and with a 16/8-h light/dark cycle under light intensity of 66 μmol m<sup>-2</sup> s<sup>-1</sup>.

The diatom community composition in each biofilm sample was analyzed according to the European Committee for Standardization (EN 14407). The biofilm was scraped from the glass disks and boiled during 4 h in HNO<sub>3</sub> hours, to remove any organic material, at 100 °C in a sand bath until the solution was colorless. Pellets were then rinsed two times in distilled water, and 300 μL of the solution was transferred to a coverslip. The dried coverslip was placed on a glass slide and fixed with Naphrax (high refractive index medium, Brunel Microscopes, Ltd., UK). Diatom species were determined optically at a ×1000 magnification (Axio Scope A.1, Zeiss, Germany), based on their siliceous skeleton, and counted on 400 valves. Diatom cell density was optically counted (BH-2, Olympus, USA) at the beginning (T0) and at the end (T4) of the experiment with a Malassez cell.

We derived an observed  $EC_{50}$  for each species of the assemblage to the mixture of herbicides on the basis of their individual growth evolution. Using the total diatom cell density in the assemblage and the relative abundance of each diatom species at T0 and T4, we assessed their individual growth evolution at the end of the experiment for each tested concentration. A dose–response curve was fitted on growth for each species and an  $EC_{50}$  was derived.

### Dose–response modeling

Dose–response curves were fitted with R software and the “drc” package (Ritz and Streibig 2005). A log-logistic model was applied to monotonically decreasing functions. In case of hormesis, which lead to a biphasic dose–response, the Cedergreen–Ritz–Streibig model was used. Dose–response

**Table 1** Mode of action, references, and properties of herbicides

Family	Mode of action	Herbicide	CAS number	Purity (%)
Phenylureas	Photosystem 2 inhibition	Diuron	330-54-1	99.5
		Isoproturon	34123-59-6	99.9
Triazines	Photosystem 2 inhibition	Atrazine	1912-24-9	99.9
		Terbutryn	886-50-0	99.3

curves were fitted for each of the 11 diatoms species for the four herbicides separately by Larras et al. (2013). A dose–response curve was fitted for the mixture exposure for each of the diatoms species found in the assemblage experiment, based on their specific growth rate evolution in the assemblage. The EC<sub>50</sub> was numerically determined from each dose–response curve, and 95 % confidence intervals were determined with delta method (Ritz and Streibig 2005). Because most of the sensitivity data in the literature are EC<sub>50</sub>, we used the same 50 % threshold for easier comparison.

To assess if the sensitivity of species may change when they are exposed in single-species or multi-species conditions, we derived from CA model (Eq. 1) the sensitivity of each species to the quaternary mixture. These modeled EC<sub>50</sub> could then be compared for each species to the EC<sub>50</sub> that was observed from the microcosm experiment where the species assemblage was exposed to a gradient of the herbicide mixture.

$$\sum_{i=1}^n \frac{c_i}{EC_{xi}} = 1 \tag{1}$$

where  $C_i$  is the concentration in the mixture of the herbicide  $i$ , and  $EC_{xi}$  is the concentration of herbicide  $i$  which leads to the effect  $x$ .

### Single-substance SSD fitting

L-SSDs and S-SSDs were fitted using a log-logistic regression (Eq. 2) for each herbicide  $i$  on single-species EC<sub>50</sub> values:

$$Fraction\ affected_{i,s} = \frac{100}{1 + 10^{(\log HC_{50i} - \log EC_{50i,s}) * slope}} \tag{2}$$

According to Chèvre et al. (2006),  $HC_{50i}$  is the hazardous concentration affecting 50 % of the species and  $EC_{50i,s}$  is the EC<sub>50</sub> of the herbicide  $i$  for species  $s$ . The slope is the slope parameter with the best fit for all EC<sub>50</sub> values in the SSD. A literature and specific HC<sub>5</sub> (L-HC<sub>5</sub> and S-HC<sub>5</sub>, respectively) were derived numerically from each curve.

### Quaternary mixture SSD fitting

We built SSDs for the mixture (SSD<sub>mix</sub>) based on the four single-substance SSDs (Online Resource 1). To build each single SSD<sub>mix</sub>, we derived a HC<sub>mix</sub> value for each level of the potentially affected fraction from Eq. 3, derived from the original CA model equation:

$$HC_{xCA} = \left( \sum_{i=1}^n \frac{P_i}{HC_{xi}} \right)^{-1} \tag{3}$$

where  $HC_{xCA}$  is the HC for a level of effect  $x$  of the mixture,  $P_i$  is the proportion of the component  $i$  in the mixture, and  $HC_{xi}$  is the HC for a level of effect  $x$  of the component  $i$ . This

process was applied to model S-SSD<sub>mix</sub> and L-SSD<sub>mix</sub> from single-substance S-SSDs and L-SSDs, respectively. HC<sub>5</sub> values, modeled to protect 5 % of the species exposed to the mixture of herbicides, were obtained from the L-SSD<sub>mix</sub> and the S-SSD<sub>mix</sub> and were defined as L-HC<sub>5mix</sub> and S-HC<sub>5mix</sub>, respectively.

## Results

### Species sensitivity for single herbicides and mixture

The EC<sub>50</sub> values for benthic diatoms obtained by Larras et al. (2013) from 44 single-species bioassays conducted in the laboratory are summarized in Table 2. These EC<sub>50</sub> values varied greatly between species and herbicides. FRUM was the most sensitive species for diuron, atrazine, and terbutryn with EC<sub>50</sub> values of 38 nM–8.89 µg/L, 491 nM–106 µg/L, and 0.41 nM–0.1 µg/L, respectively. For these herbicides, the higher EC<sub>50</sub> values were 11,180 nM–2606 µg/L (SMEN), 69,283 nM–14,943 µg/L (NPAL), and 2212 nM–534 µg/L (NPAL), respectively. For isoproturon, the EC<sub>50</sub> values varied from 247 nM–51 µg/L (CMEN) to 20,143 nM–4155 µg/L (EOMI). Predicted EC<sub>50</sub> values of each species for the quaternary mixture, based on the single-substance EC<sub>50</sub> and the molar ratio of HC<sub>50</sub>, were 15.5 nM (FRUM), 394 nM (CMEN), 666 nM (UULN), 1268 nM (FCVA), 1407 nM (ADMI), 2111 nM (MAFO), 2581 nM (ELSE), 7218 nM (GPAR), 16,226 nM (NPAL), and 19,235 nM (EOMI).

Species of the literature dataset were mainly composed of organisms developing in the water column mostly belonging to Chlorophyceae (23 species), Bacillariophyceae (6 species), Cyanobacteriaceae (5 species), macrophytes (7 species), Rhodophyceae (1 species), and Euglenophyceae (1 species) (Table 3). We observed a wide range of sensitivity between species and different levels of toxicity between herbicides. The EC<sub>50</sub> values varied between 1.50 nM–0.35 µg/L (*Chlorella vulgaris*) and 2317 nM–540 µg/L (*Ulothrix fimbriata*) for diuron and from 24 nM–5 µg/L (*Scenedesmus subspicatus* and *Chlorella pyrenoidosa*) to 461 nM–95 µg/L (*S. subspicatus*) for isoproturon. For atrazine, the EC<sub>50</sub> values varied between 51 nM–11 µg/L (*Scenedesmus acutus acutus*) and 365,353 nM–78,800 µg/L (*Euglena gracilis*), while for terbutryn, the values varied between 8.3nM–2 µg/L (*Pseudokirchneriella subcapitata*) and 97 nM–23.41 µg/L (*Chlorella vulgaris*).

The ecotoxicological data found in literature were all significantly lower than those obtained for specific benthic diatoms for atrazine ( $p$  value 0.0004), diuron ( $p$  value 0.0004), isoproturon ( $p$  value 0.00006), and terbutryn ( $p$  value 0.04).

**Table 2** Specific benthic diatoms EC<sub>50</sub> (nM) database for the four herbicides obtained from Larras et al. (2013)

Abbreviations	Species	EC <sub>50</sub> (nM)			
		Diuron	Isoproturon	Atrazine	Terbutryn
CRAC	<i>Craticula accomoda</i>	6118 [3445; 8786]	7160 [3747; 10,578]	18,931 [15,973; 21,889]	319 [0; 1450]
EOMI	<i>Eolimna minima</i>	11,180 [4359; 18,006]	20,143 [12,881; 27,404]	47,812 [25,037; 70,591]	1318 [903; 1732]
MAFO	<i>Mayamaea fossalis</i>	596 [545; 648]	2434 [1978; 2889]	4307 [3644; 4966]	261 [28; 497]
ESLE	<i>Encyonema silesiacum</i>	1227 [712; 1746]	3728 [0; 13,351]	27,796 [12,523; 43,064]	107 [3; 211]
GPAR	<i>Gomphonema parvulum</i>	6105 [0; 13,819]	10,534 [8295; 12,784]	7817 [6983; 8652]	1218 [953; 1483]
FCVA	<i>Fragilaria capucina var vaucheriae</i>	187 [116; 259]	2831 [1425; 4242]	2944 [2216; 3672]	265 [130; 399]
UULN	<i>Ulnaria ulna</i>	178 [136; 221]	640 [524; 756]	1076 [510; 1641]	280 [238; 323]
FRUM	<i>Fragilaria rumpens</i>	38 [23; 53]	751 [524; 979]	491 [246; 742]	0,41 [0; 9]
NPAL	<i>Nitzschia palea</i>	7152 [3926; 10,374]	8663 [1983; 15,348]	69,283 [30,615; 107,952]	2212 [1500; 2929]
ADMI	<i>Achnanthydium minutissimum</i>	240 [197; 287]	1769 [1338; 2196]	4595 [3445; 5740]	207 [157; 261]
CMEN	<i>Cyclotella meneghiniana</i>	210 [182; 238]	247 [205; 290]	4437 [3718; 5156]	26 [23; 29]

95 % confidence intervals are shown in square brackets

### Microcosms: species-specific growth rate evolution

Of the 11 species introduced at the beginning of the incubation, 7 were retrieved from the assemblage after the 12-day incubation period, before the contamination. In control samples, species displayed differing growth rates within the community: NPAL, ADMI, and to a lesser extent EOMI had higher growth rates under control conditions than CRAC and GPAR (Fig. 1), while CMEN and FRUM displayed the lowest growth rate. When exposed to the herbicide mixture, the different species displayed differing responses. The growth rate of FRUM was severely affected from the lowest concentration of the herbicide mixture, whereas most of the other species were only affected from the second lowest concentration. Even though the growth of all species was severely affected at the highest concentrations, it was rarely totally inhibited. As the relative abundance of the diatoms was assessed from their cleaned siliceous skeletons, some dead diatoms may have been included in the count.

The sensitivity of species for the mixture modeled from single species bioassays were compared to those observed from the multi-species exposure experiment (Fig. 1). The sensitivity under multi-species conditions was slightly underestimated by the CA model prediction for ADMI and considerably underestimated for NPAL, CRAC, GPAR, and EOMI, (Fig. 1). The sensitivities of FRUM and CMEN appeared to be similar under single- and multi-species conditions. However, the EC<sub>50</sub> of FRUM obtained under multi-species conditions was weakly reliable since the lowest concentration tested inhibited growth by already more than 50 %. Species sensitivity prediction based on data obtained under single-species conditions clearly resulted in an underestimation of the effect of the herbicide mixture.

### Literature SSD versus specific SSD

L-SSDs were built using sensitivity data for freshwater phototrophic organisms such as chlorophytes, diatoms, cyanobacteria, macrophytes, and euglenas (Fig. 2). Diuron and atrazine L-SSD curves were built using the sensitivity of 18 literature species (13 chlorophytes, 1 macrophyte, 3 cyanobacteria, and 1 rhodophyte) and 28 literature species (6 diatoms, 12 chlorophytes, 7 macrophytes, 2 cyanobacteria, and 1 euglenacea), respectively. Tolerant species were mostly benthic diatoms and a few tolerant chlorophytes, while more sensitive species were mostly chlorophytes and cyanobacteria. The three *Fragilariiales* diatom species were the most sensitive to atrazine and diuron while the euglena species was the most tolerant. Isoproturon and terbutryn L-SSD curves were built using the sensitivity of 10 (1 diatom, 8 chlorophytes, and 1 macrophyte) and 4 (chlorophytes) literature species, respectively. S-SSD curves were built using benthic diatom sensitivity data obtained in Larras et al. (2013) (Fig. 2). All S-SSDs were drawn at higher concentrations than L-SSDs, which means that more sensitive species were included in the L-SSDs. However, the lower tails of the L-SSD and S-SSD curves for terbutryn were close.

### HC<sub>5</sub> threshold derivation and protectiveness assessment

The HC<sub>5</sub>, HC<sub>50</sub>, and slopes were derived from the L-SSD and S-SSD curves (Table 4). The slopes were always steeper for the S-SSDs than for the L-SSDs. The S-SSDs produced higher HC values than the L-SSDs, especially for HC<sub>5</sub>. The HC<sub>5mix</sub> was derived from the S-SSD<sub>mix</sub> and the L-SSD<sub>mix</sub> curves. As a consequence, the S-HC<sub>5mix</sub> (106 nM) was higher by a factor of 10 than the L-HC<sub>5mix</sub> (16.71 nM) (Table 4). However, in

**Table 3** Literature organisms EC<sub>50</sub> (nM) database for the 4 herbicides obtained from Chèvre et al. (2006)

Phototrophic groups	Species	Concentrations (nM)			
		Diuron	Isoproturon	Atrazine	Terbutryn
Cyanobacteriaceae	<i>Anabaena flosaquae</i>			1020	
	<i>Anacystis nidulans</i>			112	
	<i>Anabaena variabilis</i>	24.88			
	<i>Spirulina platensis</i>	36.46			
	<i>Synechococcus</i> sp.	2.36			
Chlorophyceae	<i>Ankistrodesmus</i> sp.	25.74			
	<i>Chlamydomonas geitleri</i>				20
	<i>Chlamydomonas reinhardtii</i>		193	380	
	<i>Chlamydomonas</i> sp.	158			
	<i>Chorella fusca</i>			70	
	<i>Chorella kessleri</i>			8034	
	<i>Chlorella pyrenoidosa</i>	5.58	24		
		103	97		
			121		
	<i>Chlorella saccharophila</i>			3616	
	<i>Chlorella vulgaris</i>	1.50	109		55
		18.45			97
	<i>Chlorococcum hypnosporum</i>	107			
	<i>Dictyosphaerium pulchellum</i>	25.74			
	<i>Hormidium flaccidum</i>	2145			
	<i>Scenedesmus abundans</i>			510	
	<i>Scenedesmus acutus acutus</i>	17.55	116	51	
	<i>Scenedesmus quadricauda</i>	11.58	102	394	
	<i>Scenedesmus subspicatus</i>	12.87	24	97	
		14.16	82		
		154	102		
		4.29	102		
			461		
			145		
	<i>Scenedesmus vacuolatus</i>		228		32.36
	<i>Selenastrum capricornutum</i>	94			
<i>Pediastrum</i> sp.			2488		
<i>Pithophora oedogonia</i>			93		
<i>Pseudokirchneriella subcapitata</i>	6.65	170	927	8.29	
				11.19	
				13.67	
	<i>Stichococcus bacillaris</i>			15,165	
	<i>Ulothrix fimbriata</i>	2317			
Bacillariophyceae	<i>Cyclotella gamma</i>			1992	
	<i>Cyclotella meneghiniana</i>			4448	
	<i>Navicula pelliculosa</i>		48	278	
			63		
	<i>Stephanodiscus parvus</i>			3046	
	<i>Ulnaria ulna</i>			947	
	<i>Fragilaria radians</i>			412	
Rhodophyceae	<i>Ceramium tenuicorne</i>	14.59			
Euglenophyceae	<i>Euglena gracilis</i>			365,353	

**Table 3** (continued)

Phototrophic groups	Species	Concentrations (nM)			
		Diuron	Isoproturon	Atrazine	Terbutryn
Macrophytes	<i>Ceratophyllum demersum</i>			102	
	<i>Elodea canadensis</i>			263	
	<i>Lemna minor</i>	107	150	395	
			160		
			194		
	<i>Myriophyllum spicatum</i>			1666	
	<i>Myriophyllum heterophyllum</i>			612	
	<i>Najas</i> sp.			111	
<i>Potamogeton perfoliatus</i>			1331		

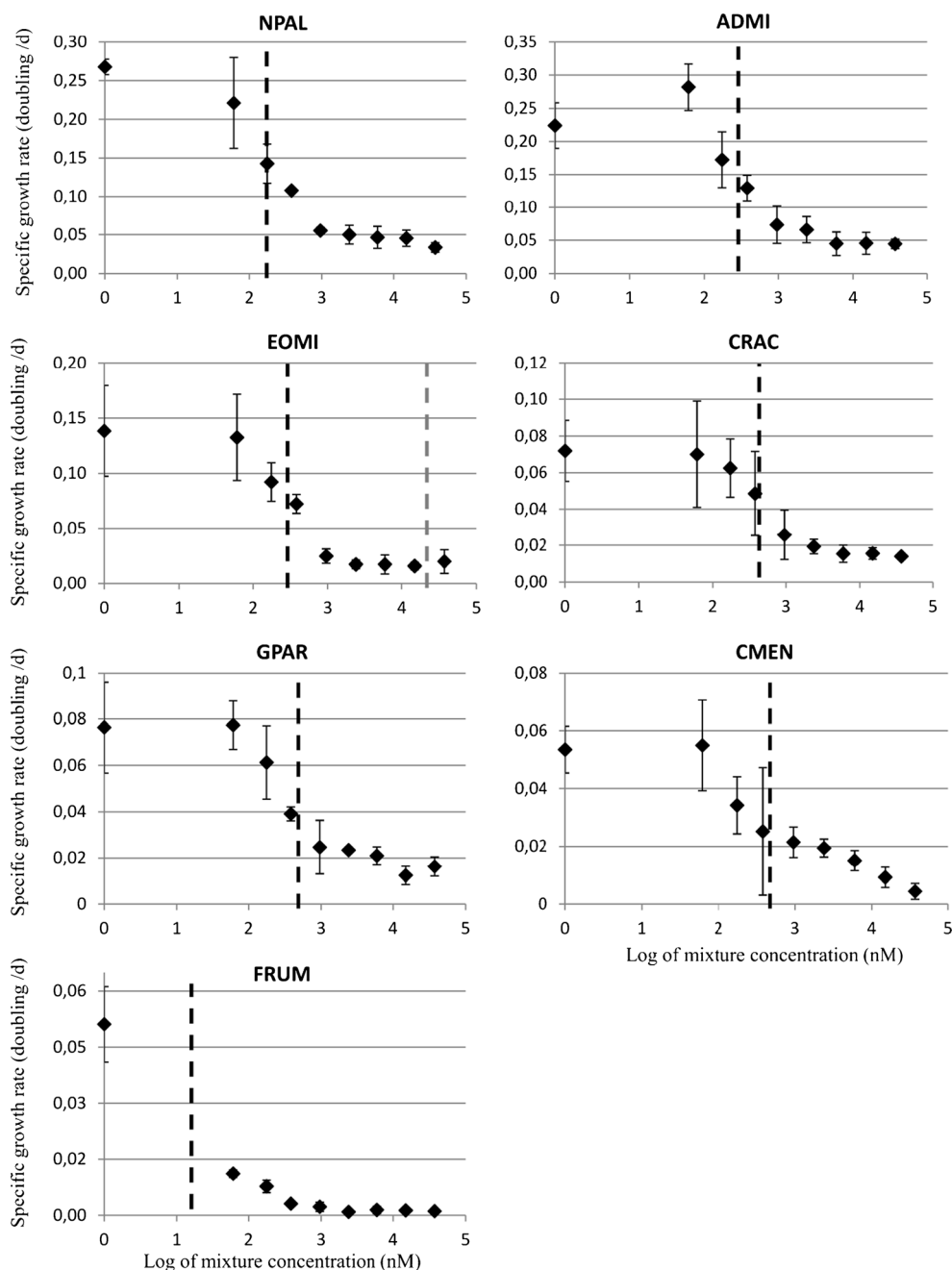
the species assemblage, the specific growth of one species (FRUM) was inhibited by at least 50 % at concentrations equal to literature and specific HC<sub>5mix</sub> thresholds (Fig. 3). All other species were inhibited at concentrations higher than these thresholds which should consequently protect them.

## Discussion

The critical point when using SSDs is that these latter are based on ecotoxicological data available in the literature, which differ for the different substances in terms of the amount of data available, the species tested, and the endpoints used. Currently available data on herbicide risk assessment for primary producers are related to organisms living in the water column. This means that attempting to derive protective concentration thresholds specific to the phytobenthos remains a complicated task. Van den Brink et al. (2006) argued that primary producers have to be used for herbicide risk assessments, because they display the greatest sensitivity to such chemicals. However, algae living in the biofilm matrix and algae living in the water column display differing biological and ecological traits and exposure ways. In this context, we observed that the responses of diatoms and other algal classes are so different that the sensitivity data may generate a bimodal distribution in the SSD curve depending on the herbicide (see isoproturon, Online Resource 2). Some authors suggested to split taxa for SSD building when organisms are not similarly exposed in the environment (Suter et al. 2002) and if sensitivity means are statistically different for different groups of organisms (Versteeg et al. 1999). However, when a consequent dataset is available, it seems more appropriate to build separate SSD curves for algae living in the biofilm matrix and algae living in the water column.

Herbicide risk assessment specific to microphytobenthos remains poorly explored. First, phytobenthos is embedded in an exopolysaccharides matrix that interacts with water constituents and could adsorb hydrophobic chemicals (Wolfaardt et al. 1994; Headley et al. 1998; Lundqvist et al. 2012). As a result, benthic growth in biofilm could modify the exposition of organisms to herbicides and their sensitivity (Dorigo et al. 2010; Larras et al. 2013). The set of species used to build the S-SSD was chosen to maximize the representativeness of the phytobenthos community of Lake Geneva in terms of taxonomy, morphology, and life history traits. Because this type of community is mainly composed of benthic diatom species (Hoagland et al. 1982; Guerrero and Rodriguez 1991), we only worked with Bacillariophyceae. To match the natural mode of growth in the environment and to take into account the presence of an exopolysaccharide matrix, species were grown under benthic conditions for both the assemblage experiment and the single-species bioassays. Second, benthic diatoms display a great diversity of biological traits, which differed from those of algae living in the water column species. For example, chlorophytes are known to be autotrophic species, highly dependent on photosynthesis and very sensitive to PSII inhibitors. Conversely, many benthic diatoms are defined as mixotrophic species (Round et al. 1990; Van Dam et al. 1994) that evolved in a benthic environment where light resources can be limited. In this context, facultative or obligatory heterotrophic species are able to metabolize other substrata (Hellebust and Lewin 1977) to decrease their dependence on photosynthesis. Previous studies evidenced that such heterotrophic benthic diatoms were more tolerant than autotrophic diatoms species to PSII inhibitors (Pérès et al. 1996; Bérard et al. 1998; Debenest et al. 2009). In our study, this was the case for NPAL, CRAC, EOMI, and GPAR species. As a consequence, the results showed that the choice of species highly influenced the derived HC<sub>5</sub> values. For the four single herbicides, the species from the literature dataset were more

**Fig. 1** Specific growth rate evolution of each species in response to the increasing gradient of herbicide mixture concentration after 96 h of exposure in microcosms. Grey and black dash lines represent the EC<sub>50</sub> (nM) predicted with CA model from the sensitivity data obtained under single-species condition and the EC<sub>50</sub> observed in community, respectively

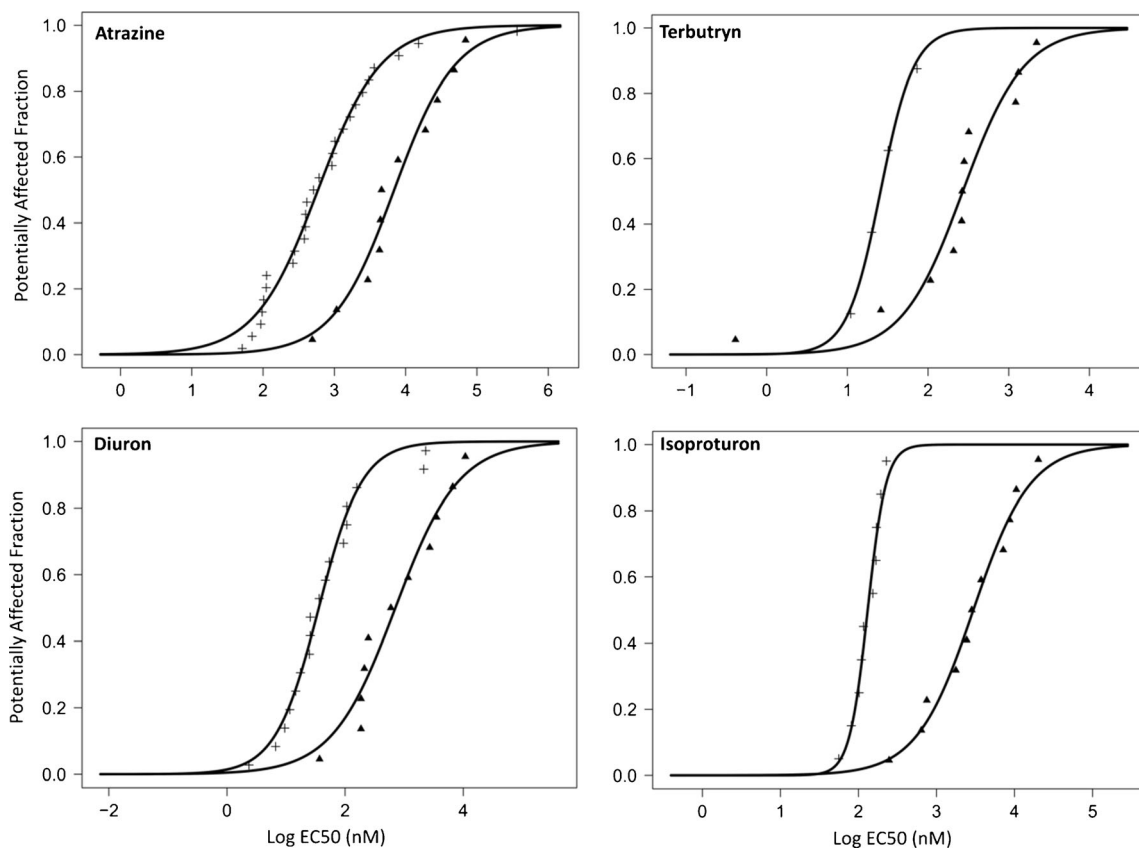


sensitive than specific benthic diatoms as confirmed by the statistical analysis. The literature dataset was mostly based on chlorophytes, which are autotrophic microalgae and more sensitive to PSII inhibitors than benthic diatoms (Tang et al. 1997; Bérard et al. 1998; Bérard and Pelte 1999). The position of the lower EC<sub>50</sub> values within the distribution modifies the lower tail fitting of the SSD curve, which influences HC<sub>5</sub> extrapolation (Versteeg et al. 1999; Wheeler et al. 2002). That was observed in our data set where lower EC<sub>50</sub> values differed strongly between the specific and literature datasets. Indeed, for each herbicide, L-SSDs were built at the right side of S-SSD, due to the lower EC<sub>50</sub> values for literature species

indicating their higher sensitivity. Conversely, because specific species were more tolerant than literature species, S-SSD curves were pulled toward higher concentrations. As a consequence, the derived HC thresholds were higher for specific species.

In the microcosm experiment, the exposed community was finally composed of 7 benthic diatom species (FRUM, ADMI, CRAC, NPAL, GPAR, EOMI, and CMEN) which were part of the 11 species used to build the S-SSD curves. One species (FRUM) exhibited a higher sensitivity to the herbicide mixture, with an EC<sub>50</sub> value in the range of the low S-HC<sub>5mix</sub> threshold. At the opposite, all other species exhibited a higher





**Fig. 2** Literature (*cross*) and specific (*diamond*) SSD curves based on the  $EC_{50}$  for each herbicide

resistance, with  $EC_{50}$  values higher than the  $S-HC_{5mix}$  threshold. Because the lower tail of the four single-substance L-SSD curves was mainly composed by sensitive chlorophytes, the derived  $L-HC_{5mix}$  threshold was lower than that of  $S-HC_{5mix}$ . However, it is clear that risk assessment approaches are theoretical and that uncertainties are linked with the calculation. It is therefore difficult to affirm that the results from the two methods are significantly different.

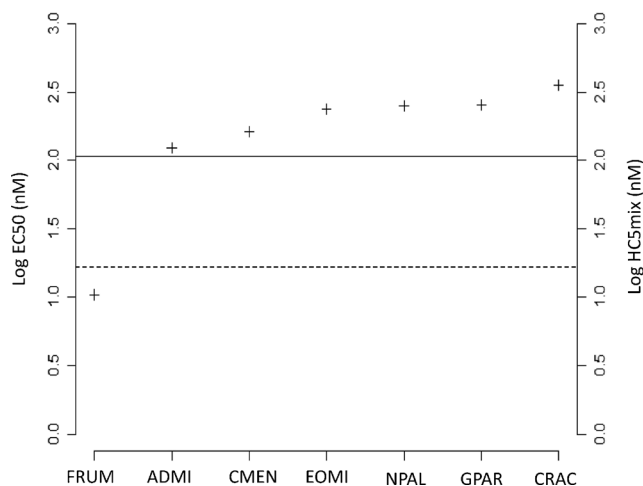
It appears that the predicted  $EC_{50}$  values of several diatoms for the mixture determined from single-species bioassays using the CA model were poorly estimated in the light of the

**Table 4**  $HC_5$ ,  $HC_{50}$ , and slopes of literature and specific SSD curves obtained for each single substance and the quaternary mixture with CA model

	Literature SSD			Specific SSD		
	$HC_5$	$HC_{50}$	slope	$HC_5$	$HC_{50}$	slope
Diuron	2.98	36	1.18	13.2	789	0.72
Isoproturon	59.26	131	3.69	250	2967	1.19
Atrazine	29.50	590	0.98	375	6904	1.01
Terbutryn	6.35	25.78	2.1	20.8	269	1.15
Mixture	16.71	146	–	106	2733	–

$HC$  values are expressed in nanomolars. A hyphen indicates no available data

$EC_{50}$  obtained under multi-species conditions. The sensitivity of benthic diatoms appeared to change with the biological complexity of the system: EOMI, CRAC, GPAR, and NPAL were highly more sensitive under multi- than under single-species conditions. Single-species bioassays are widely used in ecotoxicology, and the data obtained from these tests are supposed to reflect the sensitivity of species in their natural



**Fig. 3**  $EC_{50}$  (nM) of each species for the mixture based on specific growth rate observed in the assemblage. The *dotted line* represents the  $HC_{5mix}$  derived from the  $L-SSD_{mix}$  and the *full line* represents the  $HC_{5mix}$  derived from the  $S-SSD_{mix}$

environment, even though they are in fact conducted in the laboratory under optimum conditions. Even if these tests are useful tools for assessing the basic toxicity of chemicals and appear to be fundamental for risk assessment, many studies have shown that they are poorly representative of the environment (Cairns 1984; Chapman 1995). In the literature, the changes in sensitivity with increasing biological complexity levels are widely discussed for various macroalgae and microalgae. Coutris et al. (2011) observed no difference in sensitivity between single-species conditions and complex communities for macrophytes exposed to a mixture of atrazine, isoproturon, and alachlor for a short period of time. However, Bérard et al. (2003) reported that microalgae can be more sensitive to PSII inhibitors such as atrazine and irgarol in a community than in single-species conditions. In our study, the main difference observed between the two conditions was the level of biological complexity. Under multi-species conditions, there was probably competition for resources, especially with presence of high nutrient consumer species like EOMI, CRAC, GPAR, and NPAL. In this case, the apparent herbicide sensitivity of these species is greater than when determined under single-species conditions. Moreover, the two autotrophic species (FRUM and CMEN) used in our experiments displayed similar sensitivities under single- and multi-species conditions. In community, organisms more dependent on nutrients may be more severely affected by herbicides, because they have to compete with other species and this can make them less able to cope with the toxic effects. This competition effect should be probably less marked in high-nutrient media. However, we performed the microcosms and the single-species bioassays under similar physico-chemical conditions, especially using the same high nutrient concentration provided by the specific diatom culture media, and so the main difference appeared to be the presence or the absence of interspecies competition for resources. Consequently, the underestimation of species sensitivities evaluated under single-species bioassays may lead to a poorly estimated protection HC5 that may be under-protective in real environmental conditions in presence of biotic interactions.

## Conclusion

In summary, this study showed that the choice of incoming data for building SSD play a crucial role and may change greatly the values of the HC thresholds. In our study, SSD based on literature data were more sensitive than the one obtained with specific data. This is reassuring as threshold derived from these literature SSDs can be assumed to protect also local benthic algae in Lake Geneva. However, getting data for local species is important and one could expect local species to be more sensitive than literature data. Indeed, diatoms are complex microalgae displaying many characteristics

that have to be considered in risk assessment when their communities have to be protected. Furthermore, a discrepancy exists between the sensitivity of diatoms determined under single- and multi-species conditions, which may be attributable to interspecies interactions. Even though this was observed here at the EC<sub>50</sub> level, we would also expect sensitivity differences to occur at threshold levels widely used in risk assessment, such as the EC<sub>10</sub> and even the no-observed-effect concentration (NOEC). Consequently, if SSD models are based on data that underestimate the sensitivity of species, as a result of this discrepancy, the HC<sub>5</sub> thresholds are likely to be less protective than expected. Further studies on communities would be important to increase the predictive power of the threshold defined for risk assessment.

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