1 **Screening level mixture risk assessment of pharmaceuticals in STP effluents**

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Abstract

We modeled the ecotoxicological risks of the pharmaceutical mixtures emitted into the environment from STP effluents. The classic mixture toxicity concept of Concentration Addition was used to calculate the total expect risk of the analytically determined mixtures, compare the expected impact of seven effluent streams and pinpoint the most sensitive group of species.

Single substance-based assessments underestimate the actual risks from pharmaceutical mixtures often by more than a factor of 1 000 in several of the surveyed effluent streams, clearly indicating the need to take the joint presence of pharmaceuticals into consideration in order to provide an environmentally realistic assessment for a given water body. The mixture risk quotients regularly exceed 1, indicating a potential risk for the environment, depending on the specific environmental conditions, in particular the dilution in the recipient stream. The top 10 mixture components explain more than 95% of the mixture risk in all cases.

A mixture toxicity assessment cannot go beyond the underlying single substance data. The lack of data on the chronic toxicity of most pharmaceuticals as well as the very few data available for *in vivo* fish toxicity has to be regarded as a major knowledge gap in this context. On the other hand, ignoring Independent Action or even using the sum of individual risk quotients as a rough approximation of Concentration Addition does not have a major impact on the final risk estimate.

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43 **Keywords**

44 Pharmaceuticals in the Environment, Mixture Risks, Concentration Addition

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Highlights

- Missing chronic ecotoxicity data constitute a critical knowledge gap
- Considering mixtures is crucial for assessing the risks of pharmaceuticals in a given water body
- Mixture risk quotients based on the available data often exceed 1
- Sums of individual risk quotients adequately approximate Concentration Addition

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1. Introduction

Pharmaceuticals are detected in an ever increasing number of drinking water supplies, effluents and aquatic ecosystems, e.g. (Segura, et al., 2009; Heberer, 2002). Consequently, a range of experimental investigations has been undertaken during the last years with the aim to describe the hazards and risks of pharmaceuticals for the aquatic environment. Several studies came to the conclusion that clear ecotoxic effects are only to be expected at concentrations well above environmentally realistic levels. Hence the risk of pharmaceuticals to the environment has repeatedly been assessed as negligible, e.g. (Han et al., 2006; Miege et al., 2006; Wilson et al., 2004), or limited to specific cases, e.g. (Brain et al., 2006; Lienert et al. 2007).

However, pharmaceuticals do not occur as isolated, pure substances in an environmental compartment. A broad range of different substances is used simultaneously in human and veterinary medicine in any given area, hence pharmaceuticals often occur in the environment as multi-component mixtures (e.g. Vulliet and Cren-Olivé, 2011; Kasprzyk-Hordern et al., 2008; Moldovan, 2006, Loos et al., 2009; Gómez et al., 2007; Kolpin et al., 2002).

The joint ecotoxicity of such cocktails is typically higher than the toxicity of each individual compound (Kortenkamp et al., 2009). In particular, even if the compounds of a mixture are below their respective toxicity threshold, a joint toxic effect cannot be ruled out *a priori*. Such a pattern was observed for example in multi-component mixtures of quinolone antibiotics (Backhaus et al., 2000), a set of 14 dissimilarly acting pharmaceuticals (Backhaus et al., 2000), or a mixture of cimetidine, fenofibrate, furosemide and phenazone (Fent et al., 2006). Even mixtures of only comparatively few compounds often show a similar pattern. A mixture of

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fluoxetine and clofibric acid killed more than 50% of a daphnia population after an exposure of 6 days, although the components were present at concentrations that did not provoke significant effects individually (Flaherty and Dodson, 2005). In the same study, a significant shift in sex ratio was observed after an exposure to a three-component mixture of erythromycin, triclosan and trimethoprim - again at a mixture concentration at which all components were present at concentrations that did not provoke significant individual effects. Binary combinations of clofibric acid and carbamazepine as well as diclofenac and ibuprofen show clear mixture effects in acute Daphnia tests, although each individual component was present in a concentration below its individual no observed effect concentration (NOEC) (Cleuvers, 2003). Eguchi and colleagues demonstrated that trimethoprim, even if present only at its NOEC concentration, shifts the concentration-response curve of sulfamethoxazole and sulfadiazine in algae towards 4-5 times higher toxicities (Eguchi, 2004).

Hence, ignoring possible mixture effects might run the risk of underestimating the actual impact of pharmaceuticals in the environment, depending on the number of compounds involved, their concentrations and ecotoxicological profiles.

We have recently outlined a strategy for the compound-based environmental risk assessment of chemical mixtures (Backhaus and Faust, 2012), which is primarily based on the classical mixture toxicity concept of Concentration Addition (CA). Two possible approaches for assessing the risk of a chemical mixture were outlined:

I. The risk quotient of a given mixture is estimated as the sum of the individual EnvConc/PNEC ratios of each mixture component. EnvConc = Environmental

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Concentration, which can be modeled (Predicted Environmental Concentration, PEC), measured (Measured Environmental Concentration, MEC), or which can represent the concentration near an effluent outlet (Environmental Introductory Concentration, EIC). PNEC represents the Predicted No Effect Concentration, calculated e.g. according to the corresponding guideline of the European Chemicals Agency (ECHA, 2008). As the scenario listed in table 1 is based on a chemical monitoring campaign, we will use the term $RQ_{MEC/PNEC}$ for this type of risk quotient in the following.

II. The sum of toxic units (STU, with a toxic unit being TU=EnvConc/EC50) is calculated in a first step for each of the main trophic levels (usually algae, invertebrates, fish). The final risk quotient (RQ_{STU}) for the mixture then equals the sum of toxic units of the most sensitive trophic level multiplied with the corresponding Assessment Factor (AF), which is set to 1 000 if data represent EC50 values from short-term toxicity studies with algae, invertebrates and fish (ECHA, 2008). This risk estimate will be termed RQ_{STU} in the following.

Both approaches are based on the same input data, i.e. estimates of the environmental concentration for each compound, estimates of its toxicity to at least algae, invertebrates and fish. However, they differ in the order of the analyses. Approach I first calculates the ecosystem-level risk quotient (EnvConc/PNEC ratio) for each compound following the approach that is provided in the corresponding REACH guideline (ECHA, 2008) and then estimates the mixture risk by summing up the individual PEC/PNEC ratios. Approach II reverses this order and first estimates the mixture risk separately for each trophic level (by summing up the

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116 corresponding TUs), and only afterwards carries out the ecosystem-level extrapolation by
117 selecting the most sensitive trophic level (which, again, follows the strategy of the REACH
118 guidance documents for the assessment of individual substances (ECHA, 2008)).

119 In the following, we used these approaches for providing a screening level assessment of the
120 environmental risks of pharmaceutical mixtures previously determined in European sewage
121 treatment plant effluents. The work was based on a comparative exposure assessment of a
122 range of pharmaceuticals in 7 European STP effluents previously published by Andreozzi and
123 coworkers (table 1, Andreozzi et al., 2003). We were in particular interested to determine
124 whether the detected pharmaceutical cocktails might pose a risk to aquatic organisms
125 (warranting further studies and/or risk reductions), how this relates to the toxicities of the
126 individual pharmaceuticals, which group of organisms (trophic levels) are most sensitive and
127 which are the ecotoxicologically most important compounds. The aim was to follow standard
128 regulatory environmental risk assessment approaches for individual pharmaceuticals as closely
129 as possible.

130 ## 2. Material and Methods

131 The available single substance data for algae, invertebrates and fish were compiled from
132 reviews (Kümmerer, 2008; Jjemba, 2008; Kümmerer, 2009) and electronic databases (US EPA,
133 2012; Mistrapharma, 2012). In case no data for a particular compound / organism group was
134 found in these sources a specific search in Scopus (www.scopus.com) was conducted using the
135 search string "*substance name* AND toxicity AND (alg* OR fish* OR daph*)" where substance
136 name is the common name of the respective pharmaceutical as listed in table 1. Data from

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reviews and databases were initially used without consulting the primary literature. However, those values that were finally selected for the assessment (see table 2) were traced back to the primary publication prior to use, as far as possible.

It turned out that the amount of publically available data is currently insufficient for an analysis of the chronic toxicity of most pharmaceuticals encountered in the analyzed exposure scenarios. We hence followed the approaches as suggested by the FDA (US FDA 1998) for a Tier 1 and Tier 2 assessment of pharmaceuticals as well as the corresponding outline of the European Chemicals Agency for the initial hazard assessment of general chemicals (REACH) (European Chemicals Agency 2008) and based our analysis on acute toxicity data (EC50 values) of primary producers (algae, including blue-green algae, i.e. cyanobacteria), primary consumers (crustaceans, mainly daphnids, but also other invertebrates) and secondary consumers (fish, including data from *in vitro* studies with fish cell-lines). Under these conditions the FDA approach uses an assessment factor of 100, while the REACH guidance applies an assessment factor of 1 000 for extrapolating from the limited set of ecotoxicity data to possible effects in an ecosystem context.

If more than one EC50 was available for a given compound, the lowest value found for that particular species group was used. If no EC50 value was available for a particular compound eventual NOEC values were used. Data from limit tests that were expressed in terms of a "greater than" in the primary data sources were only considered if no exact data were available. Under these conditions the limit values were equated with the corresponding EC50.

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If absolutely no experimental toxicity data were found for a given trophic level, we used QSAR estimates for the EC50 values. These were based on the logP of the neutral species as calculated by KOW Win (vers. 1.67) and calculated by the ECOSAR program of the US EPA (vers. 1.00). The final set of toxicity data used for the modeling of expected mixture toxicities is given in table 2, the full list of initial toxicity data is provided in the supporting information.

As values for the analytical detection limits were not provided in the original publication, a concentration of zero was assumed for all pharmaceuticals that were present below the detection limit in an effluent.

3. Results and Discussion

We first briefly assess the environmental risk of the individual pharmaceuticals present in the analytical profiles of the seven European STP effluents as shown in table 1. On this basis we then estimate and assess the expected joint risk of the pharmaceutical mixtures and then identify the most sensitive species group as well as the main ecotoxicity drivers. We conclude the discussion by analyzing the limits of this screening-level analysis of the environmental risk of real-world pharmaceutical mixtures.

3.1. Environmental risks of the individual pharmaceuticals

The use of acute toxicity data is not recommended by the EMA guideline on the environmental risk assessment of human pharmaceuticals (EMA, 2006). In contrast, in an opinion on an earlier draft of the guideline the EU Commission's Scientific Committee on toxicity, ecotoxicity and the environment (CSTEE) recommend the use of the classical base set of ecotoxicological data (acute data from algae, daphnids and fish) for a first risk assessment (CSTEE, 2001), in line with

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the European approach for the risk assessment of general industrial chemicals, laid down in the REACH regulation (ECHA, 2008). The corresponding FDA guideline from 1998 starts in tier 1 with a risk assessment on the basis of acute toxicity data from one single species, using an assessment factor of 1 000 (FDA, 1998). No specific recommendation on the choice of test organism is provided, only that it should be part of the base set. If the resulting risk quotient indicates reason for concern the assessment continues in tier 2 which is based on acute toxicity data from 3 species and an assessment factor of 100. Finally, if needed, a tier 3 which uses chronic data follows.

Data on chronic toxicity were not available for most pharmaceuticals included in the analytical survey (table 1). The assessment was hence based on short-term toxicity data for algae (including cyanobacteria), daphnids (and other invertebrates) and fish (including *in vitro* data). For details on the selection of data see material methods. The final toxicity data selected for the following analyses are compiled in table 2.

No toxicity data were found for aminopyrine, fenoprofen and flurbiprofen. Data were incomplete for acetobutolol (no data for algae and fish), enoxacin (no data for invertebrates and fish), norfloxacin (no data for invertebrate) and oxprenolol (only data for invertebrates). In these cases, the toxicity was estimated by QSARs (see Material and Methods for details).

Toxic Units (TUs = MEC/EC50) for algae that exceed the critical value of 10^{-3} for tier 1 of the FDA guideline were found for the antibiotics ciprofloxacin, lomefloxacin, norfloxacin, ofloxacin and sulfamethoxazole in several of the STP effluents (see table 1, supporting information). The TUs for the algal toxicity of ofloxacin exceeded even 10^{-2} in all effluents (except L5-S), which would

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trigger the next tier assessment according to the FDA guideline. In all cases the TU was based on toxicity data from blue-green algae (cyanobacteria), which were always the most sensitive algal group. This clearly supports the suggestion of the EMA guideline to base the environmental risk assessment of antibiotics on tests with cyanobacteria (EMA, 2006). This particular group of compounds (antibiotics) has a biocidal mode of action and is assessed in a biotest battery in which the target organisms (prokaryotes) are represented. It is, however, currently unknown whether cyanobacteria, in particular the commonly used *Mycrocystis* and *Anabaena* species, are particularly sensitive or insensitive species, compared to other prokaryotes.

None of the other pharmaceuticals has a biocidal mode of action which is picked up in routine short-term assays. Although fish are pharmacologically most closely related to humans, they were in general the least sensitive organism group (table 2), and the corresponding TU never exceeded 10^{-3} (see also table 1, supporting information). One of the reasons for this pattern might be the strong prevalence of *in vitro* data in the data compilation, which were selected due to the lack of organism-level data for fish. Any subtle systemic non-biocidal effect might go unnoted in such simplified assays.

Gemfibrozil and ibuprofen had invertebrate TUs exceeding 10^{-3} in several cases (table 1, supporting information). The TU of both compounds is based on toxicity data from tests with *Hydra attenuata*, using a non-lethal endpoint (regeneration). This supports the EMA notion of basing an assessment on chronic data, and emphasizes that more reliable chronic data from organismic studies are urgently needed in order to provide a better understanding of the

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environmental effects of pharmaceuticals. It could also be questioned whether the standard ecotoxicological endpoints of chronic assays (growth, reproduction) are suited for the assessment of pharmaceuticals with a non-biocidal primary mode of action.

3.2. Environmental risks of pharmaceutical mixtures from STP effluents

The sum of toxic units (STU=∑MEC/EC50) was calculated for each organism group (algae, invertebrates, fish) within each scenario. Table 3 shows that the STU ranges from 1.95E-04 (fish, L1-F) to 4.8E-02 (algae, M1-L).

Using the full set of acute toxicity data from three trophic levels and an assessment factor of 100 (according to the FDA guideline for tier 2) results in final risk quotients (RQ_{STU}-values) between 1.6 for the effluent from L5-S and 4.8 for the M1-L effluent. That is, for all effluents the FDA guideline would recommend to continue with the next tier, as a risk for the environment is indicated. Following the methodologies laid down in the technical guidance document for the new European chemical regulation REACH (ECHA, 2008), an assessment factor of 1 000 is to be used if acute toxicity data from algae, invertebrates and fish are used for the assessment of an environmental risk, resulting in RQ_{STU}-values between 16 and 48. The REACH approach is, however, based on the use of predicted environmental concentrations (PEC's). The final assessment of the environmental risk due the total pharmaceutical load in the analysed STP effluents would hence depend on the ratio between the measured concentrations (table 1) and the resulting PECs, i.e. the dilution factor in the recipient stream.

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239 *3.2.1. Distribution of Toxic Units*

240 The distribution of the relative toxic units is shown in figure 1 for all 7 investigated scenarios
241 and for the three considered organism groups. These plots allow an easy identification of the
242 relative importance of each individual pharmaceutical in each effluent and for each organism
243 group, which might guide potential risk management and mitigation measures.

244 The figure clearly shows the uneven distribution of the toxic units in each mixture. Usually, a
245 few compounds contribute most to the overall STU, while many compounds only have a
246 negligible contribution to the overall sum of toxic units.

247 It has been previously suggested to focus the assessment of complex exposure situations on the
248 10 most important compounds (Groten et al., 2001). The analysis of the pharmaceutical
249 mixtures shows indeed that in all cases more than 95% of the total sum of TUs would covered
250 by this approach (table 3). However, it should be pointed out that the identification of the 10
251 most important compounds relies on the risk-driven ranking of the compounds in the mixture,
252 implying that initially all compounds of the analytical profile are to be included in the TU-
253 analysis. Although the "top 10" rule might therefore not simplify the initial assessment of a
254 complex exposure, it might serve as a valuable guide for risk management and mitigation.

255 Figure 1 furthermore furthermore highlights that the ranking strongly dependents on the
256 considered group of organisms. Even within a particular effluent the "most risky" compound
257 can therefore only be identified in relation to a specific group of organisms. For example,
258 ofloxacin provides 69% of the STU for algae in the S1-F effluent, but only 0.1% of the STU for

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fish. This pattern reflects the different ecotoxicological profiles of the different pharmaceuticals.

3.2.2. Relative sensitivities of the three trophic levels

Despite pronounced differences in the composition of the pharmaceutical mixture in the seven effluents (table 1, figure 1), a ranking of the three considered trophic levels clearly indicates that algae are the most sensitive group (table 3): in all seven analysed scenarios they rank highest, followed by invertebrates. Fish were always least sensitive. This pattern is consistent with the toxicity profiles of the individual compounds and is most likely due to the fact that – although EC50 values of the standard 72hrs reproduction assays are generally counted as "acute data" – the test itself is a chronic assay using reproduction as a classical chronic endpoint. Additionally, a number of antibiotics are included in the analytical profiles, compounds to which blue-green algae are particularly sensitive.

3.2.3. Mixture risk quotients based on TU analyses versus risk quotients based on the sum of PEC/PNEC ratios

Summing up the Toxic Units trophic level by trophic level in order to calculate a RQ_{STU} follows the conceptual idea of CA more closely than the sum of MEC/PNECs, which yields the $RQ_{MEC/PNEC}$ (Backhaus & Faust, 2012). However, the ratio between those two mixture risk quotients never exceeds 1.3 in all seven effluents included in the monitoring campaign (table 3), if identical assessment factors are used. This provides additional support for the suggestion to use a $RQ_{MEC/PNEC}$, (or $RQ_{PEC/PNEC}$) if the data on the toxicity of all compounds towards all different

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trophic levels are not readily available and the assessment is therefore based on aggregated PNEC values in a first tier (see discussion in (Backhaus and Faust, 2012)).

3.2.4. Use of Independent Action

The application of CA to the pharmaceutical mixtures as given in table 1 can be criticised for the obvious violation of one of the main CA-assumptions (similar mode or mechanism of action). Unfortunately, the application of IA would require knowledge on the actual effects caused by the concentrations of each pharmaceutical in each effluent. Such information is not at hand, as underlying individual concentration-response curves are most often not reported in the available study reports.

Hence, instead of actually calculating the IA-expected mixture toxicity, we estimated the maximum error that occurs by simply ignoring IA. The relationship between the EC50s predicted by CA and IA is as follows:

$$\frac{EC50^{IA}}{EC50^{CA}} \leq \frac{\sum_{i=1}^{n} \frac{c_i}{EC50_i}}{\max_{i\in(1\ldots n)}\left(\frac{c_i}{EC50_i}\right)} \qquad \textit{(eq. 1)}$$

(Junghans et al, 2006). The results are given in table 3 and show that the ratio between CA- and IA-predicted EC50 values for the mixture are at maximum between 4.2 (fish, S1-F) and 1.2 (fish, L2-Gr). For algae, the most sensitive group of organisms which drives the final risk estimate, the ratio is below 2 for all analysed effluents. Given the uncertainty of the hazard and exposure estimates of the individual pharmaceuticals (i.e. quality, quantity and spread of the individual toxicity data and the expectable fluctuations of the concentrations of the individual

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298 pharmaceuticals), a possible maximum error of less than 2 might be considered acceptable. In
299 particular it would not change the regulatory implications of the analyses, i.e. the risk quotient
300 would still exceed 1 even if IA could have been explicitly calculated.

301 *3.2.5. Relation between the predicted environmental risks of the individual pharmaceuticals*
302 *and the pharmaceutical mixtures*

303 The ratio STU/max(TU) (eq. 1) has also been termed the Maximum Cumulative Ratio (MCR) and
304 has been suggested as a measure whether a mixture toxicity assessment is warranted in a
305 certain situation, (Price and Han, 2011). However, it has to be emphasised again that the
306 calculation of descriptors such as the MCR is only possible if all compounds in a given scenario
307 are initially included in the analysis, as it is otherwise not possible to determine the maximum
308 TU. Furthermore, the MCR only describes the *minimum* ratio between the risk of a single
309 mixture component and the complete mixture.

310 A more useful estimate of the expected risk underestimation that might result from ignoring
311 the joint presence of several compounds would hence be the ratio STU/median(TU), i.e. the
312 ratio between the risk of an average mixture component and the complete mixture. Table 3
313 shows that this ratio easily exceeds a factor of 1 000, emphasising the importance of taking
314 mixture effects into consideration in order to improve the realism of environmental risk
315 estimates.

316 **4. Conclusions**

317 In-depth analytical fingerprints as provided by Andreozzi and coworkers (Andreozzi et al., 2003)
318 are critical for improving our understanding of the environmental exposure to pharmaceuticals.

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This, in combination with ecotoxicological studies, allows us to provide a better understanding of their environmental risks. As pharmaceuticals are usually present as multi-component chemical cocktails, a mixture toxicity assessment is indispensable for an environmentally realistic risk assessment. Single substance-based assessments underestimate the actual risks from pharmaceutical mixtures by more than a factor of 1 000 in several of the surveyed effluent streams.

Obviously, any mixture toxicity assessment cannot go beyond the underlying single substance data. The lack of data on the chronic toxicity of most pharmaceuticals as well as the very few data available for *in vivo* fish toxicity has to be regarded as a major knowledge gap in this context. Ignoring Independent Action or using the sum of MEC/PNECs instead of STUs, on the other hand, does not make a major difference for the final risk estimate.

The resulting risk quotients regularly exceed 1, indicating a potential risk for the environment, depending on the specific environmental conditions, in particular the dilution in the recipient stream. It is worth noting that the compounds included in the analytical survey are most likely not be the only pharmaceuticals in the investigated STP effluents, nor will pharmaceuticals be the only ecotoxicologically relevant compounds present. The toxicity estimates presented in the present study hence do not reflect the overall toxicity of the STP streams, but instead provide an assessment of the total toxicity contribution of the investigated pharmaceuticals that were included in the analytical survey.

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References

Andreozzi, R., Raffaele, M., and Nicklas, P., 2003. Pharmaceuticals in STP effluents and their solar photodegradation in aquatic environment. Chemosphere, 50(10), 1319–30

Backhaus, T., Altenburger, R., Boedeker, W., Faust, M., Scholze, M. and Grimme, L.H., 2000. Predictability of the toxicity of a multiple mixture of dissimilarly acting chemicals to Vibrio fischeri. Environmental Toxicology and Chemistry, 19(9), 2348–2356.

Backhaus, T, Scholze, M., and Grimme, L., 2000. The single substance and mixture toxicity of quinolones to the bioluminescent bacterium Vibrio fischeri. Aquatic toxicology, 49(1-2), 49–61.

Backhaus, T., and Faust, M., 2012. Predictive environmental risk assessment of chemical mixtures: a conceptual framework. Environmental science & technology, 46(5), 2564–73.

Cleuvers, M. (2003) Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. Toxicology Letters, 142(3), 185–194.

ECHA (European Chemicals Agency), 2008. Guidance on information requirements and chemical safety assessment Chapter R . 10 : Characterisation of dose [concentration] -response for environment.

EMA (European Medicines Agency), 2006. Guideline on the Environmental Risk Assessment of Medicinal Products for Human Use Doc. Ref. EMEA/CHMP/SWP/4447/00

Fent, K., Escher, C., and Caminada, D., 2006. Estrogenic activity of pharmaceuticals and pharmaceutical mixtures in a yeast reporter gene system. Reproductive toxicology, 22(2), 175–85.

Flaherty, C.M. and Dodson, S.I., 2005. Effects of pharmaceuticals on Daphnia survival, growth, and reproduction. Chemosphere, 61(2), 200–7.

Groten, J., Feron, V., and Sühnel, J., 2001. Toxicology of simple and complex mixtures. Trends in pharmacological sciences, 22(6), 316–322.

Gómez, M.J., Martínez Bueno, M.J., Lacorte, S., Fernández-Alba, R., and Agüera, A., 2007. Pilot survey monitoring pharmaceuticals and related compounds in a sewage treatment plant located on the Mediterranean coast. Chemosphere, 66(6), 993–1002.

Heberer, T., 2002. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicology letters, 131(1-2), 5–17.

Jjemba, P.K., 2008. Pharma-ecology. John Wiley & Sons, Hoboken, New Jersey, ISBN: 978-0-470-04630-2.

Kasprzyk-Hordern, B., Dinsdale, R. M., and Guwy, A. J., 2008. The occurrence of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs in surface water in South Wales, UK. Water research, 42(13), 3498–518.

Kolpin, D. W., Furlong, E. T., Meyer, M. T., Thurman, E. M., Zaugg, S. D., Barber, L. B., Buxton, HT, 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: a national reconnaissance. Environmental science & technology, 36(6), 1202–11.

Kortenkamp, A., Backhaus, T., and Faust, M., 2009. State of the Art Report on Mixture Toxicity. Report to the European Commission, DG Environment available at http://ec.europa.eu/environment/chemicals/pdf/report_Mixture%20toxicity.pdf

PeerJ PrePrints | https://peerj.com/preprints/12 | v1 received: 26 Apr 2013, published: 26 Apr 2013, doi: 10.7287/peerj.preprints.12

381 Kümmerer, K. (ed.) (2008) Pharmaceuticals in the Environment: Sources,Fate,Effects and Risks, Springer.
382 ISBN 978-3-540-74664-5

383 Kümmerer, K. (2009) The presence of pharmaceuticals in the environment due to human use--present
384 knowledge and future challenges. Journal of environmental management, 90(8), 2354–66.

385 Loos, R., Gawlik, B. M., Locoro, G., Rimaviciute, E., Contini, S., and Bidoglio, G. (2009) EU-wide survey of
386 polar organic persistent pollutants in European river waters. Environmental, 157(2), 561–8.

387 MistraPharma (2012) Database on publicly available ecotoxicity data for active pharmaceutical
388 ingredients, http://www.wikipharma.org

389 Moldovan, Z., 2006. Occurrences of pharmaceutical and personal care products as micropollutants in
390 rivers from Romania. Chemosphere, 64(11), 1808–17.

391 Price, P. S. and Han, X., 2011. Maximum cumulative ratio (MCR) as a tool for assessing the value of
392 performing a cumulative risk assessment. International journal of environmental research and
393 public health, 8(6), 2212–25.

394 Segura, P., François, M., Gagnon, C., and Sauvé, S., 2009. Review of the occurrence of anti-infectives in
395 contaminated wastewaters and natural and drinking waters. Environmental health perspectives,
396 117(5), 675–84.

397 US EPA (US Environmental Protection Agency) 2012, ECOTOX Database vers. 4.0 www.epa.gov/ecotox
398 accessed July 2012

399 Vulliet, E. and Cren-Olivé, C., 2011. Screening of pharmaceuticals and hormones at the regional scale, in

400 surface and groundwaters intended to human consumption. Environmental pollution, 159(10), 2929–34

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401 **Tables**

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405 Greece; M1-I: Latina, Italy; L4-I: Naples, Italy; L5-S: Göteborg, Sweden. For further details see
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417 Toxic Unit of an individual pharmaceutical for the indicated trophic level and effluent; STU =
418 Sum of Toxic Units, i.e. =ΣMEC/EC50 for the indicated trophic level and effluent; RQ
419 (MEC/PNEC) = ΣMEC/PNEC with PNEC=min(EC50(algae), EC50(invertebrates), EC50(fish))*1
420 000; RQ (TU)= max(ΣMEC/EC50(Algae), ΣMEC/EC50(Invertebrates), ΣMEC/EC50(Fish))*1 000;
421 EC50(CA)=EC50 predicted by Concentration Addition; EC50(IA)=EC50 predicted by Independent
422 Action

423

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424 **Figures**

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426 Figure 1: Distribution of Toxic Units for the analysed STP effluents and three trophic levels

427 (algae, invertebrates and fish)

428

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Compound	CAS	S1-F	L1-F	L2-Gr	M1-I	L3-I	L4-I	L5-S
Acebutolol	37517-30-9	3.86E-04	2.38E-04	2.97E-05	1.19E-04	5.94E-05	3.27E-04	n.d.
Aminopyrine	58-15-1	1.86E-03	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Betaxolol	63659-18-7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Bezafibrate	41859-67-0	n.d.	2.96E-03	n.d.	n.d.	n.d.	2.52E-03	n.d.
Carbamazepine	298-46-4	4.15E-03	5.08E-03	4.36E-03	1.27E-03	1.44E-03	2.12E-03	3.68E-03
Ciprofloxacin	85721-33-1	1.81E-04	1.81E-04	2.11E-04	2.11E-04	1.81E-04	1.21E-04	9.05E-05
Clofibrate	637-07-0	n.d.	n.d.	3.30E-03	n.d.	n.d.	n.d.	n.d.
Clofibric Acid	882-09-7	n.d.	n.d.	n.d.	3.17E-03	n.d.	1.07E-03	2.14E-03
Diclofenac	15307-86-5	1.38E-03	8.44E-04	3.01E-03	1.59E-03	5.00E-03	1.84E-02	n.d.
Enoxacin	74011-58-8	9.37E-05	3.12E-05	9.37E-05	9.37E-05	3.12E-05	9.37E-05	3.12E-05
Fenofibrate	49562-28-9	3.33E-04	5.54E-05	4.43E-04	4.43E-04	2.77E-04	4.43E-04	n.d.
Fenoprofen	31879-05-7	1.16E-03	7.84E-04	n.d.	n.d.	n.d.	n.d.	n.d.
Flurbiprofen	5104-49-4	8.60E-04	n.d.	n.d.	n.d.	n.d.	1.39E-03	n.d.
Gemfibrozil	25812-30-0	5.35E-03	2.40E-04	2.84E-03	3.24E-03	3.36E-03	1.90E-02	8.27E-03
Ibuprofen	15687-27-1	8.82E-03	9.70E-05	2.42E-04	8.73E-04	9.70E-05	9.70E-05	3.45E-02
Ketoprofen	22071-15-4	n.d.	6.37E-03	n.d.	n.d.	n.d.	n.d.	n.d.
Lomefloxacin	98079-51-7	5.12E-04	5.41E-04	8.25E-04	9.11E-04	5.12E-04	6.26E-04	3.70E-04
Metoprolol	37350-58-6	2.99E-04	2.99E-04	3.74E-04	3.74E-05	3.74E-05	3.74E-04	1.46E-03
Naproxen	22204-53-1	7.51E-03	2.21E-03	n.d.	1.26E-03	1.78E-03	2.27E-02	9.34E-03
Norfloxacin	70458-96-7	1.57E-04	2.51E-04	2.19E-04	2.19E-04	1.88E-04	1.88E-04	9.39E-05
Ofloxacin	82419-36-1	9.13E-04	1.41E-03	1.27E-03	1.61E-03	8.03E-04	8.58E-04	3.32E-04

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Compound	CAS	S1-F	L1-F	L2-Gr	M1-I	L3-I	L4-I	L5-S
Oxprenolol	6452-71-7	1.88E-04	7.54E-05	3.77E-05	3.77E-05	n.d.	1.13E-04	n.d.
Phenazone	60-80-0	n.d.	n.d.	n.d.	n.d.	1.97E-03	n.d.	n.d.
Propranolol	525-66-6	3.86E-05	1.54E-04	3.86E-05	3.86E-05	3.86E-05	3.47E-04	3.86E-05
Sulfamethoxazole	723-46-6	3.55E-04	2.76E-04	3.55E-04	3.95E-05	n.d.	1.18E-04	7.90E-05
Trimethoprim	738-70-5	1.38E-04	6.89E-05	2.76E-04	1.38E-04	1.03E-04	4.48E-04	1.72E-04

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Table 2: Finally selected short-term toxicity estimates (EC50's) for algae, invertebrates and fish.

A full list of all data compiled from the publically available literature is provided in the supporting information.

Values in µmol/L. Values in italics are QSAR estimates (see material and methods).

Compound	Algae	Invertebrates	Fish
Acebutolol	*3.69*	136.50	*562.90*
Aminopyrine	*25.30*	*92.27*	*1221.63*
Betaxolol	*3.32*	300.00	*45.20*
Bezafibrate	414.57	23.74	266.00
Carbamazepine	133.75	15.91	149.83
Ciprofloxacin	0.05	181.08	301.80
Clofibrate	49.44	116.19	2.00
Clofibric Acid	414.64	335.44	65.22
Diclofenac	45.58	70.51	19.00
Enoxacin	61.50	*275928.78*	*678389.32*
Fenofibrate	54.98	138.57	9.00
Fenoprofen	*132.74*	*122.54*	*164.09*
Flurbiprofen	*151.16*	*145.11*	*196.92*
Gemfibrozil	60.68	3.60	90.80
Ibuprofen	11.15	8.00	207.40
Ketoprofen	7.87	9.05	157.70
Lomefloxacin	0.53	370.00	483.85
Metoprolol	11.54	32.91	115.95
Naproxen	16.07	11.38	218.90
Norfloxacin	0.17	*332940.79*	3131.55
Ofloxacin	0.04	48.18	2767.27
Oxprenolol	*8.85*	38.06	*222.32*
Phenazone	2603.26	5312.77	531.28
Propranolol	2.26	0.85	4.67
Sulfamethoxazole	0.11	61.24	108.00
Trimethoprim	37.89	188.76	344.45

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		S1-F	L1-F	L2-Gr	M1-I	L3-I	L4-I	L5-S
Max TU	algae	2.28E-02	3.53E-02	3.18E-02	4.01E-02	2.01E-02	2.14E-02	8.30E-03
	invertebrates	1.49E-03	7.04E-04	7.88E-04	8.99E-04	9.32E-04	5.28E-03	4.31E-03
	fish	7.29E-05	4.44E-05	1.65E-03	8.35E-05	2.63E-04	9.69E-04	1.66E-04
STU	algae	3.32E-02	4.51E-02	4.24E-02	4.80E-02	2.61E-02	2.97E-02	1.61E-02
	invertebrates	3.66E-03	1.67E-03	1.26E-03	1.32E-03	1.33E-03	8.25E-03	7.76E-03
	fish	3.04E-04	1.95E-04	1.94E-03	2.48E-04	3.64E-04	1.46E-03	3.81E-04
	Ranking	A-I-F	A-I-F	A-I-F	A-I-F	A-I-F	A-I-F	A-I-F
Contribution of the first 10 compounds to the total STU (%)	algae	99.5	99.8	99.8	99.9	99.9	99.6	99.8
	invertebrates	99.3	98.9	99.5	99.6	99.8	99.8	100.0
	fish	97.3	96.8	99.9	99.1	99.7	99.6	99.9
RQ (MEC/PNEC)		35.5	45.8	45.2	49.2	27.3	36.3	19.9
RQ (TU)		33.2	45.1	42.4	48.0	26.1	29.7	16.1
Ratio RQ(MEC/PNEC) / RQ (TU)		1.07	1.01	1.06	1.02	1.05	1.22	1.24
Maximum ratio EC50(IA)/EC50(CA)	algae	1.5	1.3	1.3	1.2	1.3	1.4	1.9
	invertebrates	2.5	2.4	1.6	1.5	1.4	1.6	1.8
	fish	4.2	4.4	1.2	3.0	1.4	1.5	2.3
Sum of TU / median TU	algae	**1.41E+03**	**5.24E+03**	**5.27E+03**	**6.12E+03**	**8.75E+03**	**2.42E+03**	6.36E+03
	invertebrates	9.40E+02	8.98E+02	1.17E+03	1.42E+03	3.30E+03	3.08E+03	**3.93E+07**
	fish	3.19E+02	3.52E+02	6.15E+03	7.22E+02	1.84E+03	1.74E+03	2.53E+04

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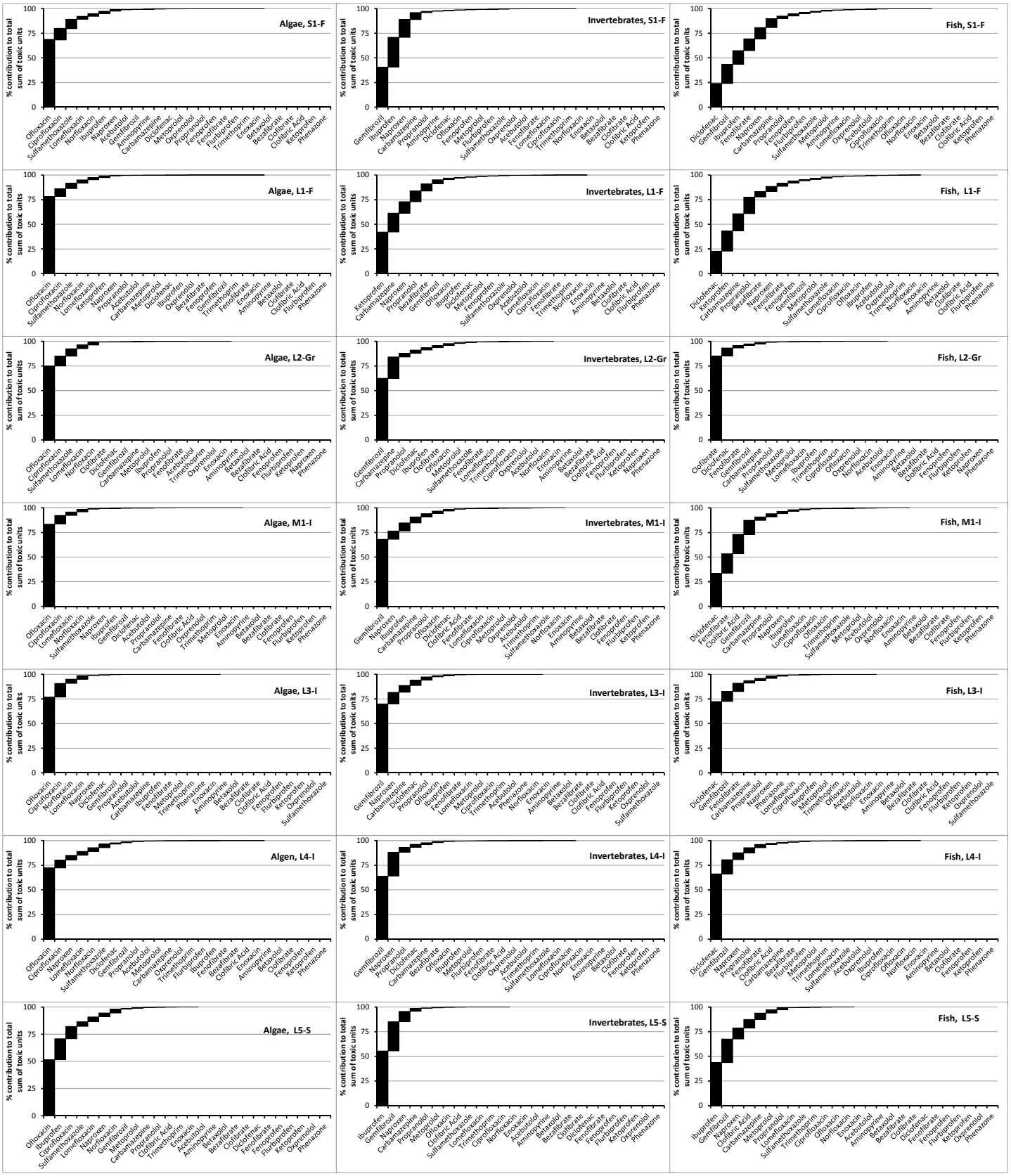
Figure 1: Distribution of Toxic Units for the analysed STP effluents and three trophic levels (algae, invertebrates and fish)



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