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In Vitro Assessment of Modes of Toxic Action of Pharmaceuticals in Aquatic Life

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An ecotoxicological test battery based on a mode-of-action approach was designed and applied to the hazard identification and classification of modes of action of six pharmaceuticals (carbamazepine, diclofenac, ethinyl estradiol, ibuprofen, propranolol, and sulfamethoxazole). The rationale behind the design of the battery was to cover the relevant interactions that a compound may have with biological targets. It is thus not comprehensive but contains representative examples of each category of mode of toxic action including nonspecific, specific, and reactive toxicity. The test battery consists of one test system for nonspecific toxicity (baseline toxicity or narcosis), two test systems for specific effects, and two test systems for reactive toxicity. The baseline toxicity was quantified with the Kinspec test, which detects membrane leakage via measurements of membrane potential. This test system may also be used to detect the specific effects on energy transduction, although this was not relevant to any compound investigated in this study. As examples of specific receptor-mediated toxicity, we chose the yeast estrogen screen (YES) as a specific test for estrogenicity, and the inhibition of chlorophyll fluorescence in algae to assess specific effects on photosynthesis. Reactive modes of action were assessed indirectly by measuring the relevance of cellular defense systems. Differences in growth inhibition curves between a mutant of *Escherichia coli* that could not synthesize glutathione and its parent strain indicate the relevance of conjugation with glutathione as a defense mechanism, which is an indirect indicator of protein damage. DNA damage was assessed by comparing the growth inhibition in a strain that lacks various DNA repair systems with that in its competent parent strain. Most compounds acted merely as baseline toxicants in all test systems. As expected, ethinylestradiol was the only compound showing estrogenic activity. Propranolol was baseline-toxic in all test systems except for the photosynthesis inhibition assay, where it surprisingly showed a 100-fold excess toxicity over the predicted baseline effect. The exact mode of toxic action could not be confirmed, but additional chlorophyll fluorescence induction experiments excluded the possibility of direct interference with photosynthesis through photosystem II inhibition. Mixture experiments were performed as a diagnostic tool to analyze the mode of toxic action. Compounds with the same mode of toxic action showed the expected concentration addition. In the photosynthesis inhibition assay, agreement between experimental results and prediction was best for two-stage predictions considering the assigned modes of action. In a two-stage prediction, concentration addition was used as a model to predict the mixture effect of the baseline toxicants followed by their independent action as a single component combined with the specifically acting compound propranolol and the reference compound diuron. A comparison with acute toxicity data for algae, daphnia, and fish showed generally good agreement for the nonspecifically acting compounds but also that the proposed test battery offered better diagnostic value in the case of the specifically acting compounds.

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Introduction

Pharmaceuticals and their metabolites are ubiquitous in the environment. Most of them are neither truly persistent nor highly bioaccumulative and many, but not all, are quite well eliminated in wastewater treatment plants. However, they are continuously emitted into the wastewater streams and therefore contribute to the burden of chemical pollutants even though their concentrations in receiving water are quite low (for an overview on recent literature see refs *1*−*3*).

Their environmental presence triggered a proposal to include an environmental risk assessment in the registration procedure for medicinal products (*4*). In 2000, a guideline on the assessment of the environmental impact of veterinary medicines was issued by the European Medicines Evaluation Agency (EMEA) (*5*). The counterpart for human pharmaceuticals is still in draft (*6*). Although the U.S. guidance document for the environmental assessment of human drugs was issued in 1998 (*7*), the registration of pharmaceuticals is not dependent on the results of this assessment (*4*).

The exposure assessment for pharmaceuticals takes account of the specific pattern of use; i.e., only the aquatic component is considered (*6*, *8*). According to the draft document issued by EMEA (*6*), the effect is assessed in an analogous way to industrial chemicals and biocides (*9*); i.e., a predicted no-effect concentration (PNEC) is derived from acute or chronic toxicity data for aquatic organisms after division by an uncertainty or extrapolation factor. Since acute toxicity toward aquatic organisms is relatively low (*10*, *11*), most risk assessments for single pharmaceuticals came to the conclusion that no risk reduction measures are necessary (*12*).

However, pharmaceuticals are designed to be bioactive. Their therapeutic use is to elicit a specific beneficial mode of action in humans. At higher doses, adverse side effects may be encountered in humans. It can be expected that any effect, beneficial or adverse, could also occur in aquatic organisms with similar biological functions and receptors. A well-known example is the endocrine disrupting effect of steroid hormones on fish (*13*, *14*), which already occurs at very low concentrations. Moreover, it cannot be excluded that pharmaceuticals act through additional unknown modes of toxic action on nontarget organisms. These effects might again be rather subtle and pass unrecognized in the case of single compounds due to low exposure concentrations.

Pharmaceuticals never occur alone in the environment but always in combination with other compounds—not only with other pharmaceuticals but also with their own metabolites or other environmental pollutants, e.g., industrial

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chemicals, pesticides, or personal care products. Possible mixture effects are therefore relevant.

For compounds that share a common mode of toxic action, cumulative exposure must be considered because such compounds typically act in a concentration additive manner (*15*, *16*). For traditional environmental pollutants, with the exception of specifically acting pesticides, the common mode of toxic action often amounts to baseline toxicity (*17*, *18*), which constitutes the minimum toxicity exhibited by any compound due to accumulation in biological membranes (*19*). In contrast, it is very likely that specific modes of toxic action need to be considered in assessing the risk of mixtures of pharmaceuticals (*20*), although a first quantitative structure−activity relationship (QSAR) analysis showed that selected nonsteroidal antiinflammatory drugs act as baseline toxicants and are concentration-additive in mixtures (*21*). Therefore, the identification of modes of toxic action toward nontarget organisms is a crucial step in assessing the effect of pharmaceuticals *(20*, *22*).

A pharmaceutical that is optimized for its action on a human receptor (or biological function) does not necessarily exhibit similar activity on an analogous receptor (or biological function) in a nontarget organism (*23*). Furthermore, it is also possible that similar targets govern different processes in different species (*23*). This option is especially relevant for invertebrates, which are genetically very remote from humans but still share highly conservative metabolic features. Highly conservative mechanisms are often related to the basic functioning of cells, such as ATP formation, and are crucial for toxicity. Several authors have proposed to make the best use of pharmacokinetic and pharmacodynamic information to derive a potential activity profile for ecological effects of pharmaceuticals that then guides the selection of appropriate ecotoxicity test systems (*20*, *23*, *24*).

In addition, we propose to complement this approach with a comprehensive test battery for screening potential ecological hazards. Such a test battery should identify basic interactions of pharmaceuticals with biota and classify their mode of toxic action in aquatic life. We have recently proposed a scheme for mode-of-action classification (*25*, *26*). Here, we apply this concept to develop a mode-of-action-based test battery that is by no means complete but represents a first step toward attaining this goal. We investigated the quantitative response of the suggested test battery on six pharmaceuticals. The compounds investigated comprise carbamazepine, diclofenac, ethinylestradiol, ibuprofen, propranolol, and sulfamethoxazole. They were selected on grounds of abundant usage, occurrence in the environment, and diversity of therapeutic classes (*2*, *3*). Additional criteria were diversity of physicochemical properties, ranging from persistent to biodegradable, including compounds of low and high hydrophobicity, as well as the wish to include acids, bases, and neutral compounds. Analysis of the toxicity and mode of toxic action of single compounds is complemented by mixture studies. The mixture studies serve as a diagnostic tool to support the mode-of-action analysis by testing if a set of chemicals act according to similar or dissimilar mechanisms (*27*). Finally, we compare our results with literature data and discuss the utility of our approach for hazard assessment.

Test Battery Based on Mode of Action

Given the complexity of biological structures and cellular functions, it is virtually impossible to cover every mode of toxic action in a screening test battery. Therefore, we designed a test battery that covers several types of molecular interactions that a pharmaceutical or any other environmental pollutant may have with a biological target. A biological target might be a target molecule, such as an enzyme or receptor, or a target site such as the biological membrane.

Basically, there are three types of interactions between a pollutant and a biological target (*25*): nonspecific and specific interactions as well as chemical reactions. Pollutants may "partition" into biological target sites, e.g. biological membranes, by nonspecific and nondirected van der Waals and H-donor/H-acceptor interactions. If the same forces are spatially directed or ionic interactions are possible, we speak of specific interactions or steric fit. These may include enzyme inhibition or specific receptor binding. Chemical reactions between pollutants and biological target molecules are often irreversible and can severely damage lipids, proteins, or DNA.

Figure 1 shows these interactions and the resulting primary toxic mechanisms. Each of these interactions is related to one or more modes of toxic action (for definition of terms and more details on the derivation of this classification scheme, refer to ref *25*). The most important nonspecific mode of action is baseline toxicity, also termed narcosis (*19*), which can be detected with the "Kinspec" test system (*28*). This detects the disturbance of membrane structure and functioning by measuring the decay of the membrane potential in isolated photosynthetic membrane vesicles (*28*). With slight modifications, this test system can also be used to assess specific effects on energy transduction such as uncoupling of the electron transfer from the ATP synthesis and specific inhibition of the electron transfer and the ATP synthesis, which falls in the class of specific effects discussed below (*29*, *30*).

The second class of modes of action includes all specific and receptor-mediated effects. Since there is a myriad of possible receptors and enzymes whose activity can be affected by specific interactions with pollutants, they cannot all be covered with specific test systems. Therefore we limit the selection to a few representatives relevant to potential nontarget effects of pharmaceuticals. Two specific modes of action that are particularly relevant in aquatic environments are inhibition of photosynthesis and endocrine disruption. A background level of herbicides is present in many freshwater systems, and if pharmaceuticals additionally affect photosynthesis, primary producers may become impaired. We used chlorophyll fluorescence measurements in green algae to detect specific and nonspecific effects on photosynthesis (*31*, *32*). Receptor−ligand mediated modes of action are still considered the major pathway for endocrine disruption, although other mechanisms have also been observed, e.g., a ligand-independent pathway, a pathway independent of DNA binding and nongenomic pathways (*33*). Here, we selected the yeast estrogen screen (YES) (*34*) as a test system for receptor-mediated estrogenic activity. We are aware that the proposed test battery has major shortcomings on receptor-mediated mechanisms and plan to include a wider range of effects, e.g. inhibition of acetylcholine esterase, androgen receptors, and aryl-hydrocarbon receptors, in future projects.

The third class of modes of action is reactive toxicity. This class includes direct reaction by electrophilic pollutants and indirect reactions by reactive oxygen species. Pharmaceuticals are unlikely to produce reactive oxygen species unless they occur in mixtures with heavy metals or produce indirectly reactive oxygen species after blocking mitochondrial or photosynthetic electron flow (*35*). However, certain pharmaceuticals, such as cytostatic drugs, and possibly some of the metabolites, may react as electrophiles with biological nucleophiles. Again, this mechanism is not very likely to occur but must be checked to definitely exclude this possibility. Both DNA and proteins contain nucleophilic sites and may therefore be the target of reactive chemicals. Both subclasses of reactive mechanisms can be detected indirectly through cellular defense mechanisms. Growth inhibition differences between an *Escherichia coli* (*E. coli*) strain that cannot synthesize glutathione (GSH) and the corresponding parent

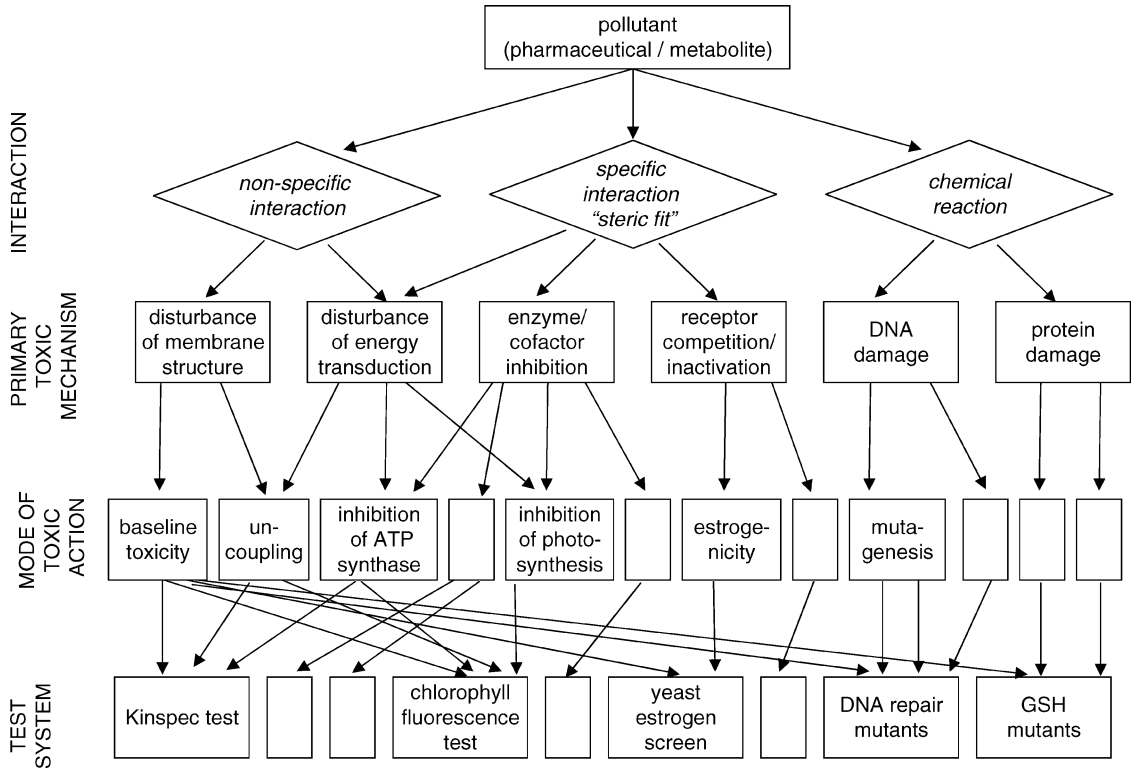

FIGURE 1. Classification of modes of toxic action according to interactions between pollutants and biomolecules, and an overview of the test systems selected in this study. The empty fields refer to additional modes of toxic action. Corresponding test systems are still to be identified and included.

strain gives an impression of the importance of conjugation with GSH as a detoxification step as well as of the importance of nonspecific protein damage (*36*). Analogously, the difference between concentration-effect curves for growth inhibition of an *E. coli* strain that lacks many important DNA repair systems (SOS response, adaptive response, and repair of N-3 adenine) and its parent strain is a good indicator of DNA damage (*36*).

Baseline toxicity is typically detected in all test systems as the baseline effect of a given test system. However, it can directly be assessed only in the Kinspec system, whereas results in the other test systems can only be attributed to baseline toxicity by deduction. We have therefore experimentally determined a baseline in most test systems and added a positive control as a reference, i.e., a compound that clearly exhibits the specific effect targeted by the given test system.

Additionally, certain primary toxic mechanisms can be linked to different modes of toxic action, and these are often not clear-cut but can include several primary interactions. An example is endocrine disruption, which may be a direct, receptor-mediated mechanism or can act indirectly by affecting the hormone metabolism. This is a caveat that cannot be avoided. However, we feel it is important to introduce ordering principles and structures when deriving a test battery based on mode of action.

As can be seen from Figure 1, the test battery is not exhaustive but covers relevant interactions and examples of action mechanisms of crucial cellular functions. Since it has a modular structure, it can be readily extended, as indicated by the blank boxes in the figure. Some of these blank boxes could readily be filled, although certain modes of toxic action are still unknown or no specific test system has yet been developed for them. This refers mainly to specific and receptor-mediated mechanisms. It will never be possible to include them all in a test battery. At this stage, therefore, we opted to include only selected mechanisms that are known to be relevant for environmental pollutants and are likely to be encountered when screening pharmaceuticals. Whenever this test battery is used for applications other than assessing the hazards of pharmaceuticals, additional receptor-mediated mechanisms, such as acetylcholine esterase inhibition if insecticides are investigated, should be considered and corresponding test systems should be added to the battery. Note also that the actual test systems function on different levels of biological organization, from the subcellular (Kinspec) via reproduction in bacteria (DNA and GSH mutants) to physiological measures in living organisms (chlorophyll fluorescence in algae). Nevertheless, the test systems share the feature of responding specifically to one or more modes of toxic action. In this respect, the battery is similar to test batteries proposed earlier for the mode-of-action classification (*37*, *38*).

Materials and Methods

Chemicals. The pharmaceuticals propranolol (CAS RN 525-66-6, >98%), sulfamethoxazole (CAS RN 723-46-6, no purity reported), 17-α-ethinylestradiol (CAS RN 57-63-6, >98%), diclofenac (CAS RN 15307-86-5, no purity reported), ibuprofen (CAS RN 15687-27-1, >98%), and carbamazepine (CAS RN 298-46-4, no purity reported) were obtained from Sigma (Buchs, Switzerland). The positive controls were diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea (CAS RN 330-54-1, >99.4%), from Riedel-de Haen, Buchs, Switzerland) for the chlorophyll fluorescence assay, 17-β-estradiol (CAS RN 50-28-2, from Sigma) for the yeast estrogen screen, hydroxyethylacrylate (CAS RN 818-61-1, ≥97%, from Fluka) for the GSH assay, and ethyl methane sulfonate (EMS, CAS RN 62-50-1, ≥98%, from Fluka, Buchs, Switzerland) for the DNA damage assay. Baseline toxicants are listed in ref *28*. All solvents and salts were obtained from Fluka. Stock solutions were prepared in water, acidified for the bases, and made slightly alkaline for the acids to improve solubility. If they could not be dissolved in water, 0.1 M solutions in ethanol

TABLE 1. Physicochemical Parameters and Effect Concentration of the Six Pharmaceuticals Investigated in the Different Test Systems of the Test Battery Based on Mode of Action

	octanol–water partition coefficient	liposome–water distribution ratio at pH 7	acidity constant	baseline toxicity (Kinspec)[a]	inhibition of PSII quantum yield[b]	yeast estrogen screen[c]	biosensor GSH+ MJF276	biosensor GSH− MJF335	biosensor DNA+ MV1161	biosensor DNA− MV4108
	log K_{ow}	log D_{lipw} at pH 7	pK_a	log(1/EC(M))	log(1/EC_{50}(M))	log(1/EC_{50}(M))	log(1/EC_{50}(M))	log(1/EC_{50}(M))	log(1/EC_{50}(M))	log(1/EC_{50}(M))
propranolol	3.48[d]	2.77[a]	9.24[a]	3.58	5.61	<2.00	3.35	3.32	3.04	3.05
sulfamethoxazole	0.89[f]	1.30[g]	5.7[a]	<3.3	3.08	<3.00	<3.00	<3.00	<3.00	<3.00
ethinylestradiol	3.67[e]	3.81[a]		4.46	4.38	9.6	<3.00	<3.00	<3.00	<3.00
diclofenac	4.51[d]	2.65[a]	3.99[a]	3.17	3.30	<2.00	<3.00	<3.00	<2.70	<2.70
ibuprofen	3.97[d]	1.91[a]	4.45[a]	3.03	3.35	<2.00	<2.00	<3.00	2.23	2.27
carbamazepine	2.45[f]	2.73[g]		nd	<3.00	<3.00	<3.00	<3.00	<3.00	<3.00
ethanol	−0.31[d]	nd[h]		nd	−0.47	nd	0.33	0.34	nd	nd

[a] Data from ref 28. [b] Data from ref 40. [c] EC_{50} for the estrogenic effect, cytotoxicity is described in the text. [d] Data from ref 60. [e] Data from ref 61. [f] Data from ref 62. [g] Calculated from K_{ow} with the QSAR equation log K_{lipw} = 0.9 log K_{ow} + 0.52 for polar narcotics (*47*, *48*) and with the following ion correction for ionogenic compounds: $D_{lipw}(pH7) = f_{neutral}K_{lipw}$. [h] nd = not determined.

were prepared, and ethanol was either evaporated in the test vials (YES) or accounted for by adding the same amount of ethanol to the controls. In addition, concentration-effect curves were measured for ethanol (EC_{50} values are listed in Table 1) and concentration additivity with ethanol was checked. The reported concentrations refer to nominal values.

Kinspec Test. The Kinspec test is performed with membrane vesicles isolated from the photosynthetic bacterium *Rhodobacter sphaeroides*. Short flashes of light are applied to induce the electron transport and buildup of a membrane potential. In the presence of baseline toxicants, the membrane becomes leaky and the membrane potential is decreased. This effect can be visualized by following the absorbance change at 505 nm, which is directly correlated to the membrane potential. The details of the method and the experimental results for pharmaceuticals have been reported elsewhere (*28*).

Chlorophyll Fluorescence Test. The green unicellular algae *Desmosdesmus subspicatus* (*D. subspicatus*, Chodat) SAG 86.81, obtained from the "SAG-Sammlung von Algenkulturen" at the University of Göttingen, Germany, were grown in batch cultures in the medium of the OECD test guideline 201 for the alga growth inhibition test (*39*). The standard test was performed as reported in ref *40*. Fluorescence was measured after 24 h of exposure with a ToxY-PAM fluorometer (prototype manufactured by Gademann Instruments, Würzburg, Germany; series production by Heinz Walz, Effeltrich, Germany). The effective quantum yield of energy conversion at photosystem II reaction centers, *Y*, was calculated with eq 1, where *F* is the momentary fluorescence yield and $F_{M'}$ the maximum fluorescence yield induced by a saturation pulse (*31*). The inhibition of the photosystem II quantum yield was calculated using eq 2 (*32*).

$$Y = \left(\frac{F_{M'} - F}{F_{M'}}\right) \quad (1)$$

$$\text{photosynthesis inhibition (\%)} = \left(1 - \frac{Y_{sample}}{Y_{control}}\right) \times 100\% \quad (2)$$

During the development of the method, growth was additionally determined by counting cell numbers in a Neubauer cell (Brand, Wertheim, Germany) and by measuring the optical density at 685 nm (spectrophotometer Uvikon 930, Kontron Instruments, Munich, Germany).

For propranolol and the reference compound diuron, the induction kinetics of fluorescence in dark-adapted algae were additionally measured as described in ref *32* according to the method of Schreiber et al. (*41*).

Yeast Estrogen Screen. The recombinant yeast estrogen screen was kindly provided by J. Sumpter (Brunel University, Uxbridge, U.K.). The culture and exposure of the yeast cells were performed as described by Routledge and Sumpter (*34*) with minor changes and data evaluation as reported by Rutishauser et al. (*42*). For those compounds that did not show estrogenic effects, growth inhibition was detected by light scattering measured as the optical density at 620 nm.

GSH Test. The detoxifying effects of GSH on the population growth of *E. coli* were evaluated by comparing the growth inhibition of the MJF335 strain, which cannot synthesize GSH, and its parent strain MJF276 (*36*, *43*) after incubation with pharmaceuticals. These strains were obtained from Stéphane Vuilleumier (University of Strasbourg, France). The determination and calculation of growth inhibition of MJF335 and MJF276 were performed as described by Harder et al. (*36*), but the incubation time with the pharmaceuticals was increased to 24 h because the compounds are more stable and less toxic than the electrophiles tested earlier. Growth was monitored by the difference in optical density at 600 nm between the start and end of the exposure period ΔOD_{600}. Growth inhibition was calculated according to

$$\text{growth inhibition (\%)} = \left(1 - \frac{\Delta OD_{600,sample}}{\Delta OD_{600,control}}\right) \times 100\% \quad (3)$$

EC_{50} values were derived from concentration-growth inhibition curves as described below. If there is a significant difference (e.g., a factor of 2.4 or higher (*36*)) between the EC_{50} values of MJF276 and MJF335, detoxification by GSH plays a role, which is also an indication of the potential for protein damage. Hydroxyethylacrylate, which has EC_{50} values of 7.1×10^{-4} M with MJF 335 and 1.5×10^{-4} M with MJF276, corresponding to a relative potency factor of 4.6 (*36*), was used as a positive control.

DNA Damage Test. The *E. coli* strains MV1161 (*44*) and MV4108 were used to test for DNA damage (*36*). These strains were kindly provided by Michael Volkert (University of Massachusetts, Worcester, MA). MV1161 is the parent strain of MV4108, which is deficient in several DNA repair mechanisms (SOS response (*recA*, *uvrA*), adaptive response (*ada*, *alkB*, *alkA*), and repair of 3-methyladenine (*tag*)) and is therefore very sensitive toward any genotoxic chemical. Experiments were performed according to ref *36* with a 1 h exposure time and using colony-forming units (cfu) as a measure of growth inhibition (eq 4). If the EC_{50} value of a

given chemical with MV4108 is more than 10 times smaller than that with MV1161, DNA damage is a likely mode of toxic action (*36*).

$$\text{growth inhibition (\%)} = \left(1 - \frac{\text{cfu}_{\text{sample}}}{\text{cfu}_{\text{control}}}\right) \times 100\% \quad (4)$$

Since the reproducibility of this test was not very good due to the large number of mutations in the MV4108 strain, a positive control of 2 μL of EMS/(5 mL of *E. coli* suspension) (resulting in 3.8×10^{-3} M EMS) was performed in parallel to each assay. This concentration of EMS resulted in 100% inhibition of MV4108 growth and 0% inhibition of MV1161 growth. Only data from those experiments that yielded this result in the presence of EMS were further evaluated. We identified approximately 30% of the experiments as not reproducible with this procedure but were unable to determine the cause of these outliers.

Evaluation of the Concentration Effect Curves. The concentrations resulting in a 50% effect (EC_{50}) were derived from a log−logistic fit of the concentration-effect curves (eq 5) using the Prism 4.0 software (GraphPad, San Diego, CA) by computing the best fit for experimental data with the adjustable parameters of slope m and EC_{50}.

$$\text{effect (\%)} = \frac{100\%}{1 + 10^{m}(\log \text{EC50} - \log \text{concn})} \quad (5)$$

Asymmetric logistic equations (*32*) and other models (*45*) for describing the concentration-effect curves were also tested but did not generally improve the quality of the fit, so all the experimental data were evaluated with the simple two-parameter symmetric logistic model. Errors for EC_y at effect levels y other than 50% (eq 6) were derived by error propagation (eq 7).

$$\log \text{EC}_y = -\frac{\log((1-y)/y)}{m} + \log \text{EC}_{50} \quad (6)$$

$$\sigma_{\log \text{EC}_y} = \sqrt{\left(\log\left(\frac{1-y}{y}\right)m^{-2}\right)^2 \sigma_m{}^2 + \sigma^2_{\log \text{EC}_{50}}} \quad (7)$$

Baseline Toxicity versus Specific Mode of Toxic Action. The toxic ratio (TR, eq8) is defined as the ratio of the $EC_{50,baseline}$ of a given compound to the experimentally determined $EC_{50,exptl}$. TR indicates whether a compound acts according to baseline toxicity or according to a specific mode of toxic action (*46*). As a reference for baseline toxicity, $EC_{50,baseline}$, either the measured EC in the Kinspec assay may be used or it may be derived from a QSAR of baseline toxicity in the respective organism. TR < 10 corresponds to baseline toxicity, and TR ≥ 10 indicates a specific mode of toxic action (*46*).

$$\text{TR} = \frac{\text{EC}_{50,\text{baseline}}}{\text{EC}_{50,\text{exptl}}} \quad (8)$$

Since we are dealing with ionogenic compounds whose octanol−water partition coefficient K_{ow} (Table 1) does not represent a good model for their affinity to biological membranes, the QSAR equations were based on the liposome−water distribution coefficients K_{lipw} of the neutral species or the liposome−water distribution ratios D_{lipw}(pH7) for ionogenic compounds (Table 1). If no experimental D_{lipw} values were available, the K_{lipw} of the neutral species was estimated by the log K_{ow} to log K_{lipw} ratio published by Vaes et al. for nonpolar and polar narcotics (*47*, *48*).

Mixture Experiments. Mixture experiments in the Kinspec system are reported elsewhere (*28*). In the chlorophyll fluorescence test, one such experiment was performed with a mixture of five pharmaceuticals, mixed in the ratio of their EC_{50}s. Another experiment was performed with the five pharmaceuticals plus the PSII inhibitor diuron (*49*). The fraction p_i of a mixture component i is defined as follows:

$$p_i = \frac{\text{EC}_{50_i}}{\sum_{j=1}^{n} \text{EC}_{50_j}} \quad (9)$$

The concentration-effect curves and $EC_{50_{mix}}$ values were derived according to eq 5 with a total concentration c_{mix} on the concentration axis. c_{mix} is the sum of the concentration of the n components i, c_i. If compounds act according to a similar mechanism, they obey concentration addition (*15*). Concentration addition means that the sum of all toxic units, i.e., the ratios of c_i to the effect concentration at any effect level y, EC_{y_i} (eq 6), must equal 1 (eq 10).

$$\sum_{i=1}^{n} \frac{c_i}{\text{EC}_{y_i}} = 1 \quad (10)$$

Consequently, the effect concentration $EC_{y_{mix}}$ is calculated for each effect level y from

$$\text{EC}_{y_{\text{mix}}} = \left(\sum_{i=1}^{n} \frac{p_i}{\text{EC}_{y_i}}\right)^{-1} \quad (11)$$

By incrementally applying eq 11 for effects y from 0 to 100%, a concentration-effect curve for concentration addition can be predicted. The standard deviation of $EC_{y_{mix}}$ was derived by error propagation of eq 11 with the standard deviation of the EC_{y_i} derived by error propagation (eq 7) and ignoring any error in p_i. Alternatively, the error of log $EC_{y_{mix}}$ was approximated by a resampling method using a log-normal distribution of the errors of all input parameters and 1000 random resampling steps of eq 11. The routine was written in Mathematica (Version 5.0, Wolfram Research). The error σEC_{y_i} was derived with error propagation from eq 6 and the error of p_i, σp_i, was estimated to be a 5% pipetting error. Both methods yielded similar error bars, but preference was given to the bootstrap method as it yielded errors with a log-normal distribution.

The prediction of the alternative mixture concept of independent action (*50*) can be calculated with

$$E(c_{\text{mix}}) = 1 - \prod_{i=1}^{n}(1 - E(c_i)) \quad (12)$$

where $E(c_{mix})$ corresponds to the predicted effect of the mixture and $E(c_i)$ to the effect of mixture component i. Again, error analysis was performed both by error propagation and by a bootstrap method as described above. The confidence intervals obtained with the resampling method are recorded in Figure 4.

Results and Discussion

Baseline Toxicity and Specific Effects on Energy Transduction (Kinspec Test). The effects of pharmaceuticals on energy transduction were evaluated in an earlier study (*28*). The EC values are reported in Table 1. Ibuprofen and diclofenac are weak organic acids (for their acidity constants pK_a, see Table 1). Some weak organic acids are known to be uncouplers of oxidative phosphorylation and photophosphorylation (*51*). We therefore tested the potential of pharmaceuticals to act as uncouplers.

In contrast to our expectations, their experimental effect in the Kinspec system could be clearly attributed only to

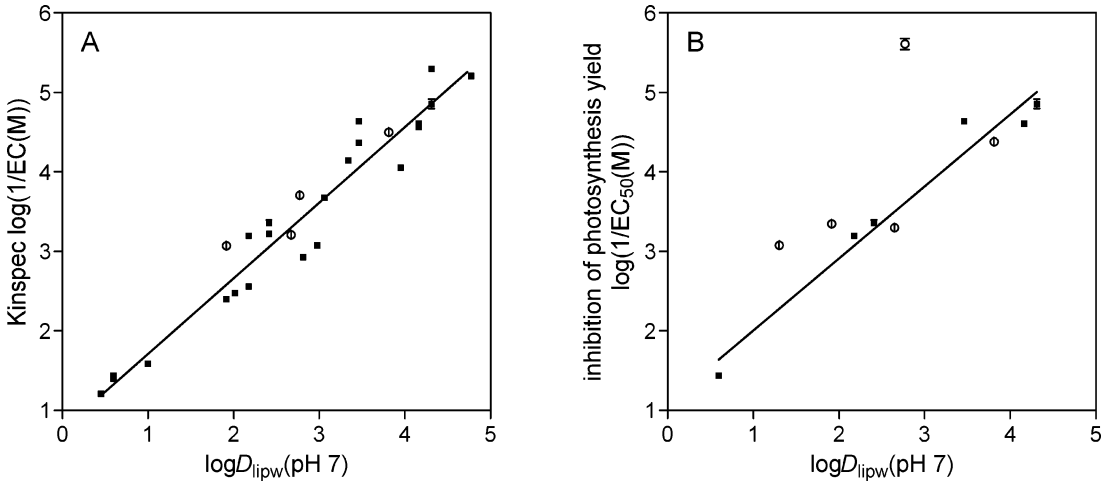

FIGURE 2. QSAR for baseline toxicants (■) based on D_{lipw}(pH7) for (A) EC values in the Kinspec test and (B) EC_{50} values from the chlorophyll fluorescence test. The ○ marks the pharmaceuticals; error bars correspond to standard mean errors.

baseline toxicity (*48*). As Figure 2A shows, the EC values of all the pharmaceuticals are near those predicted from a QSAR model for baseline toxicity. In addition, all the investigated pharmaceuticals showed constant effective membrane burdens that were indistinguishable from those of known baseline toxicants (*48*). From the kinetics of the electron transfer, it was obvious that inhibition of electron transfer was not relevant. Since no specific effects were evident in the bioluminescence inhibition test (*40*), which is particularly sensitive to disturbance of ATP production, we did not test for additional endpoints in the Kinspec system (*29*, *30*).

In mixtures, the pharmaceuticals were concentration additive with each other and with other baseline toxicants, confirming the same mode of toxic action in this test system (*48*).

Specific and Nonspecific Inhibition of Photosynthesis. The Supporting Information presents a detailed evaluation of the experimental method and compares the results with the alga growth inhibition test according to the OECD test guideline 201 (*39*). The EC_{50} values of all the pharmaceuticals toward the green algae *D. subspicatus* were independent of the end point, be it the cell number, cell density, or inhibition of photosystem II quantum yield determined with the chlorophyll fluorescence assay (see Supporting Information). Only the specific PSII inhibitor diuron, which was used as a reference compound, clearly showed higher sensitivity to the inhibition of photosystem II quantum yield as compared to the other end points (see Supporting Information).

The EC_{50} values of the pharmaceuticals deduced from the inhibition of photosystem II quantum yield were only weakly influenced by the exposure time, and no clear temporal trend could be deduced from them (see Supporting Information). In contrast, the effect of diuron decreased with time. However, standard errors of the percent inhibition data were often rather high after 2 and 5 h exposures. Therefore, 24 h exposure and chlorophyll fluorescence measurements were chosen as standard test conditions. This combination offers the advantage that both indirect effects on photosynthesis (baseline toxicity or general cytotoxicity) and specific inhibition of photosynthesis can be detected. However, these different effects cannot be distinguished quantitatively from each other. Consequently, all experimental data were compared with a prediction for baseline toxicity as described below. If there was any indication of specific effects on photosynthesis, a more thorough investigation of chlorophyll fluorescence was undertaken.

The EC_{50} values of the pharmaceuticals from the chlorophyll fluorescence assay after 24 h exposure are listed in Table 1. The concentration effect curves are presented in the Supporting Information (Figure S3), and the statistics are given in Table 2. The EC_{50} values for ibuprofen, diclofenac, and carbamazepine agree well (i.e. within a factor of 3) with previously reported 72 h EC_{50} values for growth inhibition of the same algae species determined according to the OECD test guideline (*11*). However, in the 72 h growth inhibition test, Cleuvers observed an $EC_{50} = 20\,\mu M$ for propranolol (*11*), while we observed 2.5 μM with the chlorophyll fluorescence assay after 24 h, which increased after 72 h to 7.8 and 9 μM with growth inhibition and chlorophyll fluorescence, respectively.

TABLE 2. Descriptors of the Concentration-Effect Curves (Equation 6) and Statistics of the Single Pharmaceuticals[a] and the Baseline Toxicants in the Chlorophyll Fluorescence Assay[b]

compound	log(1/EC_{50}(M))	std error	slope *m*	std error	n	r^2
propranolol	5.61	0.07	0.98	0.14	17	0.708
sulfamethoxazole	3.08	0.01	11.94	2.33	11	0.874
ethinylestradiol	4.38	0.01	6.90	0.01	11	0.639
diclofenac	3.30	0.01	22.49	16.19	16	0.750
ibuprofen	3.35	0.02	11.46	5.26	15	0.384
carbamazepine	<3.00					
diuron	7.42	0.55	1.19	0.16	12	0.907
3-butoxyethanol	1.44	0.01	10.04	2.79	52	0.755
2-nitrotoluene	3.36	0.03	2.58	0.55	18	0.869
3-nitroaniline	3.20	0.02	4.29	1.02	20	0.780
4-*n*-pentylphenol	4.86	0.06	0.84	0.11	61	0.761
2-phenylphenol	4.64	0.03	1.60	0.12	16	0.976
2,4,5-trichloroaniline	4.61	0.01	3.05	0.28	20	0.956

[a] Data from ref *40*. [b] The specific PSII inhibitor diuron was used as the reference compound.

A comparison of the EC_{50} values of the pharmaceuticals from the chlorophyll fluorescence assay with the results from the baseline toxicity assay (Figure 3) shows good agreement between the two tests (TR varies only from 0.6 to 2.1) for ethinylestradiol, ibuprofen, diclofenac, and carbamazepine. This confirms that baseline toxicity is also the mode of toxic action of these chemicals in algae. In contrast, propranolol is more than 2 orders of magnitude more toxic than the corresponding baseline toxicity (TR = 107).

The baseline toxicants in the 24 h chlorophyll fluorescence test listed in Table 2 (for concentration-effect curves, see Figure S4 in the Supporting Information) show a linear regression with the liposome−water distribution coefficients

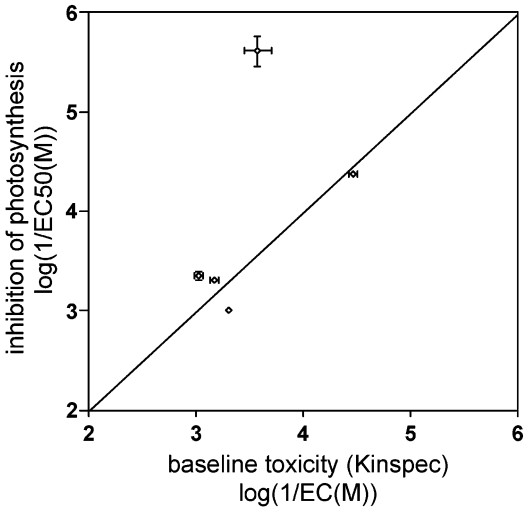

FIGURE 3. Comparison of EC_{50} values for photosynthesis inhibition with the chlorophyll fluorescence test and EC baseline toxicity values determined in the Kinspec system. The error bars correspond to 95% confidence intervals.

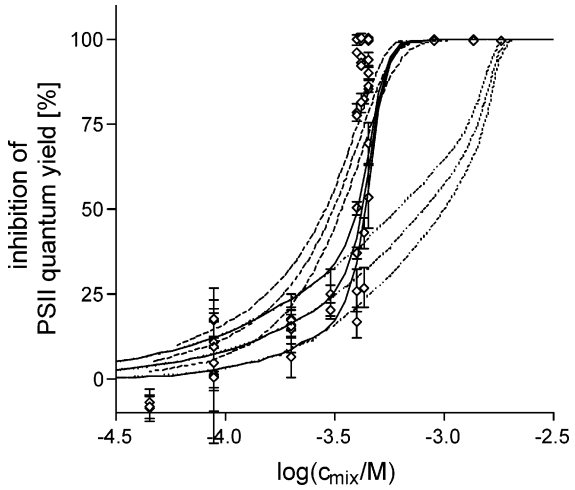

FIGURE 4. Concentration-effect curves of the mixtures of five pharmaceuticals and diuron in the chlorophyll fluorescence test. The compounds were mixed in the ratio of their EC_{50} values and the concentration. The diamonds correspond to the experimental data, and the error bars are the 95% confidence intervals. The broken lines refer to the prediction for concentration addition, the dotted lines refer to the prediction for independent action, and the drawn lines refer to the two-stage prediction. For each prediction model, the lower 95% confidence limit is shown on the left, the actual prediction in the middle, and the upper 95% confidence limit on the right.

log K_{lipw} or log D_{lipw}(pH7) (Figure 2B), yielding a QSAR for baseline toxicity of

$$\log(1/EC_{50}(M)) = (0.91 \pm 0.09)\log K_{lipw} + (1.10 \pm 0.28) \quad (13)$$

log K_{lipw} is equal to log D_{lipw}(pH7) for all baseline toxicants but needed to be corrected for speciation for most pharmaceuticals. Sulfamethoxazole (TR = 6.3), ethinylestradiol (TR = 0.7), diclofenac (TR = 0.6), and ibuprofen (TR = 3.3) fall on this QSAR line (Figure 2B), and their TR values lie well within the threshold of the baseline toxicity (TR ≤ 10). In contrast, propranolol has a TR of 101, indicating a specific mode of toxic action in algae. In conclusion, both ways of deriving TR indicate the specific toxicity of propranolol and confirm the baseline toxicity of the other pharmaceuticals investigated. A more detailed QSAR analysis and the list of TR values are given in the Supporting Information.

In view of the high TR value, and since the experiments with the PSII inhibitor diuron have shown that the chlorophyll fluorescence assay is particularly sensitive to PSII inhibitors and that this effect is more pronounced after a shorter exposure time, we additionally investigated the induction kinetics of fluorescence in dark-adapted algae (*32*, *52*). Herbicides such as diuron dramatically accelerate the rise of fluorescence in the measurement of rapid induction kinetics by inhibiting the electron transport chain in photosystem II (*52*). This effect was evident in dark-adapted algae after less than 5 min of incubation with diuron. The instantaneous PSII inhibition did not become more severe with longer exposure (up to 2 h). Unlike diuron, 10^{-4} M propranolol did not affect the initial rise of the fluorescence, but only caused a decrease in the maximum chlorophyll fluorescence signal. This effect was visible immediately after incubation and increased dramatically with increasing time up to 3 h in the dark. After 24-h exposure in light followed by 2-h incubation in the dark and measurement of the rapid induction kinetics, decreased maximum fluorescence was observed in relation to the unexposed cultures and the effect increased with increasing concentration. Under these experimental conditions, diuron also showed decreased maximum fluorescence but still exhibited the typical curve form of a PSII inhibitor. More detailed information and kinetic traces are given in the Supporting Information. The induction pattern of propranolol is clearly not the result of direct interference with components of photosystem II but is similar to observations with reactive chemicals, where both the initial rise and maximum of the fluorescence is decreased (*32*). The fact that the effect of propranolol on chlorophyll fluorescence occurred only after a longer incubation time indicates a disturbance of a biosynthesis pathway, because reproduction and growth must occur before the effect is visible. Further work would be required to clearly unravel the mode of toxic action of propranolol.

In the chlorophyll fluorescence inhibition test, additional mixture experiments were performed as a diagnostic tool for mode-of-action analysis. The mixture of propranolol, sulfamethoxazole, ethinylestradiol, diclofenac and ibuprofen, mixed in the ratios of their EC_{50} values for photosynthesis inhibition, showed higher toxicity than predicted from the independent action of all the components in the chlorophyll fluorescence test, in particular at high effect levels, where the experimental data were almost congruent with the prediction for concentration addition (*40*). Since not all pharmaceuticals have a common mode of toxic action, we evaluated a two-stage prediction procedure, treating the four baseline toxicants as concentration additive. The resulting prediction for concentration addition was then coupled to the propranolol and diuron response in the model of independent action. Such an approach has been successfully applied to the prediction of effects of a mixture of 40 compounds that exhibit four different modes of toxic action (*53*). Note that such an analysis becomes easier and more robust as the number of components in a mixture increases.

Nevertheless, it served as an exploratory tool in the present study. Indeed, the two-stage model (Figure 4) improved the prediction at low effect levels and was indistinguishable from concentration addition at high effect levels. It thus described the experimental data better. Since the experimental concentration-effect curve is very steep and the experimental data vary considerably, we cannot clearly confirm that this is the true model. Nevertheless, this exercise indicates the importance of a reliable analysis of the dominant mode of toxic action for a prediction of mixture effects.

If the five pharmaceuticals were modeled as concentration additive or, alternatively, if their experimental mixture toxicity

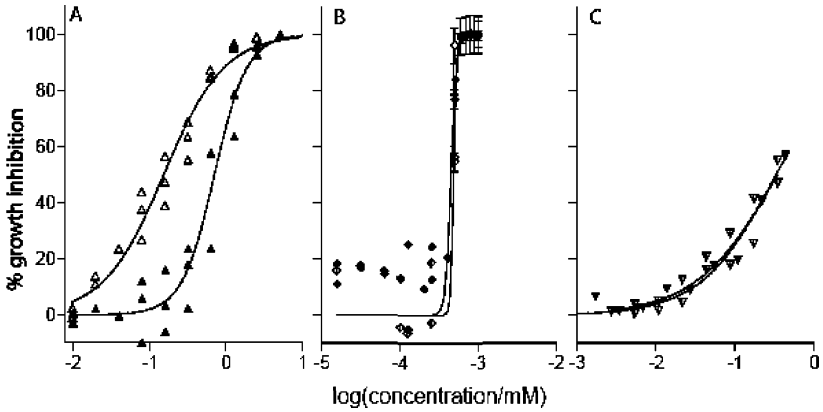

FIGURE 5. Concentration-effect curves in MJF 276 (GSH+) (filled symbols) and MJF335 (GSH−) (open symbols): (A) (△,▲) positive control hydroxyethylacrylate; (B) (◇,◆) propranolol; (C) (▼,▽) negative control ethanol. The error bars correspond to the standard deviations of the experimental data. The lines are the best fit to eq 6 with the adjustable parameters listed in Table 3.

curve was taken as the basis for the two-stage model, and this mixture of pharmaceuticals was treated in the second stage as a two-component mixture with diuron using the model of independent action, the quality of prediction was lower. In particular, the slope was much smaller than that obtained from the experimental data (data not shown). These results are a further indication that propranolol has a specific effect on photosynthesis but that does not have the same mode of toxic action as diuron. All the other compounds are baseline toxicants in algae.

Estrogenic Effects. As expected, propranolol, sulfamethoxazole, ibuprofen, and diclofenac did not show any estrogenic effect in the YES up to millimolar concentrations (Table 1). However, they were cytotoxic at the highest concentrations tested. The EC_{50} values for growth inhibition in this yeast strain were 13 mM for propranolol and 89 mM for diclofenac. Ibuprofen showed 35% growth inhibition at the highest nominal concentration tested (20 mM), whereas sulfamethoxazole showed approximately 20% growth inhibition at 1 mM. These values are not very precise, because at such high concentrations probably not the entire quantity of the compounds in the 96 well plates was redissolved by the yeast suspension. Carbamazepine was not cytotoxic nor caused receptor gene induction up to 1 mM. The EC_{50} value for the estrogenic activity of ethinylestradiol (Table 1) and the reference compounds 17-β-estradiol ($\log(1/EC_{50}(M)) = 9.54 \pm 0.04$) agreed well with earlier reported results (*42*).

Reactive Toxicity. The EC_{50} values of the *E. coli* strains used for the assessment of reactive toxicity are listed in Table 1. For sulfamethoxazole, ethinylestradiol, diclofenac, and carbamazepine, the 50% effect level could not be reached in the concentration range investigated. Higher concentrations could not be achieved due to solubility problems and because the solubility enhancer ethanol had to be kept well below 2% (v/v) due to its baseline-toxic effect (EC_{50} corresponds to 1.92% ethanol for MJF 276 (GSH+) and 1.96% ethanol for MJF 335 (GSH-)).

Propranolol showed a difference in growth inhibition neither between the GSH+ and the GSH− strain nor between DNA+ and DNA− (Table 3). In each pair, the 95% confidence intervals of the EC_{50} values overlapped (Figure 5B). In contrast, the positive controls showed a statistically significant difference. Hydroxyethylacrylate was 4.6 times more toxic toward the GSH− strain than toward the GSH+ strain (Figure 5A) (*36*). The negative control ethanol, which acts as a baseline toxicant, had the same toxicity toward both strains (Figure 5C). Overall, we can conclude that propranolol is not toxic through reactivity toward GSH.

The positive control for DNA damage, 1,2-epoxybutane, was 57 times more toxic toward the MV 4108 (DNA−) strain

TABLE 3. Descriptors of the Concentration-Effect Curves (Equation 6) for Propranolol in the *E. coli* Biosensors

strain	log(1/EC_{50}(M))	std error	slope *m*	std error	n	r^2
MJF 276 (GSH+)	3.35	0.02	12.37	4.25	20	0.914
MJF 335 (GSH−)	3.32	0.04	22.64	60.35	21	0.939
MV 1161 (DNA+)	3.04	0.05	2.28	0.96	6	0.676
MV 4108 (DNA−)	3.05	0.11	5.91	10.93	5	0.403

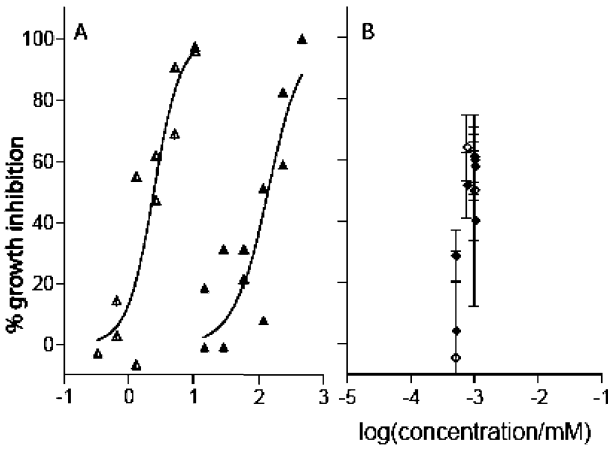

FIGURE 6. Concentration-effect curves in MV 1161 (DNA+) (filled symbols) and MV4108 (DNA−) (open symbols): (A) (△,▲) positive control 1,2-epoxybutane; (B) (◇,◆) propranolol. The error bars correspond to the standard deviations of the experimental data. The lines are the best fit to eq 6 with the adjustable parameters listed in Table 3.

than toward MV 1161 (DNA+) (Figure 6A). For propranolol (Figure 6B) and ibuprofen, in contrast, the EC_{50} values of the MV 1161 (DNA+) strain were only higher than that of MV 4108 (DNA−) by a factor of 1.01 and 1.37, respectively, which is not large enough to indicate DNA damage (*36*). The negative control ethanol did not show a response in this test, which can be explained by the test design with its short incubation time.

Since the *E. coli* are presumably only affected by baseline toxicity, we directly compared the EC_{50} values in the different test systems for propranolol. The GSH strains are more sensitive toward propranolol than the DNA strains by a factor of 2 (but not statistically significantly) because the incubation time is much longer for the GSH test (24 h) than for the DNA damage test (1 h). A different exposure regime had to be chosen for the DNA damage test than the GSH test because

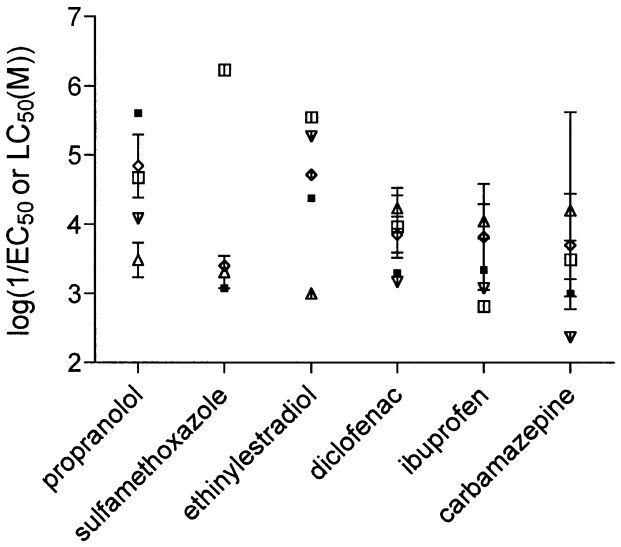

FIGURE 7. Comparison of our results in the chlorophyll fluorescence assay (■) with acute toxicity data of (△) *Vibrio Fischeri*, (□) algae, (◇) daphnia, and (▽) fish from the literature (*1, 11, 40, 54*−*59*).

MV4108 formed filaments in the presence of toxicants which rendered the OD-based measurements useless. We therefore used the experimental setup with short exposure and plating recommended by Harder et al. (*36*), although it decreases the sensitivity to nonreactive chemicals.

When the results of propranolol were compared with the other test systems, the *E. coli* strains are less sensitive than the membrane vesicles of the Kinspec assay by a factor of 1.7 (GSH) and 3.3 (DNA), respectively. These small differences are presumably caused by differences in exposure time, cell density in the assay and the composition of the cell or the subcellular fraction (all these factors causing differences in internal concentrations), and the sensitivity of the end point. They are close enough to each other to conclude that propranolol acts merely as a baseline toxicant in all *E. coli* strains investigated. When evaluating the tested concentrations of the other compounds in light of the relative sensitivity of the *E. coli* strains under the given conditions, it becomes clear that the expected EC_{50} values for baseline toxicity are only slightly higher than the tested concentrations. This indicates that baseline toxicity is the mechanism responsible for the toxicity of all other pharmaceuticals investigated and that reactive mechanisms are not relevant.

Comparison with Ecotoxicity Testing Using the Classical Test Battery of Algae, Daphnia, and Fish. We compared our results with experimental data from classical ecotoxicological test systems to explore if the test battery based on mode of action yields consistent results with classical test systems (Figure 7). The acute toxicity data for *Vibrio fischeri*, algae, daphnia, and fish were compiled from refs *1*, *11*, *40*, and *54*−*59*. Irrespective of the test system, the 25th to the 75th percentile of the EC values for acute toxicity tests for a given compound typically cover about 1 order of magnitude. The chlorophyll fluorescence test was more sensitive for propranolol for the reasons discussed above and was on the lower side of sensitivity for the other compounds, but usually well within the 95th percentile. More details and all the data used are given in the Supporting Information. This comparison is very crude but nevertheless gives an indication of the sensitivity, although the dependence on the exposure time is not considered and all the tests used here have a shorter exposure time than the classical acute tests. The advantage of the test battery based on mode of action lies more in the identification of specific modes of toxic action than in giving an absolute estimate of acute effects. It will therefore complement rather than replace acute toxicity data and will be a tool for prioritizing test procedures. Nevertheless, the proposed test battery is suitable for identifying hazards and making a preliminary classification of the mode of toxic action in nontarget aquatic organisms.

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Supporting Information Available

More detailed description of the results of the chlorophyll fluorescence assay, the concentration-effect curves of the single compounds, the QSAR analysis, rapid induction kinetics on chlorophyll florescence and the comparison with literature data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Supporting Information for "Modes of Toxic Action of Pharmaceuticals in Aquatic Organisms" by Escher et al.

Specific and non-specific inhibition of photosynthesis – evaluation of the experimental method

The experimental method for determining the specific and non-specific inhibition of photosynthesis was developed in stages. The starting point was the 72-h alga growth inhibition test according to the OECD test guideline 201 (*1*). The results obtained in this study were within a factor of six to the literature data (Figure S1). There was no difference between the different endpoints, cell number, cell density, or inhibition of photosystem II quantum yield determined with the chlorophyll fluorescence assay (Figure S1), as was shown by a one-way ANOVA. Only the specific PSII inhibitor diuron, which was used as the reference compound, clearly showed higher sensitivity to the inhibition of photosystem II quantum yield as compared to the other endpoints (Figure S1). The EC50 values derived from the inhibition of the photosystem II quantum yield were approximately six times lower than those of the other endpoints. However, the concentration-effect curves were significantly steeper with the chlorophyll fluorescence assay for most compounds, even if the EC_{50} values were very similar (data not shown).

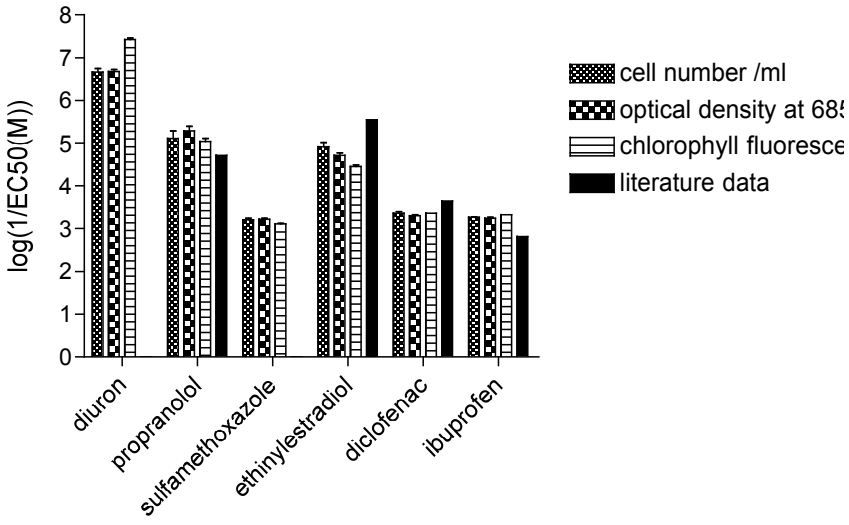

Figure S1. Comparison of different endpoints after 72–h exposure and comparison of the 72-h alga growth inhibition with literature data

The time dependence of the effects was small. The inhibition of the photosystem II quantum yield was more sensitive by a factor of approximately two towards the reference compound diuron after 2 and 5-hour exposures than after 24 and 72 hours (Figure S2). In

contrast, the EC50 values of the other pharmaceuticals were only weakly influenced by the exposure time and no clear temporal trend could be deduced.

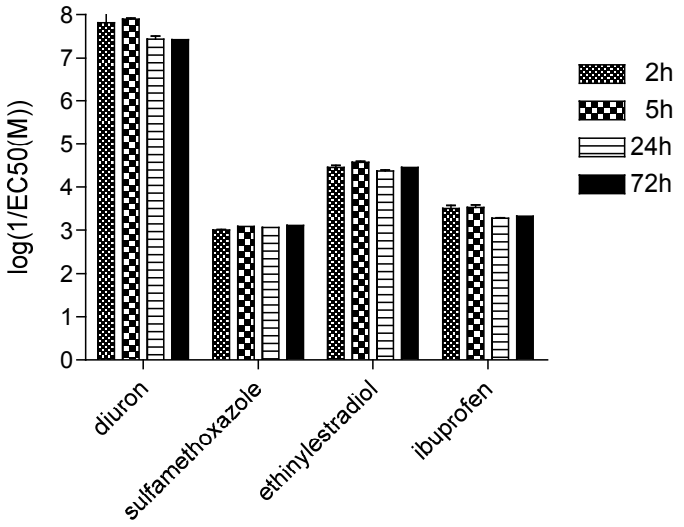

Figure S2. Time dependence of EC50 for the inhibition of the photosystem II quantum yield determined with the chlorophyll fluorescence assay.

Concentration-effect curves in the chlorophyll fluorescence test

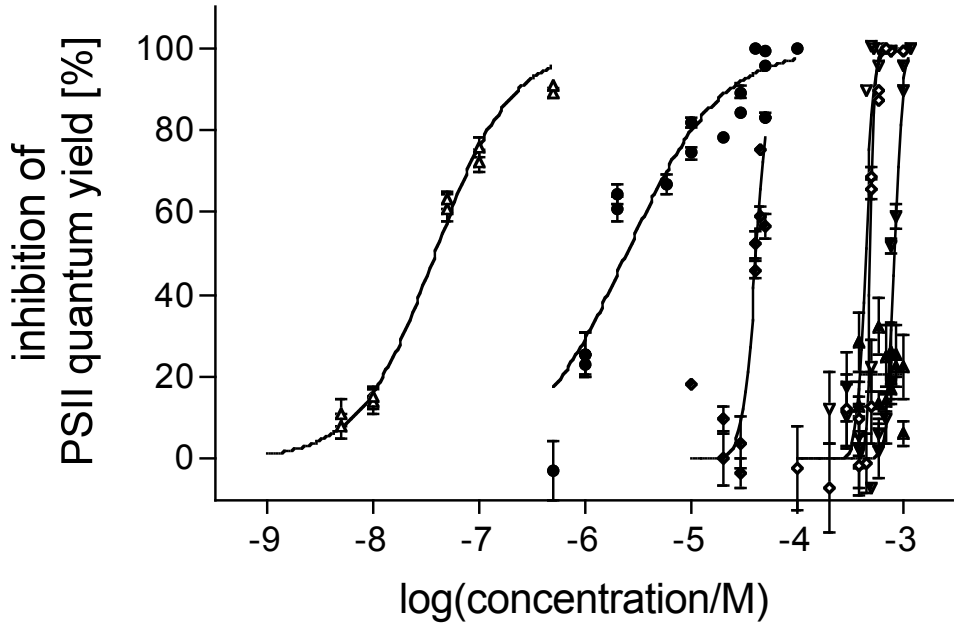

Figure S3. Concentration-effect curves of the pharmaceuticals and the reference compound diuron in the chlorophyll fluorescence test; ● propranolol, ▼ sulfamethoxazole, ◆ ethinylestradiol, △ diuron, ◇diclofenac, ▽ ibuprofen, ▲ carbamazepine. The error bars correspond to the standard deviations of the experimental data. The lines are the best fit to Equation 6 with the adjustable parameters listed in Table 2.

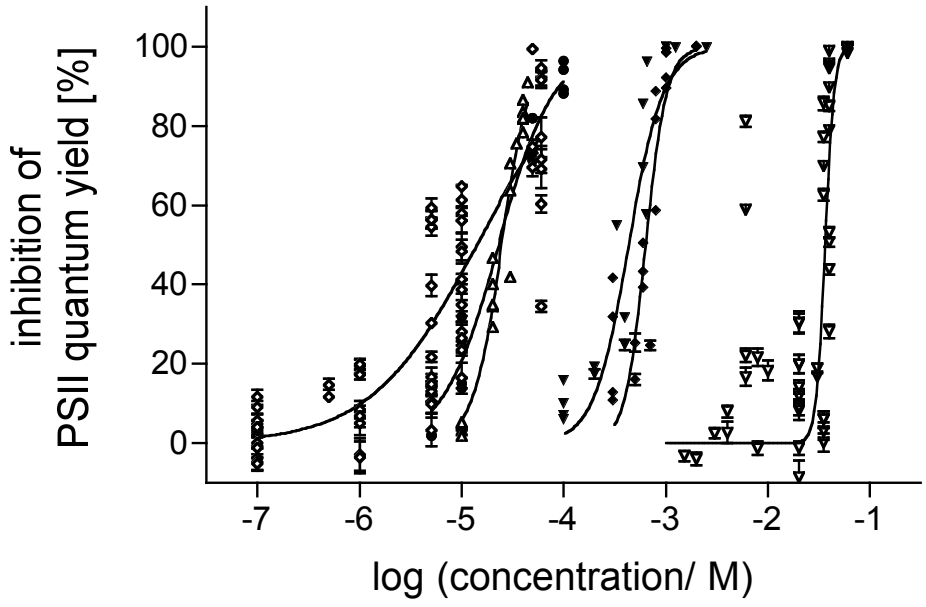

Figure S4. Concentration-effect curves of the baseline toxicants in the chlorophyll fluorescence test; ▽butoxyethanol, ▼ 2-nitrotoluene, ◆ 3-nitroaniline, △ 2,4,5-trichloroaniline, ◇4-n-pentylphenol, ● 2-phenylphenol. The error bars correspond to the standard deviations of the experimental data

QSAR analysis of the baseline toxicity in the chlorophyll fluorescence test

Baseline QSARs are commonly based on log K_{ow}. However, differences between polar and non-polar narcotics disappeared if the liposome-water partition coefficient log K_{lipw} was used instead as the descriptor (*2*). The log K_{ow}-based and the log K_{lipw}-based QSAR for the six baseline toxicants investigated (Equation S-1 and S-2) differ only slightly, but the errors were greater for the log K_{ow}-based QSAR.

$$\log(1/EC_{50}(M)) = (0.91\pm0.18) \cdot \log K_{ow} + (1.33\pm0.52) \quad \text{(S-1)}$$

$$\log(1/EC_{50}(M)) = (0.91\pm0.09) \cdot \log K_{lipw} + (1.10\pm0.28) \quad \text{(S-2)}$$

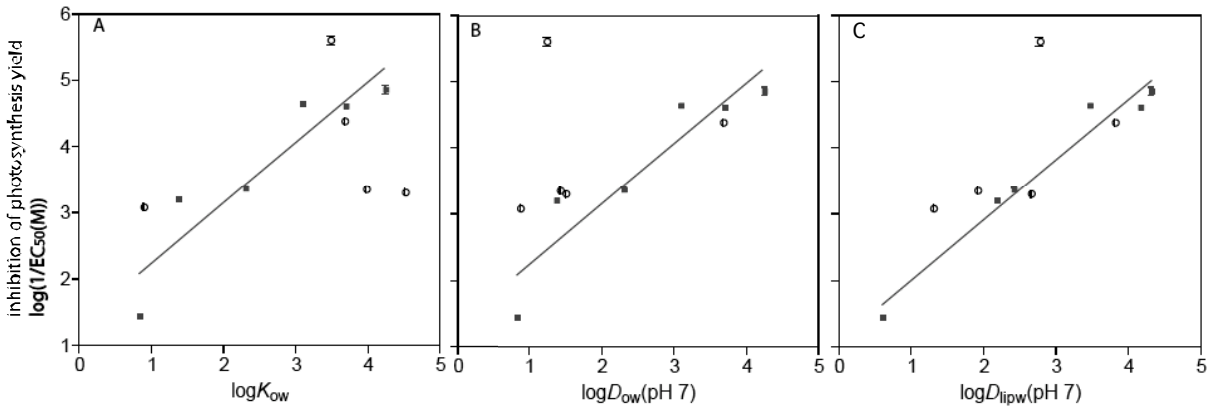

Figure S5. QSAR of EC_{50} values from the chlorophyll fluorescence test for baseline toxicants (■)based on (A) log K_{ow}, (B) logD_{ow}(pH 7) (C) logD_{lipw}(pH 7). The ❍ marks the pharmaceuticals, error bars correspond to the standard mean errors.

Log K_{ow} is not a good descriptor for the ionogenic pharmaceuticals (Figure S5A). If the apparent octanol-water partition ratio is calculated from the fraction of neutral species $f_{neutral\ species}$ at pH 7 (Equation S-3; neutral species corresponding to the acid form for the acids diclofenac and ibuprofen and the base form for the bases propranolol and sulfamethoxazole), all the pharmaceuticals with the exception of propranolol fall on the baseline (Figure S5B, Table S-1). Propranolol had a TR of 1400 with D_{ow}(pH 7) as the input parameter for the QSAR prediction.

$$D_{ow}(\text{pH } 7) = f_{neutral\ species} \cdot K_{ow} \quad \text{(S-3)}$$

However, as has been shown previously for substituted phenols (*3*), the liposome-water distribution ratio at pH 7, D_{lipw}(pH 7) (Equation S-4) is a more appropriate descriptor for such an analysis because D_{ow}(pH 7) may overestimate the specific toxicity by completely neglecting the role of the charged species.

$$D_{lipw}(\text{pH } 7) = f_{neutral\ species} \cdot K_{lipw,\ neutral\ species} + f_{charged\ species} \cdot K_{lipw,\ charged\ species} \quad \text{(S-4)}$$

Sulfamethoxazole, estradiol, diclofenac and ibuprofen were again identified as baseline toxicants, and propranolol was identified as producing a specific action, but the TR is reduced to a more realistic value of 101 (Table S-1). This is also consistent with the TR analysis using the Kinspec EC as a baseline value.

Compound	$\log(1/EC_{50}(M))$	$\log(1/EC_{50,baseline}(M))$ from Eq. S-1	$TR_{Dow\text{-}based}$	$\log(1/EC_{50,baseline}(M))$ from Eq. S-2	$TR_{Dlipw\text{-}based}$
Propranolol	5.61	2.46	1426	3.61	101
Sulfamethoxazole	3.08	2.12	9.1	2.28	6.3
Ethinylestradiol	4.38	4.67	0.5	4.55	0.7
Diclofenac	3.30	2.69	4.0	3.50	0.6
Ibuprofen	3.35	2.62	5.4	2.62	3.3

Table S-1. TR values of the pharmaceuticals, with $EC_{50,\ baseline}$ estimated from equations S-1 and S-2.

Induction kinetics of fluorescence in dark-adapted algae

In the dark-adapted state, the primary electron acceptors of photosystem II, Q_A, are fully oxidized, and the reaction centres are 'open'. This phase is characterized by the minimum fluorescence F_0 and can be detected in the Pulse-Amplitude Modulated Fluorometer (PAMF) using non-actinic (photosynthetically inactive) light. When actinic light is switched on, fluorescence emission increases to reveal a characteristic induction curve as shown for the unexposed algal cultures in Figure S-6 (control). The maximum fluorescence F_M can be determined when a saturated light pulse causes complete reduction of the primary electron acceptors. The maximum fluorescence quantum yield Y_{max} can now be calculated using equation S-5.

$$Y_{max} = \left(\frac{F_M - F_0}{F_M} \right) \qquad \text{(S-5)}$$

The herbicide diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea) causes a dramatic acceleration of fluorescence due to inhibition of the electron transport chain in photosystem II (Figure S-6A). This effect is instantaneous and occurs at concentrations around the EC50 for the ToxY-PAM assay. In contrast, $2 \cdot 10^{-5}$M propranolol, which had an effect of 82.5 % after 24 h of incubation in the ToxY-PAM, only showed an approximately 10% effect in the PAMF after incubation in the dark for 1 hr. If a fully inhibitory concentration of $1.1 \cdot 10^{-4}$ M propranolol is added to the algae, the effect on the rapid induction kinetics starts immediately but continues to increase in the next three hours until it becomes stable (Figure S-6B). This time dependence of the effect confirms that propranolol does not interfere directly with photosynthesis but that some growth is required to elicit the effect.

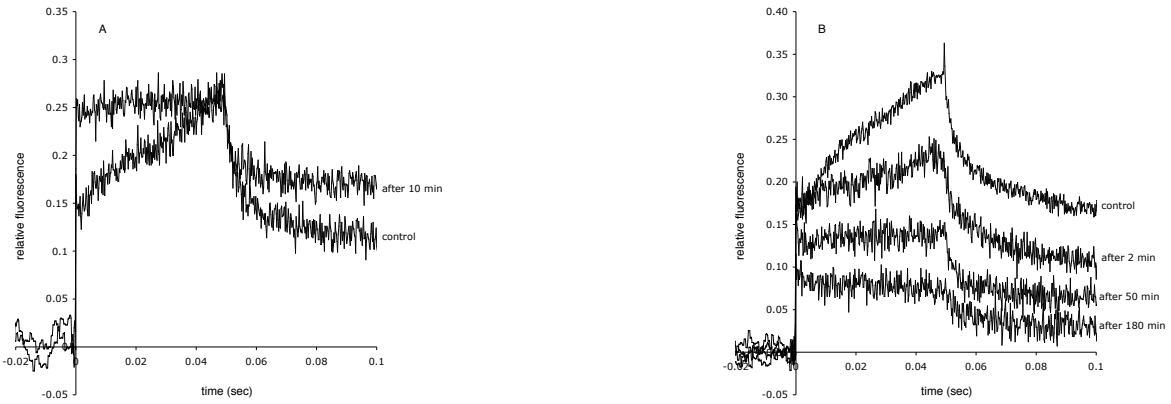

Figure S6. Rapid fluorescence induction kinetics in *Desmodesmus subspicatus* after exposure to (A) 10^{-7} M diuron and (B) $1.1 \cdot 10^{-4}$ M propranolol. The time indicated in the plot refers to the time between the addition of the compound to dark-adapted algae and the measurement of the kinetic trace.

If the samples grown for 24 h in the light and Y determined with the ToxY-PAM are dark-adapted for another two hours and the rapid induction kinetics are then measured, a strong effect can also be seen in the algae exposed to propranolol, the values of F_{max} and F_0 decreasing with increasing concentration. It is evident, however, that the kinetic

pattern remains distinctly different: diuron still shows the characteristic closing of the reaction centers due to the immediate reduction of Q_A. In contrast, the kinetics of propranolol remain qualitatively constant, only the Y_{max} drops.

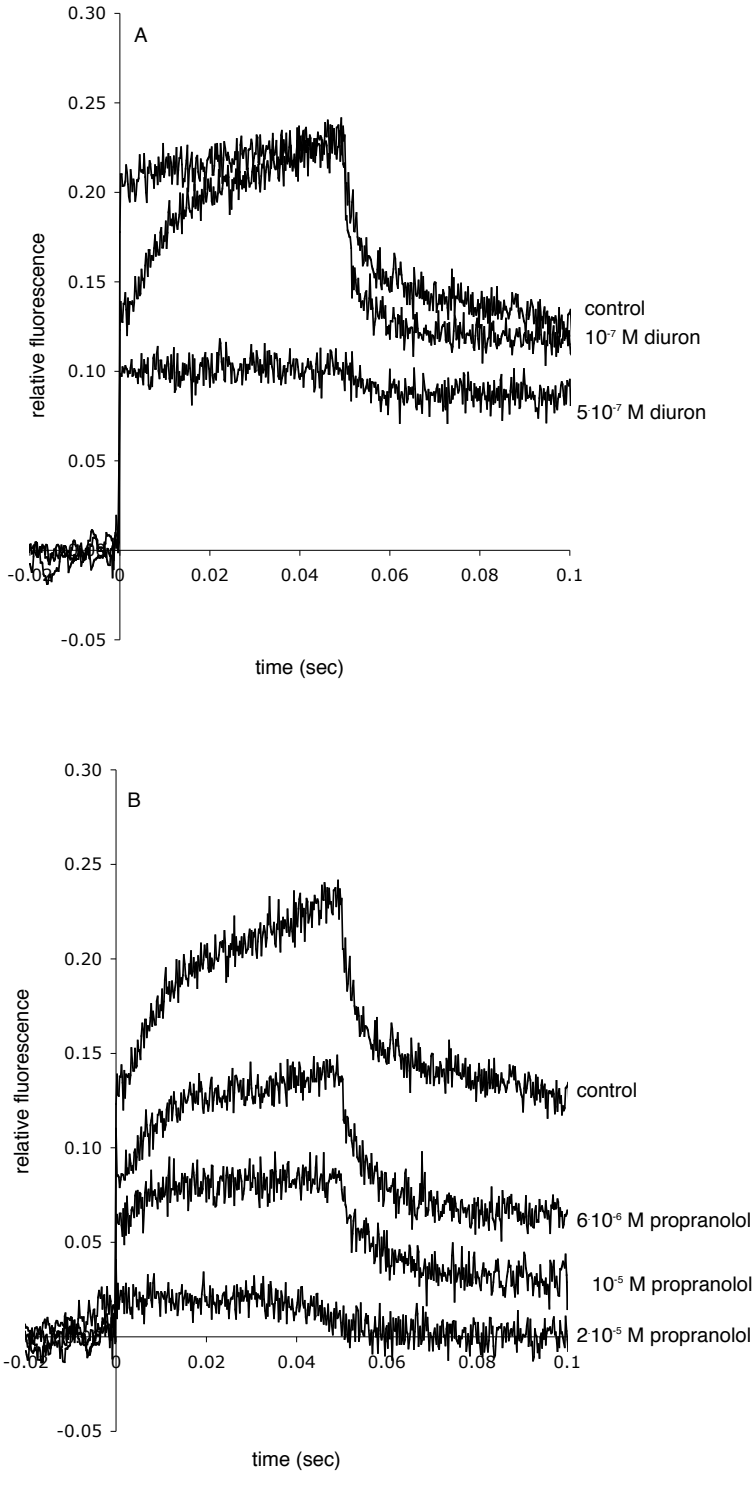

Figure S7. Rapid fluorescence induction kinetics in *Desmodesmus subspicatus* after 24-h exposure to (A) diuron and (B) propranolol in the light followed by a 2-h period of dark adaptation before measurement of the kinetic traces

Comparison with literature data from acute toxicity tests with algae, daphnia and fish

compound	EC(mg/L)	Log1/EC(M)	test	Ref.
propranolol	24.3	4.09	48h mortality LC50 *Oryzias latipes*	(4)
ethinyl estradiol	1.61	5.27	96 h mortality LC50 fathead minnow	(5)
diclofenac	214	3.17	96 h mortality LC50 zebra fish	(6)
ibuprofen	173	3.08	96 h mortality LC50 *Lepomis machochirus*	(6)
carbamazepine	25	3.98	10 d ELS NOEC	(7)
carbamazepine	1000	2.37	96 h mortality LC50 *Brachydanio rerio*	(6)

Table S-2. Literature data for acute toxicity tests with fish.

compound	EC(mg/L)	log1/EC (M)	test	reference
propranolol	7.5	4.60	48h immobility EC50 *Daphnia magna*	(*8*)
propranolol	2.75	5.03	48h mortality EC50 *Daphnia magna*	(*9*)
propranolol	1.6	5.27	48h mortality EC50 *Daphnia magna*	(*4*)
propranolol	1.5	5.29	48h mortality EC50 *Ceriodaphnia dubia*	(*9*)
propranolol	2.7	5.04	48h mortality EC50 *Daphnia magna*	(*9*)
propranolol	0.8	5.57	48h mortality EC50 *Ceriodaphnia dubia*	(*4*)
propranolol	17.7	4.22	48h mortality EC50 *Daphnia magna*	(*5*)
propranolol	3.1	4.98	48h mortality EC50 *Daphnia magna*	(*5*)
propranolol	7.5	4.60	48h mortality EC50 *Daphnia magna*	(*6*)
sulfamethoxazole	>100	3.40	48h mortality EC50 *Daphnia magna*	(*9*)
ethinyl estradiol	5.7	4.72	48h mortality EC50 *Daphnia* (not specified)	(*6*)
diclofenac	68	3.67	48h immobility EC50 *Daphnia magna*	(*8*)
diclofenac	22.43	4.15	48h mortality EC50 *Daphnia magna*	(*9*)
diclofenac	56	3.75	48h mortality EC50 *Daphnia magna*	(*6*)
ibuprofen	108	3.28	48h immobility EC50 *Daphnia magna*	(*8*)
ibuprofen	9.06	4.36	48h immobility EC50 *Daphnia magna*	(*6*)
carbamazepine	157	3.18	48h immobility EC50 *Daphnia magna*	(*10*)
carbamazepine	>13.8	4.23	48h immobility EC50 *Daphnia magna*	(*9*)

Table S-3. Literature data for acute toxicity tests with daphnia.

compound	EC(mg/L)	logEC (M)	test	Ref.
propranolol	5.80	4.70	96h growth EC50 *Desmodesmus subspicatus*	(*8*)
propranolol	5.30	4.74	96h growth EC50 *Pseudokirchn. subcapitata*	(*10*)
propranolol	7.40	4.60	96h growth EC50 *Pseudokirchn. subcapitata*	(*9*)
sulfamethoxazole	0.15	6.24	96h growth EC50 *Pseudokirchn. subcapitata*	(*9*)
ethinyl estradiol	0.84	5.55	96h growth EC50, species not specified	(*5*)
diclofenac	72.00	3.65	96h growth EC50 *Desmodesmus subspicatus*	(*8*)
diclofenac	16.3	4.29	96h growth EC50 *Pseudokirchn. subcapitata*	(*9*)
ibuprofen	315	2.82	96h growth EC50 *Desmodesmus subspicatus*	*(8)*

Table S-4. Literature data for acute toxicity tests with algae.

(1) OECD *Test guideline 201. Alga, Growth Inhibition Test (Test guideline 201, updated, adopted 7th June 1984)*; Organization for Economic Co-Ordination and Development; 1984. TG 201.

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