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Emerging contaminants in Belgian marine waters: Single toxicant and mixture risks of pharmaceuticals

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ABSTRACT

Knowledge on the effects of pharmaceuticals on aquatic marine ecosystems is limited. The aim of this study was therefore to establish the effect thresholds of pharmaceutical compounds occurring in the Belgian marine environment for the marine diatom *Phaeodactylum tricornutum*, and subsequently perform an environmental risk assessment for these substances. Additionally, a screening-level risk assessment was performed for the pharmaceutical mixtures.

No immediate risk for acute toxic effects of these compounds on *P. tricornutum* were apparent at the concentrations observed in the Belgian marine environment. In two Belgian coastal harbours however, a potential chronic risk was observed for the β-blocker propranolol. No additional risks arising from the exposure to mixtures of pharmaceuticals present in the sampling area could be detected. However, as risk characterization ratios for mixtures of up to 0.5 were observed, mixture effects could emerge should more compounds be taken into account.

1. Introduction

The occurrence of pharmaceutical compounds in the aquatic environment has received increasing attention in recent years as concerns have risen about their environmental persistence and biological activity (Fent, 2008). Indeed, drug residues have been shown to occur in many freshwater (as reviewed by for example Kümmerer, 2008) and marine ecosystems (Weigel et al., 2002; Wille et al., 2010b). These compounds end up in the environment mainly through municipal wastewater, but also due to disposal of unused medicines (Bound and Voulvoulis, 2004), wastewater from drug manufacturers and hospitals and landfill leachates (Holm et al., 1995). Moreover, many of these compounds are not readily degraded in sewage treatment plants (Fent, 2008). Pharmaceuticals occurring in the environment include antibiotics, painkillers, lipid regulators, β-blockers and neuroactive compounds (Kümmerer, 2008).

In the freshwater environment, pharmaceuticals are generally detected at concentrations in the ng L^{-1} to µg L^{-1} range. Much higher concentrations (up to 31 mg L^{-1}) have been found in for example discharges of drug manufacturing facilities (Larsson et al., 2007). In the marine environment, reported concentrations are generally in the low ng L^{-1} range. Thomas and Hilton (2004) reported concentrations up to 0.928 µg L^{-1} of the analgesic ibuprofen, and up to 0.57 µg L^{-1} of the antibiotic trimethoprim in UK estuaries. Wille et al. (2010b, 2011b) studied the occurrence of 13 pharmaceutical compounds in the Belgian coastal zone and reported concentrations of salicylic acid up to 0.855 µg L^{-1} within a Belgian coastal harbour, and up to 0.660 µg L^{-1} at open sea stations close to the shore. This compound was still detected at sampling stations located roughly 20 km off shore, at concentrations up to 0.237 µg L^{-1} and was also found in the bivalve *Mytilus edulis* at levels up to 490 ng g^{-1} dry weight. The neuroactive compound carbamazepine occurred at concentrations up to 12 ng L^{-1} at roughly 10 km off shore and was detected regularly in *M. edulis*. The remaining pharmaceuticals were only detected in the coastal harbours with a single occurrence of the β-blocker propranolol (at 1 ng L^{-1}) and the lipid regulator bezafibrate (at 8 ng L^{-1}) close to the shoreline. Propranolol was sporadically detected in *M. edulis* at levels up to 52 ng g^{-1} dry weight.

The above illustrates that contamination of the aquatic environment by pharmaceutical compounds is certainly not limited to freshwater ecosystems. Despite this, little is known about the risks these substances pose to the marine environment. Therefore, the objective of this study was to study the toxicity of pharmaceuticals occurring in the Belgian marine environment (as studied by Wille et al., 2010b) to a marine species – the diatom *Phaeodactylum tricornutum* – and subsequently perform an environmental risk

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assessment for this environment, including the potential risks arising from mixture toxicity of the detected compounds.

2. Materials and methods

2.1. Chemicals

In total, seven pharmaceutical compounds were used for ecotoxicity testing (Table 1). Salicylic acid (⩾99%), paracetamol (⩾99%), carbamazepine (>90%), atenolol (⩾98%), propranolol (⩾99%), bezafibrate (⩾98%) and trimethoprim (⩾98%) were all purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Toxicity testing

The marine diatom *P. tricornutum* Bohlin was obtained from the Culture Collection of Algae and Protozoa (CCAP 1052/1A, Oban, United Kingdom). A subculture was maintained in the laboratory in growth medium prepared as described in the ISO 10253 standard (ISO, 2006). Three days prior to the start of a growth inhibition test, a pre-culture was prepared by adding algal stock culture to fresh growth medium to obtain a cell density between 2000 and 10,000 cells/mL. The pre-culture was allowed to grow on a rotary shaker at 20 ± 1 °C under continuous illumination.

Stock solutions were prepared by dissolving the pharmaceutical compounds in growth medium with the aid of ultrasonication where necessary. For each pharmaceutical compound, a series of five different test concentrations was prepared in 200 mL of growth medium by adding the correct amount of stock solution. The test solutions (including a 200 mL control medium) were allowed to equilibrate overnight at 20 °C in the dark. Subsequently, each solution was divided in 50 mL portions and transferred to a 100 mL conical flask. Three flasks were inoculated with 10,000 cells/mL of the 3 day old culture and one was used for a background correction. All flasks were incubated at 20 ± 1 °C under continuous white light (6000–10,000 lx) and were shaken manually three times a day. The algal cell density was measured after 24, 48 and 72 h using an electronic particle counter (Coulter Counter model DN, Harpenden, Herts, UK). The temperature and pH of the test medium were measured daily.

2.3. Chemical analysis

Test concentrations were measured using the method by Wille et al. (2010b). Briefly, samples of the test concentrations were diluted and subsequently brought to a pH 6–8. Isobutcar 61 was added to each sample as an internal standard. Solid-phase extraction of the samples was performed using Chromabond HR-X cartridges (6 mL, 200 mg, Macherey–Nagel, Düren, Germany) followed by elution using 5 mL acetone and two times 5 mL methanol. Extracts were dried using nitrogen and the residues redissolved in acetonitrile/0.02 M formic acid (50/50). Analysis was carried out using high-performance liquid chromatography. The equipment consisted of an 1100 series quaternary gradient pump and autosampler (Hewlett Packard, Palo Alto, CA, USA) and a Nucleodur® C18 Isis HPLC column (5-µm particle size, 250 mm 4.0 mm, Macherey–Nagel, Düren, Germany). Analytes were detected with an LCQ DECA ion trap mass spectrometer equipped with an electrospray ionization (ESI) interface (Thermo Finnigan, San Jose, CA, USA). Further details can be found in Wille et al. (2010b).

2.4. Data analysis

To estimate the EC50 and EC10 (the concentrations inducing a growth inhibition of 50% and 10%, respectively), the average specific growth rate μ was calculated for each test culture using Eq. (1) (ISO, 2006):

$$\mu = \frac{\ln N_L - \ln N_0}{t_L - t_0} \quad (1)$$

with t_0 as the time of the test start, t_L as the time of test termination (72 h), N_0 as the nominal initial cell density and N_L as the measured cell density at time t_L. Subsequently a logistic response model was fitted to the concentration–response data (De Schamphelaere and Janssen, 2004):

$$\mu = \frac{1}{1 + \left(\frac{x}{exp(a)}\right)^{\ln(1/9)/(a-b)}} \quad (2)$$

with x as the exposure concentration, a as ln(EC50) and b as ln(EC10). For parameter estimation and calculation of the 95% confidence limits, the Levenberg–Marquardt method was used (Levenberg, 1944; Marquardt, 1963). All statistics were performed using the Statistica® software program (Statsoft, Tulsa, OK, USA).

2.5. Environmental risk assessment

The ecotoxicity data from this study were combined with literature data and subsequently used to calculate predicted no effect concentrations (PNECs) for the marine environment. To this end, an appropriate assessment factor was applied to the lowest available acute or chronic toxicity value following the rules described in the most recent guidelines relating to the European REACH legislation (EU, 2006; ECHA, 2008a). The measured environmental concentrations (MECs) used for the risk assessment were taken from Wille et al. (2010b) and are summarized in Table 2. In this study, water samples were collected four times over a timespan of 3 years (2007–2009) in coastal harbours, off-shore locations along the Belgian coastal zone and locations on the river Scheldt (see Fig. 1) and subsequently analyzed for the presence of a set of 13 pharmaceuticals. Paracetamol was also detected at the sampling stations used in this study, but the concentrations could not be quantified due to technical difficulties (unpublished data). Whenever a pharmaceutical could not be detected, the MEC was set at half the limit of quantification (LOQ). In such a case, often

Table 1
Physico-chemical properties of the target compounds. *References*: Dal Pozzo et al. (1989), Yalkowsky and Dannenfelser (1992), Hansch et al. (1995), Granberg and Rasmuson (1999), McFarland et al. (2001), Bones et al. (2006), and Paschke et al. (2007).

Compound	Type	$\log K_{ow}$	Solubility in water at 20–25 °C (mg L^{-1})
Salicylic acid	NSAID	2.26	2240
Paracetamol	Analgesic	0.46	12,780
Carbamazepine	Neuroactive compound	2.45	112
Atenolol	β-blocker	0.16	13,300
Propranolol	β-blocker	3.48	61.7
Bezafibrate	Lipid regulator	3.85	0.355
Trimethoprim	Antibiotic	0.91	400

Table 2
Ranges of the pharmaceutical concentrations (ng L^{-1}) measured along the Belgian coastal zone as adapted from Wille et al. (2010b). SAL: salicylic acid; CAR: carbamazepine; ATE: atenolol; PRO: propranolol; BEZ: bezafibrate; TRI: trimethoprim; ND: not detected.

Station	SAL	CAR	ATE	PRO	BEZ	TRI
W01	102–660	11–19	ND	ND–1	ND–8	ND
W02	26–412	ND–14	ND	ND	ND	ND
W03	ND–106	ND–4	ND	ND	ND	ND
W04	65–227	7–12	ND	ND	ND	ND
W05	18–237	ND	ND	ND	ND	ND
W06	ND–60	ND	ND	ND	ND	ND
NP1	44–306	19–68	ND	ND–12	ND	ND
NP2	ND–94	7–54	ND	ND–12	ND	ND–17
NP3	11–177	ND–37	ND	ND–7	ND	ND
OO1	203–598	21–31	ND	ND–5	ND–5	ND
OO2	74–855	19–119	ND–88	6–24	7–18	ND–29
OO3	43–374	32–36	ND	3–11	7–12	ND–13
OO4	ND–161	16–36	ND–80	ND–12	6–11	ND
ZB1	16–136	10–30	ND	ND–3	ND	ND
ZB2	87–312	10–25	ND	ND–3	ND	ND
ZB3	80–310	11–23	ND	ND–4	ND	ND
ZB4	ND–197	11–24	ND	ND–3	ND	ND
S01	51–307	5–27	ND	ND–3	ND–6	ND
S22	71–372	129–321	ND–293	10–22	ND–16	ND

half the limit of detection (LOD) is used. However, as Wille et al. (2010b) did not report LODs, we used the reported LOQs. This was not considered a problem, since using the LOQ instead of the LOD makes the risk assessment more conservative (as LOQ > LOD). Hence, there was no danger of underestimating the risk. Based on the PNEC and PEC values, the risk characterization ratio (RCR) was calculated as:

$$\mathrm{RCR} = \frac{\mathrm{MEC}}{\mathrm{PNEC}} \tag{3}$$

An RCR higher or equal to unity indicates that the ecological risks associated with the respective chemical are not adequately controlled (ECHA, 2008b).

Additionally, a screening level assessment of the risk posed by the pharmaceutical mixtures was performed using the stepwise approach proposed by Backhaus and Faust (2012). These authors propose to use the concept of concentration addition (CA) as a precautious first step in a mixture risk assessment as it generally provides the more conservative risk estimate (as compared to the concept of independent action). The risk quotient based on CA (RQ_{STU}) is calculated as:

$$\begin{aligned} \mathrm{RQ}_{STU} &= max(\mathrm{STU}_{algae}, \mathrm{STU}_{daphnid}, \mathrm{STU}_{fish}) \times \mathrm{AF} \\ &= max\left(\sum_{i=1}^{n}\frac{\mathrm{MEC}_i}{\mathrm{EC50}_{i,algae}}, \sum_{i=1}^{n}\frac{\mathrm{MEC}_i}{\mathrm{EC50}_{i,daphnid}}, \sum_{i=1}^{n}\frac{\mathrm{MEC}_i}{\mathrm{EC50}_{i,fish}}\right) \\ &\quad \times \mathrm{AF} \end{aligned} \tag{4}$$

with STU as the sum of toxic units for the respective trophic level or organism group and AF as the assessment factor. As can be seen from the formula, it is a calculation in two steps in which the STU of the most sensitive trophic level – i.e. the trophic level exhibiting the highest STU – (step 1) is used to calculate the final RQ_{STU} (step 2). AF was set at 10,000 for the marine sampling points (ECHA, 2008a). Backhaus and Faust (2012) also propose the use of $RQ_{MEC/PNEC}$, which is based on the RCR of the individual mixture components and is calculated as:

$$\mathrm{RQ}_{MEC/PNEC} = \sum_{i=1}^{n}\frac{\mathrm{MEC}_i}{\mathrm{PNEC}_i} = \sum_{i=1}^{n}\mathrm{RCR}_i \tag{5}$$

with RCR_i as the RCR of the ith of n pharmaceuticals in the mixture. While some discourage the use of $RQ_{MEC/PNEC}$ (e.g. SCHER, 2012), it is more conservative and less dependent on the availability of a full ecotoxicity dataset than RQ_{STU} and therefore it serves well as a first screening (Backhaus and Faust, 2012). Therefore, in this study $RQ_{MEC/PNEC}$ was calculated first for each pharmaceutical mixture. For each case in which $RQ_{MEC/PNEC}$ exceeded unity, RQ_{STU} was calculated as well.

3. Results and discussion

3.1. Acute toxicity to P. tricornutum

Table 3 presents the acute toxicity of the target compounds to the marine diatom *P. tricornutum*. For bezafibrate, no effect was observed up to its limit of solubility and hence no effect concentration could be derived. The β-blocker propranolol and the antibiotic trimethoprim were the most toxic substances for the test organism with 72 h EC50 values of 0.288 and 5.1 mg L^{-1}, respectively. Moreover, *P. tricornutum* seemed to be more sensitive to these substances than other (phytoplankton) species (see Fig. 2), even though the difference is relatively small. For propranolol, this was also observed for the marine diatom *Cyclotella meneghiniana* for which an EC50 value of 0.244 mg L^{-1} was reported in a 96 h growth inhibition test (Ferrari et al., 2004). *P. tricornutum* was much less sensitive to the other tested β-blocker atenolol and showed only an average sensitivity compared to other phytoplankton (see Fig. 2). The fact that zooplankton generally also exhibit a greater sensitivity towards propranolol compared to atenolol, has been attributed to the strong membrane stabilizing properties of the former (Fent, 2008). As such, (marine) diatoms may be more sensitive than green algae to adverse effects on membrane stability, but this is at this point speculative. For trimethoprim, no data for diatoms could be found in literature, but green algae in general seem to be sensitive to antibiotics as well. Indeed, in one study the green alga *Pseudokirchneriella subcapitata* exhibited EC50 values for antibiotics between 0.002 mg L^{-1} (clarithromycin) and 0.52 mg L^{-1} (ofloxacin; Isidori et al., 2005). Yang et al. (2008) reported an EC50 of 40 mg L^{-1} for trimethoprim for the same species. If *P. tricornutum* would display a similar sensitivity pattern towards antibiotics, this would imply that antibiotics other than trimethoprim (e.g. clarithromycin) could be highly toxic to this marine diatom. Further studies are warranted to confirm this hypothesis.

All other tested pharmaceuticals showed moderate (carbamazepine) to low (salicylic acid, paracetamol and atenolol) acute toxicity towards *P. tricornutum*. In general, the same observation has been made for (mainly freshwater) organisms of other trophic levels. Indeed, Fent (2008) summarized that the majority of the most studied pharmaceuticals have E/LC50 values above 1 mg L^{-1} and about 38% exhibit E/LC50 values above 100 mg L^{-1}. As noted above, antibiotics in general and the β-blocker propranolol in specific form exceptions.

3.2. Environmental risk assessment

When comparing the MECs (Table 2) to the ecotoxicity data generated for *P. tricornutum* in this study, no acute toxicity is expected at any of the sampling stations for the test species. Indeed, as the highest measured concentrations of the pharmaceuticals are between roughly 130,000 (for carbamazepine) and 4000 (for propranolol) times lower than their respective EC10 values, any acute toxic effects towards the test species are highly unlikely. This finding is similar to the conclusion by Fent (2008) who stated that acute toxicity of pharmaceuticals to aquatic organisms in general, is unlikely to occur at the measured concentrations.

Table 4 presents the PNEC values for the marine environment as derived from the data of this study and data from literature. For

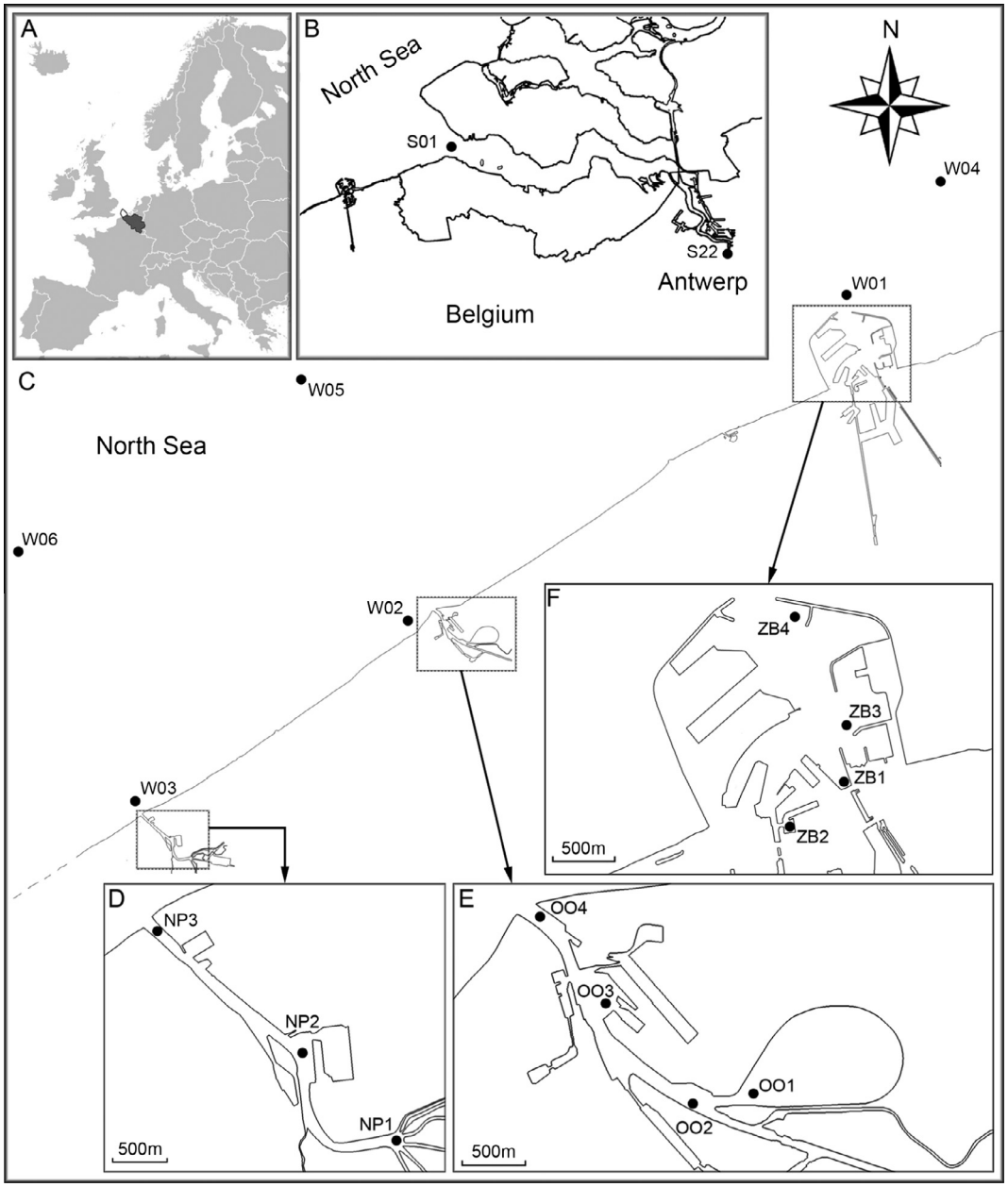

Fig. 1. Overview of the sampling stations in the Belgian coastal zone (adapted from Wille et al. (2010b)). A: overview map showing the location of Belgium in Europe; B: map showing the additional sampling stations on the Scheldt river (S01 and S22); C: overview of the Belgian coast depicting the six offshore stations (W01–W06); D: detail of Nieuwpoort harbour with three sampling stations (NP1–NP3); E: detail of Oostende harbour with four sampling stations (OO1–OO4); F: detail of Zeebrugge harbour with four sampling stations (ZB1–ZB4).

Table 3
Effect concentrations of the pharmaceutical compounds obtained with the 72 h growth inhibition test with *P. tricornutum* (95% confidence limits are given between parentheses). WS: water solubility.

Substance	EC50 (mg L^{-1})	EC10 (mg L^{-1})
Salicylic acid	255.5 (242.2–269.6)	96.7 (84.9–110.2)
Paracetamol	265.8 (239.4–295.1)	93.4 (72.1–121)
Carbamazepine	62.5 (58.8–66.6)	42.2 (38.4–46.4)
Atenolol	311.9 (262.4–370.7)	6.9 (3.3–14.4)
Propranolol	0.288 (0.252–0.329)	0.09 (0.066–0.124)
Bezafibrate	>WS	>WS
Trimethoprim	5.1 (4.7–5.5)	2.4 (2–2.9)

sampling station S22 (located far upstream the Scheldt river; see Fig. 1) separate PNEC values for freshwater were derived using a lower assessment factor (AF). This AF was generally a factor 10 lower than the AF used for the marine aquatic environment (ECHA, 2008a). The maximum RCRs determined for the different sampling periods are presented in Table 5. The RCRs indicated a potential ecological risk from chronic exposure to propranolol at five sampling stations: two in the harbour of Nieuwpoort, three in the harbour of Oostende. At stations NP1, NP2, OO3 and OO4 the RCR of propranolol exceeded unity only once over the four sampling periods. At station OO2 this occurred three times (in May 2007, April 2008 and June 2009). For all other pharmaceuticals, no potential chronic risk could be identified. Similar risk assessments are scarce and have been performed exclusively for the freshwater environment. Cleuvers (2005) for example, studied the risk of three β-blockers (including atenolol and propranolol) in freshwater environments, of which only propranolol exhibited an RCR close to 1.

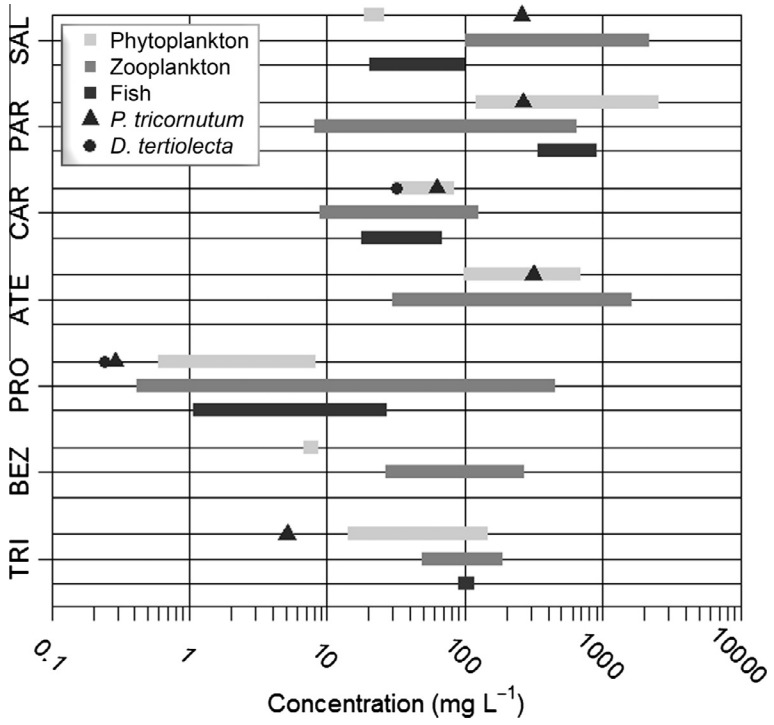

Fig. 2. Acute toxicity of the target compounds to aquatic organisms. The bars represent ranges of toxicity data from different organisms and/or experiments. Data of the marine diatoms *Phaeodactylum tricornutum* and *Dunaliella tertiolecta* are from this study and from Ferrari et al. (2004), respectively. *Other references*: Knie et al. (1983), Kühn et al. (1989), Wang and Lay (1989), Calleja et al. (1994), Broderius et al. (1995), Lilius et al. (1995), Henschel et al. (1997), Lützhøft et al. (1999), Halling-Sørensen et al. (2000), Bachmann (2002), Huggett et al. (2002), Jos et al. (2003), Cleuvers (2003), Cleuvers (2005), Eguchi et al. (2004), Ferrari et al. (2004), Hernando et al. (2004), Marques et al. (2004), Fraysse and Garric (2005), Kamaya et al. (2005), Park (2005), Han et al. (2006), Stanley et al. (2006), Isidori et al. (2007), Kim et al. (2007), Choi et al. (2008), Dussault et al. (2008), Kim et al. (2009), Park and Choi (2008), Yang et al. (2008), De Andrés et al. (2009), De Liguoro et al. (2009), Liu et al. (2009), Nassef et al. (2009), Küster et al. (2010), and Rosal et al. (2010). A full list of the literature ecotoxicity data can be found in Table A.1. SAL: salicylic acid; PAR: paracetamol; CAR: carbamazepine; ATE: atenolol; PRO: propranolol; BEZ: bezafibrate; TRI: trimethoprim.

Table 4
PNEC values of the test compounds for the marine environment derived from this study and literature review. The ecotoxicity values used for the PNEC derivation, are presented. AF: assessment factor (selected according to ECHA (2008a)), PNEC: predicted no effect concentration.

Pharmaceutical	Concentration ($mg\ L^{-1}$)	Exposure duration	Assessement endpoint	Organism	Species	Reference	AF	PNEC ($ng\ L^{-1}$)
Salicylic acid	5.6	21 d	Reproduction, NOEC	Invertebrate	*D. longispina*	Marques et al. (2004)	500	11,200
Paracetamol	9.2	48 h	Immobility, EC50	Invertebrate	*D. magna*	Kühn et al. (1989)	10,000	920
Carbamazepine	0.025	7 d	Reproduction, NOEC	Invertebrate	*C. dubia*	Ferrari et al. (2004)	100	250
Atenolol	3.2	28 d	Growth, NOEC	Fish	*P. promelas*	Winter et al. (2008)	100	32,000
Propranolol	0.001	27 d	Reproduction, NOEC	Invertebrate	*H. azteca*	Huggett et al. (2002)	100	10
Bezafibrate	0.023	7 d	Reproduction, NOEC	Invertebrate	*C. dubia*	Isidori et al. (2007)	1000	23
Trimethoprim	2.4	72 h	Growth, EC10	Diatom	*P. tricornutum*	This study	500	4800

Halling-Sørensen et al. (2000) studied the environmental risks of three antibiotics and came to a similar freshwater RCR for trimethoprim (i.e. 9.4×10^{-3}). Han et al. (2006) performed an environmental risk assessment for seven pharmaceuticals (including salicylic acid, paracetamol and carbamazepine) in the effluent of wastewater treatment plants and did not identify a risk. And finally, in a case study involving atenolol in the EU (Küster et al., 2010), a maximum RCR of 0.003 was observed in freshwater under a worst case scenario. This is similar to the RCR values for atenolol observed in this study (see Table 5).

Table 6 presents the $RCR_{MEC/PNEC}$ values of the pharmaceutical mixtures at the different sampling stations. Overall, trimethoprim and atenolol combined contributed less than 1% and propranolol and bezafibrate combined contributed roughly 77% to the toxicity of the mixtures (see Table B.1). This was due to the high and low respective PNEC values of these two pairs of pharmaceuticals. Indeed, even at the six sampling stations at sea propranolol and bezafibrate were the two most dominant chemicals despite being mainly present at levels below their respective LOQs. Besides the seven occasions identified above for which there was already a risk caused by an individual pharmaceutical compound, three additional cases were identified posing a potential risk originating from the mixture of pharmaceuticals. For these three cases, RCR_{STU} was calculated (see Table 7) which no longer indicated a potential risk posed by the pharmaceutical mixtures. However, given that only six compounds were included in the mixture risk assessment, the RCR_{STU} values – which ranged from 0.33 to 0.50 – were nonetheless relatively high. Indeed, as in the studied area many more chemicals

Table 5
Maximum values of the risk characterization ratios (RCRs) determined for the different sampling periods. Bold values emphasis stations at which the RCR was higher than 1. SAL: salicylic acid; CAR: carbamazepine; ATE: atenolol; PRO: propranolol; BEZ: bezafibrate; TRI: trimethoprim.

Station	SAL	CAR	ATE	PRO	BEZ	TRI
W01	0.059	0.076	0.001	0.100	0.348	0.001
W02	0.037	0.056	0.001	0.050	0.109	0.001
W03	0.009	0.016	0.001	0.050	0.109	0.001
W04	0.020	0.048	0.001	0.050	0.109	0.001
W05	0.021	0.010	0.001	0.050	0.109	0.001
W06	0.005	0.010	0.001	0.050	0.109	0.001
NP1	0.027	0.272	0.001	**1.200**	0.109	0.001
NP2	0.008	0.216	0.001	**1.200**	0.109	0.004
NP3	0.016	0.148	0.001	0.700	0.109	0.001
OO1	0.053	0.124	0.001	0.500	0.217	0.001
OO2	0.076	0.476	0.003	**2.400**	0.783	0.006
OO3	0.033	0.144	0.001	**1.100**	0.522	0.003
OO4	0.014	0.144	0.003	**1.200**	0.478	0.001
ZB1	0.012	0.120	0.001	0.300	0.109	0.001
ZB2	0.028	0.100	0.001	0.300	0.109	0.001
ZB3	0.028	0.092	0.001	0.400	0.109	0.001
ZB4	0.018	0.096	0.001	0.300	0.109	0.001
S01	0.027	0.108	0.001	0.300	0.261	0.001
S22	0.003	0.128	0.001	0.440	0.070	0.000

Table 6
The $RCR_{MEC/PNEC}$ (as determined using Eq. (5)) of the pharmaceutical mixtures detected at the different sampling stations. Bold values emphasis stations at which the $RCR_{MEC/PNEC}$ was higher than 1. Underlined values indicate cases for which no risk originating from an individual pharmaceutical was observed.

Station	Sampling campaign			
	May 2007	December 2007	April 2008	June 2009
W01	0.244	0.345	0.473	0.228
W02	0.173	0.195	0.222	0.207
W03	0.175	0.180	0.182	0.171
W04	0.194	0.207	0.216	0.209
W05	0.172	0.173	0.176	0.192
W06	0.171	0.176	0.175	0.176
NP1	0.830	**1.591**	0.606	0.264
NP2	0.473	**1.537**	0.395	0.189
NP3	0.172	0.969	0.109	0.186
OO1	0.865	0.580	0.557	0.287
OO2	**3.741**	**1.015**	**2.763**	**2.304**
OO3	–	**1.572**	**1.550**	0.916
OO4	0.641	**1.511**	**1.613**	0.241
ZB1	0.259	0.279	0.541	0.202
ZB2	0.212	0.271	0.518	0.240
ZB3	0.214	0.603	0.410	0.268
ZB4	0.258	0.524	0.404	0.225
S01	0.185	0.295	0.679	0.244
S22	0.642	0.286	0.526	0.365

Table 7
The sum of toxic units (STUs) per trophic level and RC_{STU} for the mixtures for which a potential risk was identified. Cases in which there was already a risk posed by an individual mixture constituent are not included. Bold values indicate the highest STU values, which were subsequently used for the calculation of RC_{STU} according to Eq. (4). AF: assessment factor.

Sampling event	Algae	Daphnid	Fish	AF	RC_{STU}
OO2–Dec2007	$\mathbf{4.30 \times 10^{-05}}$	1.86×10^{-05}	2.17×10^{-05}	10,000	0.43
OO3–Dec2007	$\mathbf{5.04 \times 10^{-05}}$	2.51×10^{-05}	2.02×10^{-05}	10,000	0.50
OO4–Dec2007	$\mathbf{4.49 \times 10^{-05}}$	2.55×10^{-05}	1.47×10^{-05}	10,000	0.45

(e.g. pesticides, organotins, perfluorinated compounds, polycyclic aromatic hydrocarbons, polychlorinated biphenyls) are present (Claessens et al., 2010; Wille et al., 2010a, 2011a; Verhaegen et al., 2012), it is not unlikely that a risk from mixtures might arise when taking other compounds into account as well. Moreover, as potential interactions between chemicals in the mixture are not taken into account with the present approach (Backhaus and Faust, 2012), a more profound risk assessment of the mixtures present in this sampling zone is certainly warranted. This is enforced by the results of Ginebreda et al. (2010), who calculated hazard indices (similar to RCR_{STU} of this study) for pharmaceutical mixtures occurring in a Spanish river basin. These authors found consistently higher hazard indices (HI) for algae compared to other trophic levels, with values up to 103. The antibiotic sulfamethoxazole, the lipid regulator gemfibrozil and the nonsteroidal anti-inflammatory drug ibuprofen were by far the most important contributors (out of a total of 24 pharmaceuticals) to the identified risks to algae. Of all the pharmaceuticals included in our study, only propranolol was not included in the work of Ginebreda et al. (2010). While this compound was identified as the most toxic in our study, this nonetheless illustrates that there are multiple other pharmaceuticals that can have a profound impact on the cumulative risk of these compounds. As such, more research is needed before risks of pharmaceutical mixtures to the marine aquatic environment can be confirmed or excluded. Such research should include the generation of more ecotoxicity data for marine species and studies on potential interaction between different mixture constituents.

4. Conclusions

Ecotoxicity data for seven pharmaceuticals were generated for the marine diatom *P. tricornutum* in a 72 h growth inhibition test. The resulting data indicated no immediate risk for acute toxic effects of these compounds at the concentrations present in the Belgian marine environment. At five sampling stations in two Belgian coastal harbours, a potential chronic risk was observed for the β-blocker propranolol. No additional risks arising from the exposure to mixtures of pharmaceuticals present in the sampling area could be detected. However, as RCR_{STU} values of up to 0.5 were observed, mixture effects could emerge when more compounds are taken into account. Therefore, more studies on the potential risks of pharmaceutical mixtures for the marine environment are required. Such studies should focus on the generation of more ecotoxicity data for marine species and on potential interactions between mixture constituents.

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Appendix A. Literature ecotoxicity data

See Table A.1.

Appendix B. Individual contribution of components to total mixture toxicity

The individual contribution of pharmaceutical i (IC_i) to the cumulative risk posed by the entire mixtures ($RCR_{MEC/PNEC}$), was calculated as:

$$IC_i = \frac{RCR_i}{RCR_{MEC/PNEC}} \cdot 100 \quad \text{(B.1)}$$

The results are summarized in Table B.1.

Table A.1
Literature ecotoxicity data for the target compounds.

Compound	Species	Endpoint	Concentration (mg L^{-1})	Reference
	Phytoplankton			
Salicylic acid	*S. subspicatus*	72 h, growth inhibition, EC50	>100	Henschel et al. (1997)
	P. subcapitata	72 h, growth inhibition, EC50	22.7	Kamaya et al. (2006)
	Zooplankton			
Salicylic acid	*D. magna*	48 h, immobility, EC50	111.7	Han et al. (2006)
	D. magna	48 h, immobility, EC50	118	Henschel et al. (1997)
	D. magna	48 h, immobility, EC50	870	Kamaya et al. (2005)
	D. magna	Immobility, EC50	143	Knie et al. (1983)
	D. magna	48 h, immobility, EC50	1945	Marques et al. (2004)
	D. longispina	48 h, immobility, EC50	1148	Marques et al. (2004)
	D. magna	24 h, immobility, EC50	230	Wang and Lay (1989)
	D. longispina	21 d, reproduction, NOEC	5.6	Marques et al. (2004)
	D. magna	21 d, reproduction, NOEC	>10	Marques et al. (2004)
	Fish			
Salicylic acid	*D. rerio*	48 h, mortality, LC50	24.6	Bachmann (2002)
	B. rerio	48 h, mortality, LC50	37	Henschel et al. (1997)
	L. idus	Mortality, LC50	90	Knie et al. (1983)
	Phytoplankton			
Paracetamol	*S. subspicatus*	72 h, growth inhibition, EC50	134	Henschel et al. (1997)
	P. subcapitata	96 h, growth inhibition, EC50	2300	Yamamoto et al. (2007)
	P. subcapitata	96 h, growth inhibition, NOEC	550	Yamamoto et al. (2007)
	Zooplankton			
Paracetamol	*D. magna*	24 h, immobility, EC50	55.5	Calleja et al. (1994)
	A. salina	24 h, mortality, LC50	577.5	Calleja et al. (1994)
	S. proboscideus	24 h, mortality, LC50	29.6	Calleja et al. (1994)
	D. magna	48 h, mortality, LC50	20.1	Han et al. (2006)
	D. magna	48 h, immobility, EC50	50	Henschel et al. (1997)
	D. magna	48 h, immobility, EC50	30.1	Kim et al. (2007)
	D. magna	48 h, immobility, EC50	9.2	Kühn et al. (1989)
	D. pulex	24 h, immobility, EC50	136	Lilius et al. (1995)
	D. magna	48 h, immobility, EC50	17	Yamamoto et al. (2007)
	Fish			
Paracetamol	*P. promelas*	96 h, mortality, LC50	814	Broderius et al. (1995)
	B. rerio	48 h, mortality, LC50	378	Henschel et al. (1997)
	O. latipes	96 h, mortality, LC50	800	Yamamoto et al. (2007)
	Phytoplankton			
Carbamazepine	*D. subspicatus*	72 h, growth inhibition, EC50	74	Cleuvers (2003)
	C. meneghiniana	96 h, growth inhibition, EC50	31.6	Ferrari et al. (2004)
	S. leopolensis	96 h, growth inhibition, EC50	33.6	Ferrari et al. (2004)
	C. vulgaris	48 h, growth inhibition, EC50	36.6	Jos et al. (2003)
	P. subcapitata	96 h, growth inhibition, EC50	64	Yamamoto et al. (2007)
	C. meneghiniana	96 h, growth inhibition, NOEC	10	Ferrari et al. (2004)
	S. leopolensis	96 h, growth inhibition, NOEC	17.5	Ferrari et al. (2004)
	P. subcapitata	96 h, growth inhibition, NOEC	6.4	Yamamoto et al. (2007)
	Zooplankton			
Carbamazepine	*H. azteca*	10 d, mortality, LC50	9.9	Dussault et al. (2008)
	D. magna	48 h, immobility, EC50	13.8	Ferrari et al. (2003)
	C. dubia	48 h, mortality, LC50	77.7	Ferrari et al. (2004)
	D. magna	48 h, mortality, LC50	111	Han et al. (2006)
	D. magna	48 h, immobility, EC50	97.8	Jos et al. (2003)
	D. magna	96 h, immobility, EC50	76.3	Kim et al. (2007)
	D. magna	48 h, immobility, EC50	55	Yamamoto et al. (2007)
	C. dubia	7 d, reproduction, NOEC	0.025	Ferrari et al. (2004)
	Fish			
Carbamazepine	*O. latipes*	96 h, mortality, LC50	35.4	Kim et al. (2007)
	O. latipes	96 h, mortality, LC50	45.87	Kim et al. (2009)
	O. latipes	96 h, mortality, LC50	61.5	Nassef et al. (2009)
	O. latipes	96 h, mortality, LC50	20	Yamamoto et al. (2007)
	D. rerio	10 d, mortality, NOEC	25	Ferrari et al. (2004)
	Phytoplankton			
Atenolol	*D. subspicatus*	72 h, growth inhibition, EC50	620	Cleuvers (2005)
	P. subcapitata	72 h, growth inhibition, EC50	190	De Andrés et al. (2009)
	P. subcapitata	72 h, growth inhibition, EC50	143	De Andrés et al. (2009)
	P. subcapitata	96 h, growth inhibition, EC50	110	Yamamoto et al. (2007)
	P. subcapitata	72 h, growth inhibition, NOEC	128.8	Küster et al. (2010)
	P. subcapitata	96 h, growth inhibition, NOEC	10	Yamamoto et al. (2007)
	Zooplankton			
Atenolol	*D. magna*	48 h, immobility, EC50	313	Cleuvers (2005)
	D. magna	48 h, immobility, EC50	1450	De Andrés et al. (2009)
	D. magna	48 h, immobility, EC50	755	De Andrés et al. (2009)

(*continued on next page*)

Table A.1 (*continued*)

Compound	Species	Endpoint	Concentration (mg L^{-1})	Reference
	C. dubia	48 h, immobility, EC50	33.4	Fraysse and Garric (2005)
	D. magna	48 h, immobility, EC50	200	Hernando et al. (2004)
	D. magna	48 h, immobility, EC50	180	Yamamoto et al. (2007)
	D. magna	21 d, reproduction, NOEC	8.9	Küster et al. (2010)
	Fish			
Atenolol	*O. latipes*	96 h, mortality, LC50	>100	Kim et al. (2009)
	O. latipes	96 h, mortality, LC50	1800	Yamamoto et al. (2007)
	P. promelas	28 d, growth, NOEC	3.2	Winter et al. (2008)
	Phytoplankton			
Propranolol	*D. subspicatus*	72 h, growth inhibition, EC50	5.8	Cleuvers (2003)
	D. subspicatus	72 h, growth inhibition, EC50	0.7	Cleuvers (2005)
	C. meneghiniana	96 h, growth inhibition, EC50	0.244	Ferrari et al. (2004)
	S. leopolensis	96 h, growth inhibition, EC50	0.668	Ferrari et al. (2004)
	P. subcapitata	96 h, growth inhibition, EC50	7.4	Ferrari et al. (2004)
	P. subcapitata	72 h, growth inhibition, EC50	0.77	Liu et al. (2009)
	P. subcapitata	96 h, growth inhibition, EC50	0.66	Yamamoto et al. (2007)
	C. meneghiniana	96 h, growth inhibition, NOEC	0.094	Ferrari et al. (2004)
	P. subcapitata	96 h, growth inhibition, NOEC	5	Ferrari et al. (2004)
	S. leopolensis	96 h, growth inhibition, NOEC	0.35	Ferrari et al. (2004)
	P. subcapitata	96 h, growth inhibition, NOEC	0.1	Yamamoto et al. (2007)
	Zooplankton			
Propranolol	*S. proboscideus*	24 h, mortality, LC50	1.87	Calleja et al. (1994)
	D. magna	24 h, immobility, EC50	15.8	Calleja et al. (1994)
	A. salina	24 h, mortality, LC50	407	Calleja et al. (1994)
	D. magna	48 h, immobility, EC50	7.5	Cleuvers (2003)
	D. magna	48 h, immobility, EC50	7.7	Cleuvers (2005)
	D. magna	48 h, mortality, LC50	2.75	Ferrari et al. (2004)
	C. dubia	48 h, mortality, LC50	1.51	Ferrari et al. (2004)
	C. dubia	48 h, immobility, EC50	1.4	Fraysse and Garric (2005)
	C. dubia	48 h, mortality, LC50	0.8	Huggett et al. (2002)
	D. magna	48 h, mortality, LC50	1.6	Huggett et al. (2002)
	H. azteca	48 h, mortality, LC50	29.8	Huggett et al. (2002)
	T. platyrus	24 h, mortality, LC50	10.31	Kim et al. (2009)
	D. pulex	24 h, immobility, EC50	3.833	Lilius et al. (1995)
	D. magna	24 h, immobility, EC50	2.7	Lilius et al. (1995)
	D. magna	48 h, immobility, EC50	1.4	Stanley et al. (2006)
	D. magna	48 h, immobility, EC50	1.57	Stanley et al. (2006)
	D. magna	48 h, immobility, EC50	1.67	Stanley et al. (2006)
	D. magna	48 h, immobility, EC50	0.46	Yamamoto et al. (2007)
	C. dubia	7 d, reproduction, NOEC	0.009	Ferrari et al. (2004)
	C. dubia	7 d, reproduction, NOEC	0.125	Huggett et al. (2002)
	H. azteca	27 d, reproduction, NOEC	0.001	Huggett et al. (2002)
	Fish			
Propranolol	*O. latipes*	48 h, mortality, LC50	24.3	Huggett et al. (2002)
	O. latipes	96 h, mortality, LC50	11.4	Kim et al. (2009)
	P. promelas	48 h, mortality, LC50	1.42	Stanley et al. (2006)
	P. promelas	48 h, mortality, LC50	1.69	Stanley et al. (2006)
	P. promelas	48 h, mortality, LC50	1.21	Stanley et al. (2006)
	O. latipes	96 h, mortality, LC50	9	Yamamoto et al. (2007)
	D. rerio	10 d, mortality, NOEC	2	Ferrari et al. (2004)
	O. latipes	14 d, growth, NOEC	0.1	Huggett et al. (2002)
	O. latipes	28 d, egg production, NOEC	<0.0005	Huggett et al. (2002)
	Phytoplankton			
Bezafibrate	*Anabaena sp.*	24 h, growth inhibition, EC50	7.62	Rosal et al. (2010)
	Zooplankton			
Bezafibrate	*D. magna*	48 h, immobility, EC50	30.3	Han et al. (2006)
	T. platyurus	24 h, mortality, LC50	39.69	Isidori et al. (2007)
	D. magna	24 h, immobility, EC50	100.08	Isidori et al. (2007)
	C. dubia	24 h, immobility, EC50	75.79	Isidori et al. (2007)
	D. magna	48 h, immobility, EC50	240.4	Rosal et al. (2010)
	C. dubia	7 d, reproduction, NOEC	0.023	Isidori et al. (2007)
	Phytoplankton			
Trimethoprim	*S. carpricornutum*	72 h, growth inhibition, EC50	80.3	Eguchi et al. (2004)
	S. carpricornutum	Growth inhibition, EC50	110	Halling-Sørensen et al. (2000)
	S. carpricornutum	Growth inhibition, EC50	130	Lützhøft et al. (1999)
	M. aeruginosa	7 d, growth inhibition, EC50	112	Lützhøft et al. (1999)
	R. salina	Growth inhibition, EC50	16	Lützhøft et al. (1999)
	P. subcapitata	72 h, growth inhibition, EC50	40	Yang et al. (2008)
	S. carpricornutum	72 h, growth inhibition, NOEC	25.5	Eguchi et al. (2004)
	P. subcapitata	72 h, growth inhibition, NOEC	16	Yang et al. (2008)
	Zooplankton			

Table A.1 (*continued*)

Compound	Species	Endpoint	Concentration (mg L^{-1})	Reference
Trimethoprim	*D. magna*	96 h, immobility, EC50	120.7	Kim et al. (2007)
	D. magna	48 h, immobility, EC50	149	De Liguoro et al. (2009)
	D. magna	48 h, immobility, EC50	92	Park and Choi (2008)
	M. macropaca	48 h, immobility, EC50	54.8	Park and Choi (2008)
	D. magna	48 h, immobility, EC50	167.4	Choi et al. (2008)
	D. magna	48 h, immobility, EC50	123	Halling-Sørensen et al. (2000)
	D. magna	21 d, reproduction, NOEC	6	Park and Choi (2008)
	Fish			
Trimethoprim	*O. latipes*	96 h, mortality, LC50	>100	Kim et al. (2007)

Table B.1
Median contribution of the individual pharmaceuticals (IC in %, median calculated over the different sampling periods) to the cumulative risk posed by the mixtures expressed as the total toxicity $RCR_{MEC/PNEC}$. SAL: salicylic acid; CAR: carbamazepine; ATE: atenolol; PRO: propranolol; BEZ: bezafibrate; TRI: trimethoprim.

	SAL	CAR	ATE	PRO	BEZ	TRI
W01	7.5	20.6	0.3	21.2	46.1	0.4
W02	7.4	5.5	0.4	24.9	54.1	0.5
W03	2.9	5.8	0.4	28.2	61.2	0.6
W04	3.3	16.9	0.4	24.1	52.3	0.5
W05	2.3	5.7	0.4	28.6	62.3	0.6
W06	2.7	5.7	0.4	28.5	62.0	0.6
Offshore median	3.1	5.8	0.4	26.5	57.6	0.6
NP1	0.6	22.9	0.1	60.9	15.5	0.1
NP2	0.6	14.4	0.2	57.0	25.3	0.2
NP3	1.1	5.8	0.4	29.2	60.9	0.6
OO1	6.2	20.8	0.1	43.8	31.5	0.2
OO2	1.5	9.0	0.1	61.6	25.5	0.1
OO3	1.3	9.0	0.1	57.2	33.2	0.1
OO4	0.5	9.5	0.1	53.2	36.2	0.1
ZB1	1.8	20.0	0.3	37.3	40.5	0.4
ZB2	5.1	19.1	0.3	30.2	42.6	0.4
ZB3	4.3	21.5	0.2	36.1	33.5	0.3
ZB4	2.3	19.1	0.2	44.1	34.5	0.3
Harbour median	1.5	19.1	0.2	44.1	33.5	0.2
S01	3.3	19.4	0.3	30.5	41.5	0.4
S22	0.6	17.1	0.1	76.0	5.3	0.0
Scheldt median	1.9	18.3	0.2	53.2	23.4	0.2
Overall median	2.3	16.9	0.3	36.1	40.5	0.4

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