Mixture toxicity of the anti-inflammatory drugs diclofenac, ibuprofen, naproxen, and acetylsalicylic acid

Michael Cleuvers[*]

Department of General Biology, Aachen University of Technology, Kopernikusstraße 16, D-52056 Aachen, Germany

Abstract

The ecotoxicity of the nonsteroidal anti-inflammatory drugs (NSAIDs) diclofenac, ibuprofen, naproxen, and acetylsalicylic acid (ASA) has been evaluated using acute *Daphnia* and algal tests. Toxicities were relatively low, with half-maximal effective concentration (EC_{50}) values obtained using *Daphnia* in the range from 68 to 166 mg L^{-1} and from 72 to 626 mg L^{-1} in the algal test. Acute effects of these substances seem to be quite improbable. The quantitative structure–activity relationships (QSAR) approach showed that all substances act by nonpolar narcosis; thus, the higher the *n*-octanol/water partitioning coefficient (log K_{ow}) of the substances, the higher is their toxicity. Mixture toxicity of the compounds could be accurately predicted using the concept of concentration addition. Toxicity of the mixture was considerable, even at concentrations at which the single substances showed no or only very slight effects, with some deviations in the *Daphnia* test, which could be explained by incompatibility of the very steep dose–response curves and the probit analysis of the data. Because pharmaceuticals in the aquatic environment occur usually as mixtures, an accurate prediction of the mixture toxicity is indispensable for environmental risk assessment.
© 2003 Elsevier Inc. All rights reserved.

Keywords: Daphnia; *Desmodesmus*; Pharmaceuticals; Mixture toxicity; Concentration addition; QSAR; Narcosis

1. Introduction

During the last several years many studies have shown pharmaceutical compounds to be present in measurable concentrations in the aquatic environment (Kümmerer, 2001; Daughton and Jones-Lepp, 2001; Daughton and Ternes, 1999; Heberer, 2002; Halling-Sørensen et al., 1998). Among the detected substances, nonsteroidal anti-inflammatory drugs (NSAIDs), including compounds used as analgesics, belong to one of the most important groups of pharmaceuticals worldwide, with an estimated annual production of several kilotons. In Germany, for example, 87.5 million prescriptions for these substances were written in 2001, with a transaction volume of ~€1130 million (Schwabe and Paffrath, 2001). Additionally, some of these drugs are purchased without prescription, so that actual consumption is certainly even higher. As a result of this high application amount as well as the drugs' pharmacokinetics (half-life, urinary and fecal excretion, metabolism, etc.), analgesics

and anti-inflammatory drugs can reach detectable concentrations in the environment. Stumpf et al. (1996) identified diclofenac ($\leqslant$1.59 μg L^{-1}), ibuprofen ($\leqslant$3.35 μg L^{-1}), and ASA (1.51 μg L^{-1}) in sewage, and lower concentrations (0.01–0.5 μg L^{-1}) in river water. In very low doses (1–6 ng L^{-1}), diclofenac and ibuprofen could be detected even in drinking water. Ternes (1998; Ternes et al., 1998) reported concentrations of diclofenac, ibuprofen, naproxen, ASA, and other compounds, some >1 μg L^{-1} in wastewater treatment plants and again in lower concentrations in surface waters. In Germany, diclofenac and ibuprofen, consumed in quantities of ~75 and 180 tons per year, respectively (Ternes, 2001), have been particularly recognized as important contaminants of the water cycle. In a screening-study of waters in Berlin, Germany, Heberer et al. (1998) found that among the most prevalent NSAIDs were diclofenac, ibuprofen, and propyphenazon. In long-term monitoring investigations of sewage and surface water samples, diclofenac was identified as one of the most important pharmaceutically active compounds present in the water cycle (Heberer et al., 2002). It was found in groundwater samples (Heberer et al.,

[*]Fax: +49-241-8022602.

E-mail address: cleuvers@bio2.rwth-aachen.de.

1997; Sacher et al., 2001) and sporadically even in raw or treated drinking water (Heberer et al., 2001a). Additional findings of diclofenac and ibuprofen have been reported, for example, from Swiss lakes and rivers (Buser et al., 1998, 1999), from Brazil (Stumpf et al., 1999), Spain (Farre et al., 2001), Greece (Heberer et al., 2001a), and the United States (Heberer et al., 2001b).

In contrast to this large amount of analytical data, studies assessing the possible ecotoxicological effects of detected drug residues are sparse (Webb, 2001), and only very few contain data about mixture toxicity (Cleuvers, 2002, 2003). These studies are very important because drug residues found in the aquatic environment usually occur as mixtures, not as single contaminants. Therefore, scientific assessment of risk to the aquatic environment should definitely consider this complex exposure situation. For this purpose, ecotoxicologists use concepts originally developed by pharmacologists in the first half of the 20th century (Loewe and Muischnek, 1926; Bliss, 1939) to predict the toxicity of mixtures. In the present study, the concept of concentration addition (Altenburger et al., 2000; Faust et al., 2001) was applied. Concentration addition is based on the idea of a similar action of single compounds, which means in a strict sense that single substances should show the same specific interaction with a molecular target site in the observed test organism (Pöch, 1993). However, it has been shown that the concept of concentration addition is also applicable to nonreactive, nonionized organic chemicals, which show no specific mode of action but whose toxicity toward aquatic species is governed by hydrophobicity (Deneer et al., 1988; Van Loon et al., 1997). The nonspecific mode of action of such compounds is called narcosis or baseline toxicity (Van Leeuwen et al., 1992; Verhaar et al., 1992). The potency of a chemical to induce narcosis is entirely dependent on its hydrophobicity, generally expressed by its $\log K_{ow}$.

Mathematically, the concept of concentration addition for a mixture of n substances is described by (Berenbaum, 1985)

$$\sum_{i=1}^{n} \frac{c_i}{EC\,x_i} = 1, \tag{1}$$

where c_i represents the individual concentrations of the single substances present in a mixture with a total effect of $x\%$, and $EC\,x_i$ are those concentrations of the single substances that would alone cause the same effect x as observed for the mixture. According to Eq. (1), the effect of the mixture remains constant when one component is replaced by an equal fraction of an equally effective concentration of another. As an important point, concentration addition means that substances applied at less than their individual "no observable effect concentrations" (NOECs) can nevertheless contribute to the total mixture effect.

The aim of this study was to create a broader basis for evaluating the ecotoxicological relevance and mode of action of mixtures of pharmaceutical compounds. For that purpose, the selected NSAIDs were applied in biotests with the green alga Desmodesmus subspicatus (Chodat) Hegewald et Schmidt and the water flea Daphnia magna Strauss.

2. Materials and methods

2.1. Test substances

Acetylsalicylic acid (2-(acetyloxy)benzoic acid; CAS No. 50-78-2) and the sodium salts of diclofenac (2-[(2,6-dichlorophenyl)amino]benzeneacetic acid; CAS No. 15307-79-6), ibuprofen (α-methyl-4-[isobutyl]phenylacetic acid; CAS No. 31121-93-4), and naproxen ((S)-6-methoxy-α-methyl-2-naphtaleneacetic acid; CAS No. 26159-34-2) were supplied in analytical grade by Sigma-Aldrich (Taufkirchen, Germany). In tests with single compounds, substances were applied in six concentrations (1, 3.2, 10, 32, 100, and 320 mg L^{-1}). For assessing mixture toxicity, a quarter of the calculated effect concentrations (EC$_5$/4, EC$_{10}$/4, EC$_{20}$/4, EC$_{50}$/4, and EC$_{80}$/4) of each substance was used. If the substances follow the concept of concentration addition, according to Eq. (1) the combination effect of the mixtures should add up to a total effect of $\sim 5\%$, 10%, 20%, 50%, and 80%, respectively.

2.2. Daphnia acute immobilization tests

Daphnia tests were conducted following the European Guideline (Commission of the European Communities, 1992) using the water flea D. magna.

Daphnids were bred in ADaM, a culture medium imitating natural freshwater (Klüttgen et al., 1994). Experiments were conducted at temperatures of $20 \pm 1°C$ and photoperiods of 16 h light/8 h dark ($\sim 20 \mu E\,s^{-1}\,m^{-2}$). As controls, 20 daphnids younger than 24 h were used, and each treatment was subdivided in four replicates, each containing 5 daphnids. Culture volume was 50 mL. Immobility (the endpoint for effect calculation) was observed after 24 and 48 h.

2.3. Algal growth inhibition tests

Algae tests were conducted following the European Guideline (Commission of the European Communities, 1993). I used the planktonic chlorococcale green alga D. subspicatus (formerly Scenedesmus subspicatus Chodat, SAG 86.81 = UTEX 2594), obtained from the SAG-Sammlung von Algenkulturen at the University of Göttingen, Germany. Initial cell densities were adjusted at 10^4 cells mL^{-1} using a calibration curve of chlorophyll

fluorescence (excitation at 460 nm; emission at 685 nm) versus cell number and appropriate dilution of pre-culture. The culture medium was prepared according to the protocol using deionized water and analytical-grade chemicals. Algae were incubated at 23 ± 2°C under continuous white light ($120 \, \mu E \, s^{-1} \, m^{-2}$) and were kept in suspension by continuous shaking (~ 80 rpm). Results were quantified in terms of average growth rates calculated from cell numbers based on measurements of chlorophyll fluorescence.

2.4. Analysis of the mode of action

To determine the mode of action of the observed substances, I used QSAR (Verhaar et al., 1992; Lipnick, 1995; Schüürmann and Markert, 1998) for nonpolar narcotic chemicals proposed for *D. magna* (Verhaar et al., 1995) (Eq. (2)) and for the chlorophyte *Pseudo-kirchneriella subcapitata* (Van Leeuwen et al., 1992) (Eq. (3)), whose sensitivity is seen to be comparable to *D. subspicatus* (Blanck et al., 1984). If the EC_{50} of the tested substances is as high as or even higher than predicted by the equations, the compounds are considered to show no specific mode of action, but to act by narcosis only

$$\text{Log } EC_{50}[\text{mol L}^{-1}] = -0.95 \log K_{ow} - 1.32, \quad (2)$$

$$\text{Log } EC_{50}[\text{mol L}^{-1}] = -1.00 \log K_{ow} - 1.23. \quad (3)$$

2.5. Statistical evaluation

All calculations were conducted using the software ToxRat (ToxRat Solutions, Aachen, Germany). EC_x values were computed using probit analysis (dose–response fitting: normal sigmoid, maximum-likelihood regression). NOECs were determined using Dunnett's multiple *t*-test procedure; $\alpha = 0.05$, one-sided smaller.

3. Results

3.1. Single-compound toxicity

Tables 1 and 2 show the individual-effect concentrations and 95% confidence limits of the applied substances in the algal growth inhibition test and the acute *Daphnia*

Table 1
Effect concentrations and 95% confidence limits of the drugs tested in the algal test

Substance	EC_5 (mg L^{-1})	EC_{10} (mg L^{-1})	EC_{20} (mg L^{-1})	EC_{50} (mg L^{-1})	EC_{80} (mg L^{-1})
Diclofenac	44.2 (35.7–50.5)	49.2 (41.2–55.2)	56.1 (48.8–61.9)	71.9 (65.5–79.1)	92.2 (83.4–106.4)
Ibuprofen	72.9 (26.1–121.8)	102.7 (44.1–159.2)	155.5 (82.4–222.9)	342.2 (242.4–471.5)	753.2 (537.6–1323.2)
Naproxen	266.0 n.d.	321.5 n.d.	404.3 n.d.	625.5 n.d.	967.5 n.d.
ASA	86.4 (74.2–90.8)	90.6 (81.6–93.7)	95.8 (91.6–97.3)	106.7 (104.4–114.3)	118.9 (112.1–142.5)

n.d.: Not determinable

Table 2
Effect concentrations and 95% confidence limits of the drugs tested in the acute *Daphnia* test

Substance	EC_5 (mg L^{-1})	EC_{10} (mg L^{-1})	EC_{20} (mg L^{-1})	EC_{50} (mg L^{-1})	EC_{80} (mg L^{-1})
Diclofenac	10.0 n.d.	15.2 n.d.	25.5 n.d.	68.0 n.d.	181.3 n.d.
Ibuprofen	58.4 (43.9–77.7)	66.0 (51.7–84.1)	76.4 (62.9–92.9)	101.2 (89.2–114.9)	134.1 (117.0–153.6)
Naproxen	26.2 (11.4–60.5)	39.5 (20.2–77.4)	64.8 (39.9–105.3)	166.3 (130.5–211.9)	426.6 (274.5–663.0)
ASA	38.1 (25.4–57.1)	45.9 (32.9–63.9)	57.4 (44.6–73.9)	88.1 (72.8–106.6)	135.2 (100.4–182.1)

n.d.: Not determinable

test, respectively. The EU Directive 93/67/EEC (Commission of the European Communities, 1996) classifies substances according to the lowest measured EC_{50} value in different risk classes. An $EC_{50} < 1\,mg\,L^{-1}$ would entail the classification, "very toxic to aquatic organisms"; from 1 through $10\,mg\,L^{-1}$ "toxic to aquatic organisms", and from 11 through $100\,mg\,L^{-1}$ "harmful to aquatic organisms". Substances with an $EC_{50} > 100\,mg\,L^{-1}$ would not be classified. On the basis of this scheme, the acute toxicity of all tested substances is relatively low. Only diclofenac, with EC_{50} values of $68.0\,mg\,L^{-1}$ in the *Daphnia* test and $71.9\,mg\,L^{-1}$ in the algal test, and ASA with an EC_{50} of $88.1\,mg\,L^{-1}$ in the *Daphnia* test would be classified as potentially harmful to aquatic organisms. The lowest NOEC was $32\,mg\,L^{-1}$ (ibuprofen and ASA in the algal test, naproxen in the *Daphnia* test; see Tables 4 and 5), and the lowest EC_{10} was $15.2\,mg\,L^{-1}$ (diclofenac in the *Daphnia* test; see Table 5).

3.2. Mode of action and mixture toxicity

The high EC_{50} values obtained imply that no specific mode of action is responsible for the toxic effects on daphnids and algae. This is confirmed by a simple QSAR approach using Eqs. (2) and (3), respectively. All measured EC_{50} values are as high as or even higher than those predicted by the QSARs for narcosis, with the exception of that for ASA (Table 3).

3.3. Mixture toxicity

Drug concentrations applied in the mixtures are shown in Tables 4 and 5 in comparison to their individual NOECs for the *Daphnia* and the algal tests, respectively. In the algal test, mixture toxicity of the NSAIDs could be accurately predicted using the concept of concentration addition (Fig. 1). The situation for the *Daphnia* test differed somewhat (Fig. 2). The first three treatments (EC_5, EC_{10}, and EC_{20}) showed no effect at

Table 3
Measured EC_{50} values in comparison to those predicted in agreement with the log K_{ow} of the observed substances

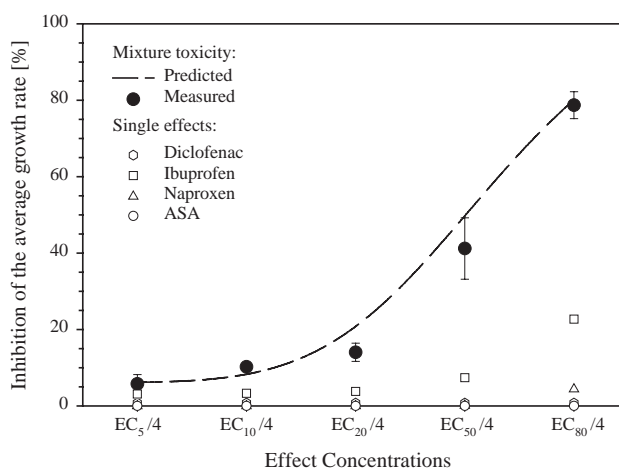| Substance	Log K_{ow}	EC_{50} (mmol L^{-1})			
		Predicted for narcosis		Measured	
		Daphnia	Algae	*Daphnia*	Algae
Diclofenac	4.4	3.16×10^{-3}	2.34×10^{-3}	0.21	0.23
Ibuprofen	3.5	0.023	0.019	0.46	1.57
Naproxen	3.3	0.035	0.030	0.66	2.48
ASA	1.2	3.47	3.72	0.49	0.59
Salicylic acid	2.3	0.31	0.30	—	—

Fig. 1. Measured mixture toxicity of the NSAIDs tested as obtained in the algal growth inhibition test in comparison to the individual toxicities of the single compounds and the mixture toxicity predicted by the concept of concentration addition.

Table 4
Concentrations of the tested drugs applied in the mixture in the algal test in comparison to the individual NOECs of the single compounds

Substance	$EC_5/4$ (mg L^{-1})	$EC_{10}/4$ (mg L^{-1})	$EC_{20}/4$ (mg L^{-1})	$EC_{50}/4$ (mg L^{-1})	$EC_{80}/4$ (mg L^{-1})	NOEC (mg L^{-1})
Diclofenac	11.1	12.3	14.0	18.0	23.1	50
Ibuprofen	18.2	25.7	38.9	85.6	188.3	32
Naproxen	66.5	80.4	101.1	156.4	241.9	100
ASA	21.6	22.6	24.0	26.7	29.7	32

Table 5
Concentrations of the tested drugs applied in the mixture in the acute *Daphnia* test in comparison to the individual NOECs of the single compounds

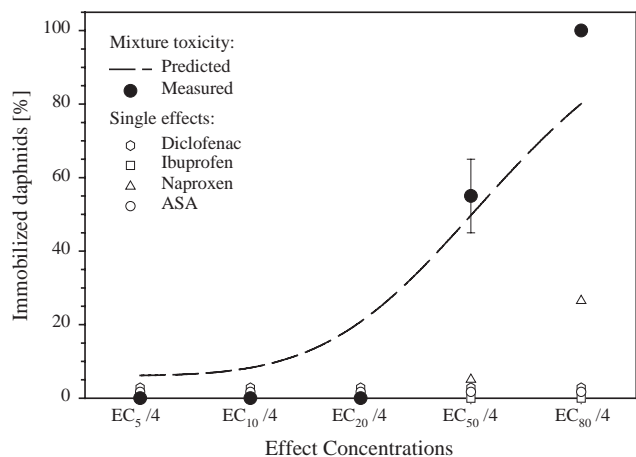| Substance	$EC_5/4$ (mg L^{-1})	$EC_{10}/4$ (mg L^{-1})	$EC_{20}/4$ (mg L^{-1})	$EC_{50}/4$ (mg L^{-1})	$EC_{80}/4$ (mg L^{-1})	NOEC (mg L^{-1})
Diclofenac	2.5	3.8	6.4	17.0	45.3	45
Ibuprofen	14.6	16.5	19.1	25.3	33.5	75
Naproxen	6.6	9.9	16.2	41.6	106.7	32
ASA	9.5	11.5	14.4	22.0	33.8	75

Fig. 2. Measured mixture toxicity of the NSAIDs tested as obtained in the acute *Daphnia* test in comparison to the individual toxicities of the single compounds and the mixture toxicity predicted by the concept of concentration addition.

all, whereas mixture toxicity at the EC_{50} and the EC_{80} doses were as high or even higher than that predicted by concentration addition.

4. Discussion

In most cases, individual EC_{50} values of the tested compounds were $> 100\,mg\,L^{-1}$. Therefore, based on the scheme used in the European Union, only diclofenac and ASA would be classified as potentially harmful to aquatic organisms. Regarding an effect threshold, the lowest NOEC was $32\,mg\,L^{-1}$, which is 10,000- to 100,000-fold higher than the highest detected concentration of that compound in the aquatic environment. In fact, the EC_x approach is generally more sensitive for detecting low effects; this is because, on the one hand, NOEC is dependent on actually selected test concentrations, and on the other hand, to compute the NOEC each treatment was separately compared to the control. In contrast, using an EC_x approach, all data points are used to determine effect concentrations. However, even the lowest measured EC_{10} (diclofenac, $15.2\,mg\,L^{-1}$ in the *Daphnia* test) is ~10,000-fold higher than concentrations detected in the field. Thus, with respect to the results obtained with daphnids and algae, acute effects of these substances are very unlikely. If we keep in mind that the tested substances are pharmaceuticals, strong acute effects caused by a specific mechanism may actually not be expected, because such an acute toxicity would surely diminish the pharmaceutical benefit of the compound.

Therefore, considering the situation in the field with mostly very low concentrations of pharmaceuticals, we must assume that generally chronic effects are more

likely than acute effects, and there may be other species that are more sensitive to these contaminants. For example, an earlier study (Cleuvers, 2003) indicated that the EC_{50} values of tested NSAIDs obtained using the duckweed *Lemna minor* were 5- to 10-fold lower than those obtained using the acute *Daphnia* or algal test. For that reason it was proposed to conduct *Lemna* tests routinely in addition to other standard tests (Cleuvers and Ratte, 2002). Additionally, it may be useful to use additional benthic organisms, like freshwater gastropods or oligochaetes, to improve the environmental risk assessment.

All drugs tested in this study are NSAIDs and are considered to have the same mode of action in humans. They inhibit the cyclooxygenases, the key enzymes catalyzing prostaglandin biosynthesis, which is partially involved in the genesis of pain and inflammation (Vane and Botting, 1998; Vane, 1971). This inhibition is responsible for the analgesic and anti-inflammatory effect of the NSAIDs. Among the other functions of prostaglandins is their ability to cause contractions or atony of muscles in different organs, with effects on blood pressure and circulation. In addition, some prostaglandins act to protect cells in the gastrointestinal tract, and their inhibition by NSAIDs with the possibility of internal bleeding is one of the most important side effects of these pharmaceuticals. Several publications have reported on the occurrence of prostaglandins in nonmammalian vertebrates (Bundy, 1985; Nomura, 1988) such as fish, amphibians, and birds, as well as in invertebrates such as corals, sponges, coelenterates, mollusks, crustaceans, insects, and in marine algae and higher plants, where they are considered to carry out different functions, for example, in defense mechanisms of plants. Unfortunately, no literature is available about the occurrence of prostaglandins in daphnids or freshwater algae, so that it is unclear whether the test species used in this study could serve as a model for a specific effect of NSAIDs. Therefore, additional biotests with vertebrates like fish could be a useful tool to assess environmental risk. The high measured EC_{50} values imply that no specific mode of action is responsible for the toxic effects found in daphnids and algae. This is confirmed by QSAR, using Eqs. (2) and (3), respectively. One problem is that the $\log K_{ow}$ values of ibuprofen sodium and naproxen sodium are not available in the literature. To allow a comparison of the toxicity of the drugs using as a baseline their $\log K_{ow}$, I used the $\log K_{ow}$ values of the pure substances, not of the sodium salts; these are indicated in Table 3. Of course, this leads to slightly different predictions of toxicity, but because of the high EC_{50} values the QSAR will in any case show that the substances are not more toxic than predicted for nonpolar narcosis. In fact, Table 3 shows that all measured EC_{50} values are equal to or even higher than

predicted by the QSARs for narcosis, with the exception of that for ASA. However, as a pro-drug, ASA is easily degraded by deacetylation into its more active form salicylic acid (Heberer, 2002), which has a higher $\log K_{ow}$. On the basis of that information, the measured EC_{50} is now higher than predicted, meaning that ASA also acts nonspecifically by narcosis.

For our results, an important point to consider is that all the drugs we tested are carboxylic acids. As a result of the pH in the biotests (7.5–8.5), as well as generally in the field, the substances will dissociate to a great extend to their polar form, reducing their bioavailability. Another significant influence is the hydrophobicity of the compounds, which directly affects the bioconcentration factor (BCF). Predictably, the higher the $\log K_{ow}$, the higher the toxicity of the substances. Again, the only exception is ASA, which, in comparison to the other compounds, is somewhat more toxic than expected. The reason for this deviation may be that, in addition to its degradation into salicylic acid, and in contrast to the other compounds, ASA has a considerable influence on pH, an additional unfavorable influence on the test organisms. The lower the pH, the higher the bioavailability of ASA.

In the algal test, due to the narcotic nature of action, the mixture toxicity of the NSAIDs could be accurately predicted using the concept of concentration addition on all effect concentrations from EC_5 to EC_{80}. This is consistent with the earlier finding that concentration addition is a valid concept for nonreactive, nonspecific compounds (Deneer et al., 1988; Van Loon et al., 1997). However, in the *Daphnia* test, the first three treatments showed no effect at all, whereas mixture toxicity at the EC_{50} and the EC_{80} doses were as high as or even higher than predicted by concentration addition. An explanation is that, because of the very steep dose–response curves of the single substances, the probit analysis overestimates the effect caused by low doses of the test substances we used. The consequence is that the mixture concentrations were too low to yield the expected effect. Therefore, additional studies with substances showing steep dose–response curves should preferably use a nonlinear regression method, for example, a four-parameter logistic fit, to calculate the EC_x values.

After comparing concentrations used in the mixtures to the individual NOECs of the single substances, it seems obvious that considerable combination effects could also occur if some or even all substances were applied in concentrations below their NOEC. This implies that the use of individual NOECs is not applicable when assessing the environmental risk of mixtures. Furthermore, more data about chronic effects, for example, on daphnids, fish, and benthic macroinvertebrates, are indispensable to estimate the environmental risk of pharmaceutical compounds.

References

Altenburger, R., Backhaus, T., Boedeker, W., Faust, M., Scholze, M., Grimme, L.H., 2000. Predictability of the toxicity of multiple chemical mixtures to *Vibrio fischeri*: mixtures composed of similarly acting chemicals. Environ. Toxicol. Chem. 19, 2341–2347.

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

Blanck, H., Wallin, G., Wängberg, S., 1984. Species-dependent variation in algal sensitivity to chemical compounds. Ecotoxicol. Environ. Saf. 8, 339–351.

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

Bundy, G.L., 1985. Nonmammalian sources of eicosanoids. Adv. Prostaglandin Thromboxane Leukotriene Res. 14, 229–262.

Buser, H.-R., Poiger, T., Müller, M.D., 1998. Occurrence and fate of the pharmaceutical drug diclofenac in surface waters: rapid photodegradation in a lake. Environ. Sci. Technol. 32, 3449–3456.

Buser, H.-R., Poiger, T., Müller, M.D., 1999. Occurrence and experimental behaviour of the pharmaceutical drug ibuprofen in surface waters and in wastewater. Environ. Sci. Technol. 33, 2529–2535.

Cleuvers, M., 2003. Aquatic ecotoxicity of selected pharmaceuticals including the assessment of combination effects. Toxicol. Lett. 142, 185–194.

Cleuvers, M., 2002. Aquatic ecotoxicology of selected pharmaceutical agents—algal and acute *Daphnia* tests (in German). UWSF—Z. Umweltchem. Ökotoxokol. 14, 85–89.

Cleuvers, M., Ratte, H.T., 2002. Phytotoxicity of coloured substances: is *Lemna* duckweed an alternative to the algal growth inhibition test? Chemosphere 49, 9–15.

Commission of the European Communities, 1992. Methods for Determination of Ecotoxicity; Annex V, C.2, *Daphnia*, Acute Toxicity to *Daphnia*. L 383A, EEC Directive 92/69/EEC, pp. 172–178.

Commission of the European Communities, 1993. Methods for Determination of Ecotoxicity; Annex V, C.3, Algal Inhibition Test. L 383A, EEC Directive 92/69/EEC, pp. 179–186.

Commission of the European Communities, 1996. Technical Guidance Document in Support of Commission Directive 93/67/EEC on Risk Assessment for New Notified Substances and Commission Regulation (EC) No. 1488/94 on Risk Assessment for Existing Substances. Part II: Environmental Risk Assessment. Office for Official Publications of the European Communities, Luxembourg.

Daughton, C.G., Jones-Lepp, T. (Eds.), 2001. Pharmaceuticals and Personal Care Products in the Environment: Scientific and Regulatory Issues. American Chemical Society, Washington, DC.

Daughton, C.G., Ternes, T.A., 1999. Pharmaceuticals and personal care products in the environment: agents of subtle change? Environ. Health Perspect. 107, 907–938.

Deneer, J.W., Sinnige, T.L., Seinen, W., Hermens, J.L.M., 1988. The joint acute toxicity to *Daphnia magna* of industrial organic chemicals at low concentrations. Aquat. Toxicol. 12, 33–38.

Farre, M., Ferrer, I., Ginebreda, A., Figueras, M., Olivella, L., Tirapu, L., Vilanova, M., Barcelo, D., 2001. Determination of drugs in surface water and wastewater samples by liquid chromatography–mass spectrometry: methods and preliminary results including toxicity studies with *Vibrio fischeri*. J. Chromatogr. A 938, 187–197.

Faust, M., Altenburger, R., Backhaus, T., Blanck, H., Boedeker, W., Gramatica, P., Hamer, V., Scholze, M., Vighi, M., Grimme, L.H., 2001. Predicting the joint algal toxicity of multi-component *s*-triazine mixtures at low-effect concentrations of individual toxicants. Aquat. Toxicol. 56, 13–32.

Halling-Sørensen, B., Nors Nielsen, S., Lanzky, P.F., Ingerslev, F., Holten Lützhøft, H.C., Jørgensen, S.E., 1998. Occurrence, fate and

effects of pharmaceutical substances in the environment—a review. Chemosphere 36, 357–393.

Heberer, T., 2002. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicol. Lett. 131, 5–17.

Heberer, T., Dünnbier, U., Reilich, C., Stan, H.J., 1997. Detection of drugs and drug metabolites in groundwater samples of a drinking water treatment plant. Fresenius' Environ. Bull. 6, 438–443.

Heberer, T., Schmidt-Bäumler, K., Stan, H.J., 1998. Occurrence and distribution of organic contaminants in the aquatic system in Berlin. Part I: drug residues and other polar contaminants in Berlin surface and groundwater. Acta Hydrochim. Hydrobiol. 26, 272–278.

Heberer, T., Fuhrmann, B., Schmidt-Bäumler, K., Tsipi, D., Koutsouba, V., Hiskia, A., 2001a. Occurrence of pharmaceutical residues in sewage, river, ground and drinking water in Greece and Germany. In: Daughton, C.G., Jones-Lepp, T. (Eds.), Pharmaceuticals and Personal Care Products in the Environment: Scientific and Regulatory Issues. American Chemical Society, Washington, DC, pp. 70–83.

Heberer, T., Verstraeten, I.M., Meyer, M.T., Mechlinski, A., Reddersen, K., 2001b. Occurrence and fate of pharmaceuticals during bank filtration—preliminary results from investigations in Germany and the United States. Water Resour. Update 120, 4–17.

Heberer, T., Reddersen, K., Mechlinski, A., 2002. From municipal sewage to drinking water: fate and removal of pharmaceutical residues in the aquatic environment in urban areas. Water Sci. Technol. 46, 81–88.

Klüttgen, B., Dülmer, U., Engels, M., Ratte, H.T., 1994. ADaM, an artificial freshwater for the culture of zooplankton. Water Res. 28, 743–746.

Kümmerer, K. (Ed.), 2001. Pharmaceuticals in the Environment. Sources, Fate, Effects and Risks. Springer, Berlin, Germany.

Lipnick, R.L., 1995. In: Rand, G.M. (Ed.), Fundamentals of Aquatic Toxicology. Taylor & Francis, London, pp. 609–655.

Loewe, S., Muischnek, H., 1926. Über Kombinationswirkungen. 1. Mitteilung: hilfsmittel der fragestellung. Naunyn-Schmiedebergs Arch. Exp. Pathol. Pharmakol. 114, 313–326.

Nomura, T., 1988. Phylogenetic aspects of prostaglandins. Seitai Bogyo 5, 67–74.

Pöch, G., 1993. Combined Effects of Drugs and Toxic Agents. Modern Evaluation in Theory and Practice. Springer, Berlin, Germany.

Sacher, F., Lange, F.T., Brauch, H.-J., Blankenhorn, I., 2001. Pharmaceuticals in groundwater. Analytical methods and results of a monitoring program in Baden–Würtemberg, Germany. J. Chromatogr. A 938, 199–210.

Schüürmann, G., Markert, B., 1998. Ecotoxicology: Ecological Fundamentals, Chemical Exposure, and Biological Effects. Wiley, Spektrum Akademischer Verlag, New York, Heidelberg, pp. 665–749.

Schwabe, U., Paffrath, D. (Eds.), 2001. Arzneiverordnungsreport. Springer, Berlin.

Stumpf, M., Ternes, T.A., Haberer, K., Seel, P., Baumann, W., 1996. Nachweis von Arzneimittelrückständen in Kläranlagen und Fließ gewässern. Vom Wasser 86, 291–303.

Stumpf, M., Ternes, T.A., Wilken, R.-D., Rodrigues, S.V., Baumann, W., 1999. Polar drug residues in sewage and natural waters in the state of Rio de Janeiro, Brazil. Sci. Total Environ. 225, 135–141.

Ternes, T.A., 1998. Occurrence of drugs in German sewage treatment plants and rivers. Water Res. 32, 3245–3260.

Ternes, T.A., 2001. In: Daughton, C.G., Jones-Lepp, T. (Eds.), Pharmaceuticals and Personal Care Products in the Environment: Scientific and Regulatory Issues. American Chemical Society, Washington, DC, pp. 39–54.

Ternes, T.A., Stumpf, M., Schuppert, B., Haberer, K., 1998. Simultaneous determination of antiseptics and acidic drugs in sewage and river water. Vom Wasser 90, 295–309.

Van Leeuwen, C.J., van der Zandt, P.T.J., Aldenberg, T., Verhaar, H.J.M., Hermens, J.L.M., 1992. Application of QSARs, extrapolation and equilibrium partitioning in aquatic assessment: I. narcotic industrial pollutants. Environ. Toxicol. Chem. 11, 267–282.

Van Loon, W.M.G.M., Verwoerd, M.E., Eijnker, F.G., van Leeuwen, C.J., van Duyn, P., van deGuchte, C., Hermens, J.L.M., 1997. Estimating total body residues and baseline toxicity of complex organic mixtures in effluents and surface waters. Environ. Toxicol. Chem. 16, 1358–1365.

Vane, J.R., 1971. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nature 231, 232–235.

Vane, J.R., Botting, R.M., 1998. Mechanism of action of nonsteroidal antiinflammatory drugs. Am. J. Med. 104, 2S–8S.

Verhaar, H.J.M., van Leeuwen, C.J., Hermens, J.L.M., 1992. Classifying environmental pollutants. 1. Structure–activity relationships for prediction of aquatic toxicology. Chemosphere 25, 471–491.

Verhaar, H.J.M., Mulder, W., Hermens, J.L.M., 1995. In: Hermens, J.L.M. (Ed.), Overview of Structure–Activity Relationships for Environmental Endpoints, Part 1: General Outline and Procedure. Report prepared within the framework of the project, QSAR for Prediction of Fate and Effect of Chemicals in the Environment, Environmental Technologies RTD Programme, European Commission, Contract No. EV5V-CT92-0211.

Webb, S., 2001. In: Kümmerer, K. (Ed.), Pharmaceuticals in the Environment. Sources, Fate, Effects and Risks. Springer, Berlin.