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# A strategy for an initial assessment of the ecotoxicological effects of transformation products of pesticides in aquatic systems following a tiered approach

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ABSTRACT

In order to conduct a fast and comprehensive toxicity screening of pesticide transformation products (TPs), this study used a tiered approach by a combination of *in silico* and experimental methods to determine the probability to be of relevance for risk assessment. The six pesticides Boscalid, Penconazole, Diuron, Terbutryn, Octhilinone (OIT), and Mecoprop were used as model compounds. Identification of corresponding environmental known and unknown TPs were done by literature analysis and photolysis experiments in combination. Aquatic solutions of the pesticides were photolysed to generate TPs which can be expected in the aquatic environment. The resulting mixtures were screened for TPs by high resolution LC-MS/MS. The herein developed approach was conducted at three different tiers: Literature review and *in silico* methods were used to predict exemplary the environmental bacterial toxicity and the genotoxicity of every single TP at tier I. In case of indications to be toxic, experiments at tier II were applied. Hereby, the photolytic mixtures containing parent compound and TPs were used for the consecutive toxicity test. Microtox assay for the parent compounds and the photolytic mixture was conducted to determine the acute and chronic toxicity and the growth inhibition of *V. fischeri*. Umu-tests were conducted to determine primary DNA damage. At tier III, single substance standards were used to conduct toxicity tests in case of toxic indication by previous tiers and availability of analytical standard. Identification of TPs revealed 45 known environmental TPs that originated from the six pesticides. The number of substances that need to be assessed was therefore more than sevenfold. By the tiered approach, it was possible to assess toxicological effects on environmental bacteria of 94% of the selected TPs. For 20% we found strong evidence to be toxic to environmental bacteria, as they were assessed at least at two tiers. For further 44% of the TPs we found slight evidence, as they could be assessed at one tier. Contrary, this approach turned out to be unsuitable to assess genotoxic effects of TPs neither by *in silico* tools nor by experiments. The number of substances that could probably pose a risk onto environment was quadrupled in comparison to the consideration of solely the parent compounds. Thus, this study demonstrates that the conducted screening approach allows for easy and fast identification of environmental relevant TPs. However, the study presented was a very first screening. Its applicability domain needs to be assessed further. For this purpose as a very next step the approach suggested here should be verified by applying additional endpoints and including additional parent compounds.

## 1. Introduction

TPs of organic pesticides that are formed by abiotic and biotic processes are increasingly identified in the environment (Burrows et al., 2002; Fenner et al., 2013). They increase the number of substances that need to be considered within risk assessment. However, TPs are still neglected even in current proposals of prospective assessments of pesticides risk (Schäfer et al., 2019). Within regulatory schemes only known and relevant TPs are considered and need to be assessed (European Union, 2009). Besides the exposed environmental concentration in the respective media, the risk of a substance is derived by its environmental properties. Studies that analyzed these properties showed that TPs are often more mobile and persistent in the aquatic environment than their parent compounds. Some of these studies also showed that TPs might pose similar or even higher toxic effects on different species (Belfroid et al., 1998; Bustos et al., 2019; Escher and Fenner, 2011; Gutowski et al., 2015c, 2015a; Sinclair and Boxall, 2009). Factors that could indicate a consistent or even increased

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toxicity of TPs compared to their parent compounds were described by Sinclair and Boxall (2009). As high mobility and persistency are well known for the majority of the known pesticide TPs, this study focused on the investigation of their toxic properties using the examples of environmental toxicity and genotoxicity.

In light of the increasing amount and diversity of chemicals and their TPs, it is questionable whether to deal with different required toxic endpoints - especially in case of the absence of an analytical standard (Kümmerer et al., 2019). To handle with that issue, there are different methods available to determine the toxicity of TPs (Escher and Fenner, 2011), e.g. by experimental effect-driven approaches. Such comparative analysis were conducted by toxicity testing of parent compounds and the corresponding reaction mixture containing TPs that were received by previous degradation experiments (Brack, 2003; Herrmann et al., 2016). Furthermore, computational methods such as QSAR/QSPR (quantitative structure-activity relationships/ quantitative structure-property relationship) were increasingly used for the assessment of environmental properties of pesticide TPs (Gutowski et al., 2015a, 2015b; Villaverde et al., 2017; Villaverde et al., 2018b). Finally, hybrid approaches were conducted in a combination of in vitro and *in silico* methods to evaluate the toxicity of pesticide TPs (Villaverde et al., 2018b). On this occasion, both the effect by a single TP and the effects of the mixture could be analyzed as it was successfully demonstrated for antibiotics (Menz et al., 2017).

By considering the high diversity of known and unknown TPs that need to be assessed, it became clear that there is a high demand for concepts that allow for a fast and comprehensive approach to identify relevant TPs (Escher et al., 2014; Menz et al., 2017). Both experimental and *in silico* methods have their specific limitations. However, to take advantage of both methodological strengths, both should be used in combination within an integrative approach. To go beyond the above-mentioned comparative methodology, this study made use of a tiered approach that allows for the gradual identification of the toxicity of pesticide TPs. At tier I, literature data and computational methods were used to identify TPs that might be toxic. In case of toxic indications an experimental approach were used to determine exemplary eco- and genotoxicity of a photolytic mixture including the parent compound and its TPs at tier II. In order to minimize the financial and temporal expense by synthesis of unknown or unavailable TPs, tier II provides a further pre-assessment of positive candidates at tier I. Finally, ecotoxicity of single TPs were only experimental examined at tier III if TPs showed toxic indication in tier II and an analytical standard was commercial available (Fig. 1). On the whole, this tiered approach provides a new strategy to assess the probability of TPs to be of relevance for risk assessment. This supports faster decision and priority setting to handle the great number of TPs which will become important in future especially in regulatory schemes.

This approach was explored by the TPs of Boscalid, Penconazole, Diuron, Terbutryn, OIT, and Mecoprop. These pesticides were chosen as model compounds as they including different substance classes and scopes of application, e.g. as plant protection agent and biocide. Additionally, they represent substances with different modes of action and levels of ecotoxicity. The parent compounds have different toxic $EC_{50}$ values between 0.05 mg $L^{-1}$ (OIT) and 59 mg $L^{-1}$ (Diuron) on *V. fischeri* (Bollmann et al., 2017b; EPA, 2010; Hernando et al., 2007; Mottier et al., 2014; Rodríguez-Cabo et al., 2018; Strachan et al., 2001; Tixier et al., 2001), indicating specifically and non-specifically acting toxicants and a high variability in effect concentration. Furthermore, the substances Diuron and Terbutryn are currently used as biocides, although their former application as plant protection agent was prohibited due to their harmful effects on aquatic organisms (European Commission, 2019). As we want to assess environmental TPs, only TPs that were formed by biological, chemical, and/or physical processes in environmental compartments were considered.

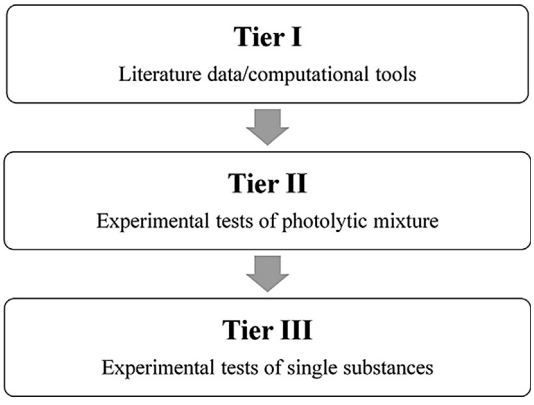


**Fig. 1.** Tiered pesticide TP assessment approach.

## 2. Methods

### 2.1. Workflow

A workflow was conducted for every test compound and their corresponding TPs in a tiered approach (Fig. 2). On this way already known and not yet known TPs could be identified. These TPs were used for subsequent assessment. In combination with the computer based *in silico* tool MultiCASE literature data was used to assess and evaluate the environmental toxicity and genotoxicity of TPs (Tier I). MultiCASE was used due to its inclusion of the required endpoints and its applicability for TPs of organic compounds as shown by previous studies (Gutowski et al., 2015c; Mahmoud et al., 2014; Menz et al., 2017; Rastogi et al., 2014). In case of toxic indications, photolysis experiments of the parent compounds were performed to produce a reaction mixture of TPs for further toxicity testing in a luminescent bacteria test (LBT) and a genotoxicity test (umu-test) (Tier II). Photolysis was chosen as transformation process due to its fast implementation unlike biodegradation experiments (OECD, 1992, 2008). This is even more underlined by the fact that TPs formed by photolysis and biodegradation are similar in many cases (Bollmann et al., 2016, 2017a, 2017b; Tixier et al., 2001). Moreover, it turned out, that TPs that are formed by photolysis experiments covers the majority of TPs that are known from literature. The respective parent compounds and the photolytic mixtures underwent a toxicity test for the comparative and initial assessment of TPs as it was done elsewhere (Escher and Fenner, 2011; Herrmann et al., 2015). In case of toxic indications by mixture analysis, single TPs were tested in our toxicity tests as long as an analytical standard was commercially available and they had not been tested in previous studies (Tier III).

Assessment of TPs was done as following (see Fig. 2): In case of toxic indication by neither the first nor the second tier, TPs were assessed to be probably not toxic. As the single toxicity test at tier III gave more evidence about their toxicity, TPs that were assessed to be most probably not toxic if test were negative. TPs that were tested positively at tier I but were not contained in the photolytic mixture were assessed to be probably toxic. If positive candidates of tier I were positively tested at tier II, TPs were assessed to be most probably tested as. Same applies for TPs that were positively tested in the single test at tier III.

### 2.2. Chemicals and reagents

Analytical standards were purchased from Sigma Aldrich, Neochema, and Dr. Ehrenstorfer and had a purity of > 97.3%. Acetonitrile (VWR) was used as organic mobile phase and for the preparation of stock solutions. Aqueous mobile phase and solutions for the implementation of photodegradation experiments were prepared with ultrapure water (Membra Pure, Germany; Q1:16.6 MΩ and Q2: 18.2 MΩ).

The freeze-dried luminescent bacterium *V. fischeri* (NRRL-B-11177)

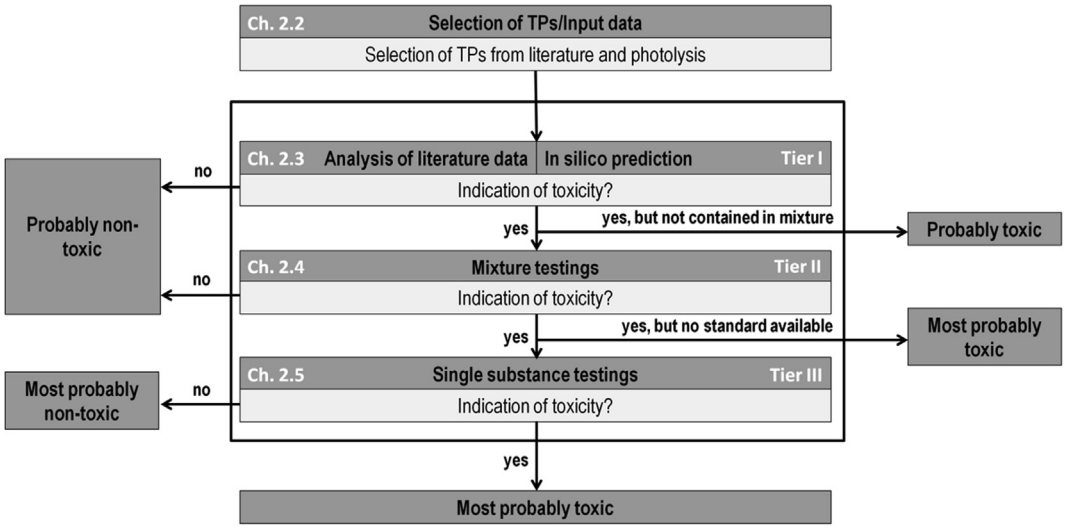


**Fig. 2.** Workflow and schedule of the tiered approach of this study. Toxicity assessment was conducted at different levels (I-III) that were described in detail in the corresponding chapters (2.2–2.5).

were purchased from Hach-Lange GmbH, Düsseldorf, and stored until usage at −20 °C. The gram-negative bacterium *Salmonella typhimurium* (TA1535pSK1002) was received from the German Collection of Micororganisms and Cell Cultures GmbH, Braunschweig, Germany.

### 2.3. Selection of environmental TPs (Input data)

Input data was generated by a comprehensive review of literature data and additional photolysis tests to receive a reaction mixture that contained TPs of every test compound.

#### 2.3.1. Identification of TPs by literature and data research

As pesticide TPs are partly considered within regulatory schemes, analysis of available literature and reports on pesticides by the European Food Safety Authority (EFSA) and the Environmental Protection Agency of the United States of America (US-EPA) was evaluated. Data of pesticide reports were often fed by unpublished data which is why not only primary literature was used here. For each compound a comprehensive literature and database analysis was performed to identify known TPs. These TPs were selected due to their potential formation by environmental processes, e.g. known transformation pathways and processes such as photolysis, biodegradation, and hydrolysis in different compartments. TPs that were only formed by AOP or other technical processes were not considered. In addition, the state of knowledge on the aquatic environmental analysis and detection of the selected TPs were reviewed to get a more comprehensive understanding for their potential risk.

#### 2.3.2. Generation of TPs and photolytic mixtures by photolysis

Each pesticide was diluted in pure water (pH 7) and approximately at their respective limit of solubility in water (S3). Photolysis was conducted with an initial volume of 800 mL using a xenon arc lamp (TXE 150, Peschl Ultraviolet, Mainz, Germany) which had an emission spectrum roughly matching the solar radiation (Yager and Yue, 1988; S1). However, cut-off wavelength is much beneath the natural border of 290 nm but was tested to be not relevant for the formation of transformation products (Hensen et al., submitted for publication). It was equipped with an ilmasil quartz immersion tube in a cylindrical batch reactor (T = 20 ± 2 °C). Photolysis experiments were performed over a time period of 8.0 h with hourly sampling (10 mL). Other test conditions and settings are listed in S2 and S3. The samples after 0, 1, 2, 3, 4, 5, 6 7, and 8 h were stored at −20 °C until one part of the samples was analyzed in mass spectrometer to identify formed TPs over the whole irradiation time. The other part was used for subsequent toxicity tests.

#### 2.3.3. Analysis and elucidation of TPs

Analysis of primary elimination of parent compound and elucidation of TPs in photolytic mixture was done by LC-MS. Analysis was conducted using a RP-column (Nucleodur 100-3, 125/2, c18 ec; Macherey Nagel, Düren, Germany) as stationary phase while 0.01% Formic acid (A) and Acetonitrile (B) were used as mobile phase with a flow of 0.4 mL $min^{-1}$ and a gradient as described in S2. Oven temperature was set to 30 °C. The structure of TPs were elucidated using an Iontrap (Dionex Ulitmate 3000 UHPLC system, Dionex, Idstein, Germany) and a LTQ Orbitrap-XL high-resolution mass spectrometer with ESI source (Thermo Scientific, Dreieich, Germany) in a full scan. Positive mode was used to analyze Boscalid, Penconazole, Diuron, Terbutryn, and OIT. Negative mode was used to analyze Mecoprop. Applied setting can be received from S2.

### 2.4. In silico prediction models (Tier I)

*In silico* analysis of the selected TPs employing QSARs was performed using different models provided by the CASE Ultra software package (v. 1.7.0.5, MultiCASE Inc.) expert system for the substructure based prediction of toxicity and bioactivity of chemicals. The model TOX_EB was used for the prediction of short-term bacterial luminescence inhibition in the Microtox assay. To analyze the bacterial mutagenicity, a statistical model (GT1_A7B) and an expert rule-based model (GT_EXPERT) were used in combination as recommended by the ICH M7 guidelines (EMA/CHMP/ICH/83812/2013). All *in silico* models used in this study have defined and validated applicability domains. More information about training sets, validity criteria, and predictive performance of the CASE Ultra software can be found elsewhere (Chakravarti et al., 2012).

Using this model, output variables were provided in a discrete categorical form that allow for the classification of chemicals. Therefore, substances could be assessed that were within a probability range of 35–55% of the model. All TPs that were found to be out of that range could only be inconclusively assessed (IN). All TPs that contain structural features that are not covered by the training set chemicals of the model were designated as “out of domain” (OD).

### 2.5. Cytotoxicity in bacteria: toxicity tests of the mixture (Tier II) and of single substances (Tier III)

A modified luminescent bacteria test (LBT) (Menz et al., 2013) was implemented to assess cytotoxic effects onto aquatic environmental bacteria (*Vibrio fischeri*) (NRRL_B_11177; Hach-Lange GmbH, Düsseldorf, Germany). In this test, three endpoints were analyzed: acute (30 min of exposure) and chronic toxicity (24 h) and growth inhibition (14 h). Due to the need of seawater conditions of luminescent bacteria, one half of initial solution was mixed with one half of a 2% (w/v) sodium chloride solution. In case of high toxic effects (about 100% luminescent inhibition) at the limit of solubility of substances, solutions were diluted until effects were reduced to 60–80% luminescence inhibition. Concentrations of parent compounds in the reaction mixture were dependent on the amount of elimination after eight hours and are depicted in S3. Concentration of TPs was not determined.

The umu-test and umu-test S9 were done to determine genotoxic effects on DNA of *Salmonella typhimurium* (TA1535 psk 1002; German Collection of Micororganisms and Cell Cultures GmbH, Braunschweig, Germany) (ISO/FDIS 13829 (1999)). The test based on measurement of induction ratio (IR) of the umuC gene. A substance is classified as genotoxic in case of IR $>$ 1.5. Test concentrations are also depicted in S3.

Single substances were diluted in water and stirred for half an hour. In some cases 1% DMSO was added to enable a better solubility of substances in water. Toxicity tests were conducted as described above.

Short-term luminescent inhibition of the conducted LBT was comparable to CASE Ultra model TOX_EB, whereas the umu-test was comparable with the models assessing bacterial mutagenicity (GT_A7B and GT-Expert) (Menz et al., 2017).

## 3. Results and discussion

### 3.1. Identification of TPs and their environmental occurrence

By data retrieved from literature and database research, and identification of TPs formed in photolysis experiments we compiled a list of 45 environmental TPs in total originating from the six pesticide parent compounds (Fig. 3). For more structural and analytical details of the TPs see S7 and S8. The TPs review demonstrated that the number of substances that need to be considered at environmental hazard and risk assessment was multiplied by a factor of 7.5 in comparison to the consideration of solely the parent compounds.

Seven TPs of Boscalid were found (Table 1). They are reported by the US-EPA (EPA, 2010) and in another study analyzing UV-treatment of Boscalid (Lassalle et al., 2014). This study was considered as an exception, since three of the TPs (TP-307(a), TP-307(b), and TP-325(a)) that were found in that study were also formed by our degradation experiments and the analysis of photolysis occurring under environmental conditions is still lacking for Boscalid. Out of the seven TPs, only the metabolites (TP-157, TP-158, TP-309, and TP-325(b)) are considered by approval reports so far. For Penconazole three studies were available that analyzed photodegradation (Hensen et al., submitted for publication; Rodríguez-Cabo et al., 2018; Schwack and Hartmann, 1994). Including three environmental relevant and already considered metabolites (TP-70, TP-130, and TP-286) from available reports, ten TPs of Penconazole were selected. Five TPs of Diuron that occurred from biodegradation and photodegradation were found. They are already known since 1982 (Ellis and Camper, 1982; Jirkovský et al., 1997; Tanaka et al., 1986). In total, twelve Terbutryn TPs were identified. Although this substance is used for decades, the elucidation of formed TPs has been done only recently (Bollmann et al., 2016, 2017a; Hensen et al., 2018). Seven OIT-TPs were found that were formed by biodegradation and photodegradation (Bollmann et al., 2017a). All four selected Mecoprop TPs are well known from various studies (Boule et al., 2002; Meunier and Boule, 2000), whereas two of them (TP-107 and TP-141) were already considered in the corresponding approval report (EFSA, 2017).

We found that 82% and 58% of the selected TPs were formed by photolytic processes and microbial degradation, respectively. 42% were formed by both processes. Under given analytical conditions (e.g. ionization and chromatography), LC-MS non-target screening indicated the presence of 27 TPs in the photolytic mixtures. In fact, 4 TPs of Boscalid, 7 TPs of Penconazole, 3 TPs of Diuron, 10 TPs of Terbutryn, 2 TPs of OIT, and 3 TPs of Mecoprop were detected in the respective photolytic mixture. Structures of all these TPs were already described in literature (Table 1). Some of the TPs that were described in literature, e.g. the majority of OIT-TPs, were not found in our photolysis experiments. One possible explanation is that other studies used different mass spectrometric devices and settings (i.e. mass spectrometer, ionization mode and source) or more likely the shorter duration time of our photolysis experiments.

Literature research (Table 1) demonstrated that one third of the identified TPs were already detected in different aquatic environments. In total, seven studies were found that detected at least one of the selected TPs. The studies were conducted from 1997 to 2018, whereby most of them were done in the past ten years. Out of the TPs that were analyzed in the aquatic environment 47% were detected in surface water and 40% in groundwater. Overall, 53% of the TPs could be detected in surface water and/or groundwater samples. However, 47% of the analyzed TPs could neither be detected nor quantified in any water sample. Hence, there is a lack of studies investigating the occurrence of TPs in the aquatic environment.

Most references were found for Diuron-TP-162, TP-205, and TP-219 and Terbutryn-TP-212 (Hydroxy-Terbutryn). Terbutryn-TP-212 (Hydroxy-Terbutryn) could also be formed by the degradation of Terbuthylazin as well. Thus, Terbutryn-TP-212 was referred to its other parent compound Terbuthylazin in most studies (Hernández et al., 2008; Reemtsma et al., 2013). None of the TPs of Boscalid and Penconazole was found in surface water or groundwater samples. The highest concentration in surface water (field runoff) was found for Diuron-TP-219 (cmax = 7.9 μg $L^{-1}$) (Field et al., 1997). In groundwater, highest concentration was found for Mecoprop-TP-141 of about $c_{max}$ = 1.36 μg $L^{-1}$ (McManus et al., 2014).

As it is well known that $\log K_{OW}$ (logP) is negatively correlated to water solubility $S_W$ (Isnard and Lambert, 1989) and this parameter shows generally the tendency of a substance to distribute into the aquatic environment, 82% of the analyzed TPs have a higher tendency to be more mobile in the aquatic environment than their corresponding parent compounds. This fact was received by the calculated logP values of TPs by QSAR (S5). Thus, there is in fact a high probability to detect them in the aquatic environment. The reason that most of the TPs were hitherto not analyzed could be explained to some extent that they were declared to be not relevant due to lacking awareness and assessment of TPs (Laabs et al., 2015). Hence, there is a great demand for the implementation of TPs in monitoring programs (Escher et al., 2014; German Federal Environmental Agency, 2019)

### 3.2. Genotoxicity of TPs

At tier I, it is shown by literature review that there were huge data gaps regarding the genotoxicity of TPs. Except a few non-specific TPs that were evaluated (Zeiger et al., 1992) none of these TPs were assessed by experiments. Prediction of TPs of Boscalid was done by the QSAR tool T.E.S.T. in a previous study and it was found that Bosclaid-TP-307(a), TP-307(b), and TP-325(b) were mutagenic in rat (Lassalle et al., 2014). This is supported by our *in silico* results, where we found that TP-307(a), TP-325(a), and TP-325(b) were genotoxic by employing the CASE Ultra statistical model GT-A7B on *S. typhimurium* (S5). No other TP was predicted to be genotoxic by QSAR. The absence of genotoxic potential could be confirmed for a variety of TPs, such as Boscalid-TP-157, Mecoprop-TP-107, Diuron-TP-162, and Mecoprop-TP-

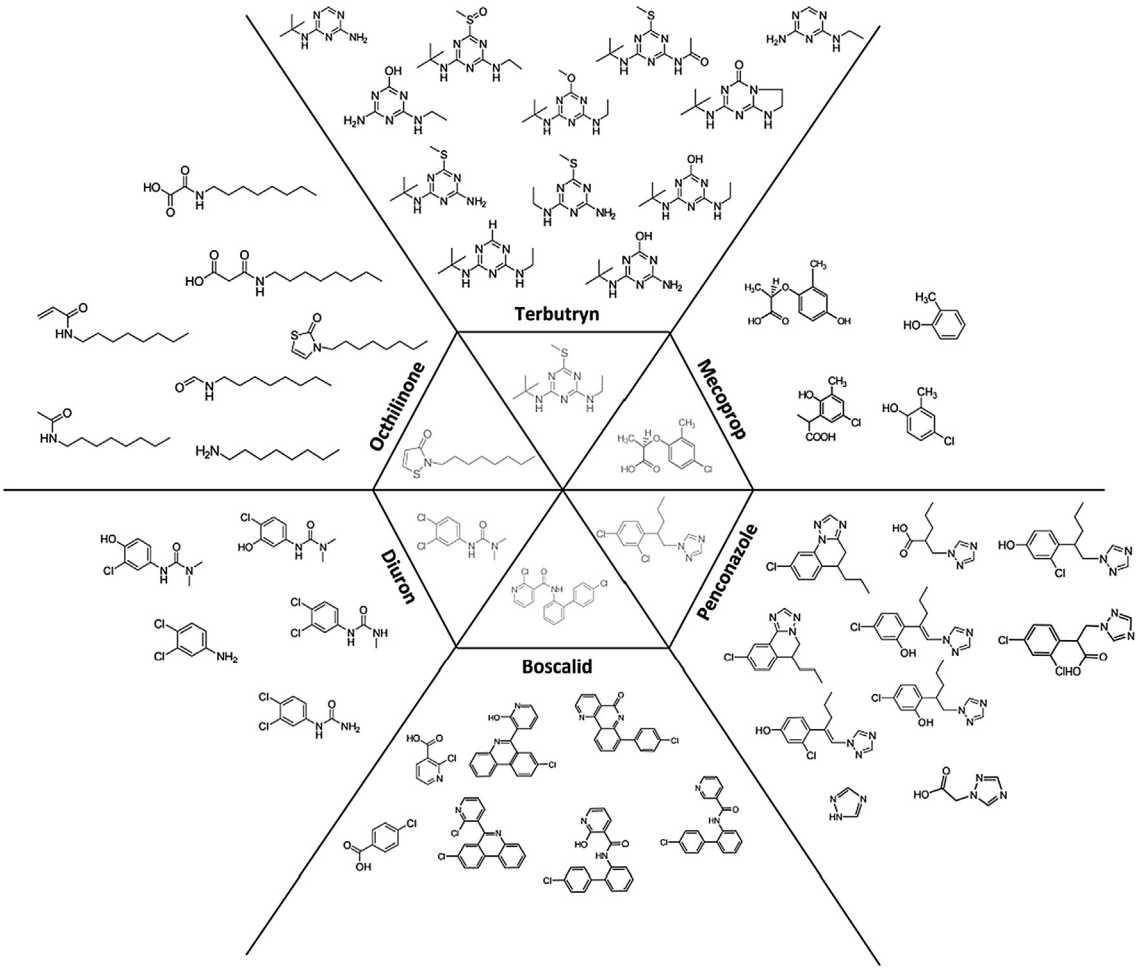


**Fig. 3.** 45 TPs originating from six pesticidal parent compounds – illustration of the multiplication of known substances that should be further investigates by an environmental risk assessment.

141 by a study that analyzed mutagenicity on *S. typhimurium* of 311 substances (Zeiger et al., 1992). At tier II, none of the TPs showed positive genotoxic effects in *S. typhimurium* in our experiments, neither the parent compounds nor the photolytic mixtures (S10). Hence, we concluded that the majority of TPs were not genotoxic to *S. typhimurium.* For this reason we did no further genotoxicity testing. Overall, it turned out that our approach was not useful to determine the genotoxicity of pesticide TPs in a photolytic mixture. This was also seen in other studies were the photolytic mixtures of different parent compounds had no genotoxic effect (Kotnik et al., 2016; Mahmoud et al., 2014; Menz et al., 2017; Toolaram et al., 2016). Thus, genotoxicity must be assessed in separate single tests in future approaches. Therefore, synthesis of single TPs that show genotoxic activity by QSAR seem to be unavoidable. In this particular case, it should be done for Boscalid-TP-307(a), TP-307(b), TP-325(a), and TP-325(b) as they were found to be probably genotoxic by *in silico* prediction.

### 3.3. Ecotoxicity of TPs

Ecotoxic properties of about 60% of the selected TPs were not known so far. No literature data was available for Boscalid-TPs. Contrary, every selected TP of Diuron was already assessed by previous studies (Ellis and Camper, 1982; Jirkovský et al., 1997; Tanaka et al., 1986). For the other TPs, ecotoxicity was done only occasionally. Overall, seven studies analyzed ecotoxicity of the selected TPs. In many studies luminescence inhibition of *V. fischeri* was used as ecotoxicological endpoint. In others test organisms such as *Pseudomonas putida* and aquatic organisms in general were used. Results of LBT are depicted in Table 2 and in S4.

#### 3.3.1. Boscalid-TPs

At tier I there was no literature data available regarding the ecotoxicity (e.g. luminescence inhibition of *V. fischeri*) of Boscalid-TPs. By QSAR, we found positive results of Boscalid and its TPs TP-307(a), TP-307(b), TP-309, TP-325(a), and TP-325(b). It was shown that one common alert was found for all structures (S6) that caused the effect. Further testing of the photolysis mixture at tier II was not possible due to the low solubility of the substance. Hence, the required test concentration could not be reached due to interferences of solvents on photolysis and toxicity tests (Parvez et al., 2006). TPs that showed toxic activity (TP-325 and TP-309) could not be analyzed by a single toxicity test due to the non-availability of an analytical standard. Hence, tier II and III were not useful for Boscalid and data availability turned out to be really scarce as the toxic indications were only based on one tier. Hence, there is an urgent need to synthesize TPs with toxic indication to get stronger evidence for their ecotoxicity or its absence. This turned out to be especially important for TPs of parent compounds that have low water solubility. These difficulties in toxicity testing were already mentioned for low soluble parent compounds (Hernando et al., 2007; Tang et al., 2013), but were never the subject of discussion for mixture toxicity tests of TPs that originated from little soluble substances. The circumstance that TPs often have a higher water solubility compared to their parent compounds is of utmost importance due to the difficulties in toxicity tests described above and due to the possibly higher

**Table 1**
Literature review of the selected TPs. Known synonyms of the TPs, formation process by biodegradation/metabolism (M) or photodegradation (P) and the corresponding reference (REF) where they were elucidated. Environmental detection (ED) of TPs in surface water (SW) and groundwater (GW). ND = not detetcted, NQ = not quantified due to missing analytical standard.

| Substance | Known Synonyms | Formation | ED SW [c in µg $L^{-1}$] | ED GW [c in µg $L^{-1}$] |
|---|---|---|---|---|
| **Boscalid** | | | | |
| TP-157 | M510F64, p-chlorobenzoic acid | M[1] | – | – |
| TP-158 | M510F47, 2-chloronicotinic acid | M[1] | – | – |
| TP-307(a)* | – | P[2] | – | – |
| TP-307(b)* | – | P[2] | – | – |
| TP-309 | M510F08 | M[1] | – | – |
| TP-325(a) | – | P[2] | – | – |
| TP-325(b)* | M510F49 | MP[1][2] | – | – |
| **Penconazol** | | | | |
| TP-70 | CGA 71019, 1,2,4-Triazole | M[3] | – | – |
| TP-130 | CGA 142,856 | M[3] | – | – |
| TP-184* | – | P[4] | – | – |
| TP-248(a) | – | P[5] | – | – |
| TP-248(b)* | – | P[6] | – | – |
| TP-264(a)* | – | P[4] | – | – |
| TP-264(b)* | – | P[4] | – | – |
| TP-266(a)* | – | P[4] | – | – |
| TP-266(b)* | – | P[4] | – | – |
| TP-286 | CGA 179,944 | M[3] | – | – |
| **Diuron** | | | | |
| TP-162 | DCA | M[7] | < 0.025 [15]; ND [15][16] | ND [15][16] |
| TP-205 | DCPU | MP[8] | < 0.01 [14]*;0.9–3 [15]; ND [15] | < 0.01 [14]*;0.6 [15];ND [16] |
| TP-215(a) | – | P[9] | – | – |
| TP-215(b) | – | P[9] | – | – |
| TP-219 | DCMPU | MP[9] | < 0.01 [14]*;1.3–7.9[15];ND [16] | < 0.01[11]; < 0.1[14]*;~1[15]; ND[16] |
| **Terbutryn** | | | | |
| TP-140 | Desthiomethyl-Desbutyl-T. | P[10] | – | – |
| TP-156 | Desbutyl-2-Hydroxy-T. | P[10] | – | – |
| TP-168 | Desthiomethyl-Desethyl-T. | MP[10] | NQ [11] | ND [11] |
| TP-184[c)] | Desethyl-2-Hydroxy-T. | MP[10] | 0.2–1.2 [15] | 1.2 [15] |
| TP-186 | – | P[11] | ND [11] | ND [11] |
| TP-196 | Desthiomethyl-T. | MP[10] | NQ [11] | ND [15] |
| TP-210 | – | P[11] | NQ [11] | ND [11] |
| TP-212[c)] | 2-Hydroxy-T.; MT13; GS 23,158 | MP[10] | 0.02 [11]; 0.023[14]*; ~0.1[16]; 0.8 [17] | 0.023 [14]*ND [13]; 0.1 [16] |
| TP-214[d)] | Desethyl-T. | MP[10] | 0.08 [11] | 0.003 [11] |
| TP-226 [e)] | Terbumeton** | MP[10] | 0.02 [11];0.006 [17]* | ND [11] |
| TP-256 | – | P[11] | NQ [11] | ND [11] |
| TP-258 | T.-Sulfoxid | MP[10] | ND [11] | ND [11] |
| **OIT** | | | | |
| TP-130 | Octylamin | MP[12] | – | – |
| TP-158 | N-Octylformamide | MP[12] | – | – |
| TP-172 | N-Octylamide | MP[12] | – | – |
| TP-184 | N-Octylprop-2-enamide | MP[12] | – | – |
| TP-202 | N-Octyloxamic acid | MP[12] | – | – |
| TP-214 | 2-Octylisothiazol-3(2H)-one | MP[12] | NQ [11] | ND [11] |
| TP-216 | N-Octyl Malonamic acid | MP[12] | – | – |
| **Mecoprop** | | | | |
| TP-107 | o-Cresol, 2-Methylphenol | MP[13] | – | – |
| TP-141 | 2-MCP, 4-Chloro-o-cresol | MP[13] | NQ [18]* | 0.005–1.36 [19] |
| TP-195 | TP-195 | P[13] | – | – |
| TP-213 | TP-213 | P[13] | – | – |

**References:** [1]: EPA, 2010; [2]: Lassalle et al., 2014; [3]: EFSA, 2008; [4] Hensen et al., 2019; [5]: Schwack and Hartmann, 1994; [6]: Rodríguez-Cabo et al., 2018; [7]: Ellis and Camper, 1982; [8]: Jirkovský et al., 1997; [9]: Tanaka et al., 1986; [10]: (Bollmann et al., 2016) (Bollmannetal.,É; [11]: Hensen et al., 2018; [12]: Bollmann et al., 2017b [13]: Boule et al., 2002; [14]: Reemtsma et al., 2013; [15]: Field et al., 1997; [16]: Hernández et al., 2008; [17]: Benvenuto et al., 2010; [18]; Laganà et al., 2002; [19]: McManus et al., 2014.

* 50 percentile.

** Only studies relating to Terbumeton as TP and not as parent compound were considered,*** As a TP of MCPA.

probability to affect the aquatic environment.

### 3.3.2. *Penconazole-TPs*

EFSA report on Penconazole concludes that TP-70 and TP-286 are highly ecotoxic to aquatic organisms (EFSA, 2008). Precise ecotoxicological endpoints could not be obtained from this report. Our QSAR results revealed that seven out of ten assessed Penconazole-TPs showed toxic effects (Table 2). As many of them were with a probability of 50.7% out of the grey zone of 35 to 55% they could only be inconclusively assessed.

At tier II, the toxicity test of the photolytic mixture of Penconazole showed that chronic effects of Penconazole to luminescent bacteria and the photolytic mixture were similarly high. There were additionally moderate acute toxicity and growth inhibition effects of the mixture (> 20%). This was, however, not seen for the parent compound. As the mixture test results verify the results obtained at tier I, there were indications that TPs were probably more toxic than Penconazole.

At tier III, the toxicity of TP-70 was examined in a single LBT and turned out to be non-toxic to *V. fischeri* at a tested concentration of 30 mg $L^{-1}$. As studies revealed aquatic toxicity to other aquatic organisms than *V. fischeri* they might be affected by this TP. Regarding the toxicity of the photolytic mixture, TPs of phenyl ethyl azolic fungicides

**Table 2**
Predicted and experimental cytotoxicity in bacteria following the workflow at three different tiers: Tier I: Literature review and calculated by CASE Ultra model TOC_EB (MultiCASE Inc.; Model Version 1.5.2.0.899.500), Tier II: Toxicity test (luminescence bacteria test, LBT) of a photolytic mixture, and Tier III: LBT of single substance standards of TPs. Positive (+), negative (−), inconclusive (IN), and results that were out of domain (OD) of QSAR (MultiCASE). TPs that were identified in photolytic mixture (PM) and experimental results of LBT corresponding to three endpoints: acute toxicity (AT), chronic toxicity (CT) and growth inhibition (GI) as well as the results of the single LBT of some TPs if an analytical standard was available (Analyt.Std.).

| Substance | Tier I | | PM | Tier II | | | Analyt. Std. | Tier III | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Literature | QSAR | | LBT mixture | | | | LBT single | | |
| | mg $L^{-1}$ (*V. fischeri*) | TOX_EB | | AT | CT | GI | | AT | CT | GI |
| **Boscalid** | $EC_{50}$ = 5.33 [1] | + | + | – | – | – | | | | |
| TP-157 | | – | – | | | | + | | | |
| TP-158 | | – | – | | | | + | | | |
| TP-307(a) | | + | + | | | | – | | | |
| TP-307(b) | | + | + | | | | – | | | |
| TP-309 | | + | – | | | | – | | | |
| TP-325(a) | | + | + | | | | – | | | |
| TP-325(b) | | + | + | | | | – | | | |
| **Penconazol** | $LC_{50}$ = 0.7 [2]* | + | + | – | + | – | | | | |
| TP-70 | toxic [2]* | OD | – | | | | + | – | – | – |
| TP-130 | | OD | – | | | | – | | | |
| TP-184 | | OD | + | + | + | + | – | | | |
| TP-248(a) | $LC_{50}$ = 4.7 [2]* | IN (+) | + | + | + | + | – | | | |
| TP-248(b) | $LC_{50}$ = 2.1[2]* | IN (+) | + | + | + | + | – | | | |
| TP-264(a) | | IN (+) | + | + | + | + | – | | | |
| TP-264(b) | | + | + | + | + | + | – | | | |
| TP-266(a) | | IN(+) | + | + | + | + | – | | | |
| TP-266(b) | | + | + | + | + | + | – | | | |
| TP-286 | toxic [3]** | + | – | | | | – | | | |
| **Diuron** | 58 [4] | – | + | – | – | – | | | | |
| TP-162 | 0.5 [4] | + | – | | | | + | | | |
| TP-205 | 15 [4] | + | – | | | | + | | | |
| TP-215(a) | 71 [4] | IN (+) | + | + | + | + | – | | | |
| TP-215(b) | 72 [4] | + | + | + | + | + | – | | | |
| TP-219 | 18 [4] | + | + | + | + | + | + | | | |
| **Terbutryn** | > 8.13 [5] [6] | OD | + | | – | – | – | | | |
| TP-140 | > 7.96 [5] | – | – | | | | – | | | |
| TP-156 | | – | – | | | | – | | | |
| TP-168 | > 7.60 [5] | OD | + | + | – | – | – | | | |
| TP-184 | | OD | + | + | – | – | – | | | |
| TP-186 | | OD | + | + | – | – | – | | | |
| TP-196 | > 6.54 [5] | OD | + | + | – | – | – | | | |
| TP-210 | | OD | + | + | – | – | – | | | |
| TP-212 | Relevant [7] | OD | + | + | – | – | + | – | – | – |
| TP-214 | | OD | + | + | – | – | +[1] | | | |
| TP-226 | 89.4 [8] | OD | + | + | – | – | + | – | – | – |
| TP-256 | | OD | + | + | – | – | – | | | |
| TP-258 | | OD | + | + | – | – | – | | | |
| **OIT** | 0.05 [5] | IN (+) | + | + | + | + | | | | |
| TP-130 | | – | + | + | + | + | – | | | |
| TP-158 | | OD | – | | | | – | | | |
| TP-172 | > 8.36 [5] | – | – | | | | – | | | |
| TP-184 | 4.51 [5] | – | – | | | | – | | | |
| TP-202 | | – | – | | | | – | | | |
| TP-214 | 1.09 [5] | IN (+) | + | + | + | + | – | | | |
| TP-216 | | – | – | | | | – | | | |
| **Mecoprop** | 91[9]*, 24 [10]*** | + | + | – | + | – | | | | |
| TP-107 | 27.1 mg $L^{-1}$ [11] | – | – | | | | – | | | |
| TP-141 | 0.29 [9]* | + | + | + | + | + | + | + | + | + |
| TP-195 | | + | + | + | + | + | – | | | |
| TP-213 | | + | + | + | + | + | – | | | |

**References:** [1]: EPA, 2010; [2]: Rodríguez-Cabo et al., 2018; [3]: EFSA, 2008; [4]: Tixier et al., 2001; [5]: Bollmann et al., 2017a; [6]: Hernando et al., 2007; [7]: EFSA, 2011; [8]: Villa et al., 2012; [9]: Mottier et al., 2014; [10]: Strachan et al., 2001; [11]: Jenning et al., 2001

* Test organism: *Daphnia magna* (by QSAR);

** Test organism: aquatic organisms;

*** Test organism: *Pseudomonas putida;* [1] Solved in ACN that influences the luminescence of *V. fischeri* in the test conducted.

that were formed by cyclization (TP-248(a) and TP-248(b) might have similar or higher $EC_{50}$ values than their parent compound (Rodríguez-Cabo et al., 2018). Derived from the *in silico* tool ECOSAR used by the authors, it was found that TP-248(a) and TP-248(b) of Penconazole were slightly less toxic than the parent compound to aquatic organisms (*Daphnia magna*, green algae, and fish). The increased effect of the photolytic mixture found in our study might be triggered by other TPs of the photolytic mixture than these both ones, e.g. TP-264(a), TP-264(b), TP-266(a) and/or TP-266(b) or could be provoked by synergistic effects of the photolytic mixture. However, our results showed for the first time that TPs of Penconazole could be ecotoxic on aquatic organisms and should receive more attention regarding their behavior

in the environment. This is especially important as TPs that were not formed by biological processes but by photolysis instead were generally not considered in studies. This can be explained by the fact that Penconazole is declared as not degradable by direct photolysis due to its absorption spectrum below the terrestrial sunlight (EFSA, 2008). As indirect photolysis could also be a relevant pathway (Remucal, 2014) and TPs of Penconazole formed by direct and indirect photolysis were found out to be similar in another study (Hensen et al., submitted for publication) these TPs need to be considered more closely in future research of risk assessment.

### 3.3.3. Diuron-TPs

The literature review (Tier I) revealed $EC_{50}$ of 58 mg $L^{-1}$(Diuron), 71 mg $L^{-1}$ (TP-215(a), 72 mg $L^{-1}$ TP-215(b), 18 mg $L^{-1}$ (TP-219), 15 mg $L^{-1}$ (TP-205), and 0.5 mg $L^{-1}$ (TP-162). Bacterial toxicity (tier I) were positively calculated for all TPs. In contradiction to the gathered literature data the results by QSAR calculation showed a difference between Diuron and the TP-215(a) and TP-215(b), and indicated a higher toxicity of these TPs (Table 2). The CASE Ultra model TOX_EB determined the dichlorophenyl group as the decisive toxic moiety, which is part of TP-162, TP-205, and TP-219 and explains their higher toxicity. The chloro-hydroxyphenyl group that is part of TP-215(a) and TP-215(b) was predicted with a lower probability to be toxic than the dichlorophenyl group. This confirmed the lower toxicity of TP-215(a) and TP-215(b) compared to the other TPs. Another study stated that the subsequent loss of the methylurea group of TP-219, TP-162, and TP-205 led to a decrease in toxicity in algae but to an increase in toxicity to daphnids (Neuwoehner et al., 2010). The results of the experimental LBT (Tier II) showed no inhibition of luminescence for the parent compound Diuron for all three endpoints. In contrast, the photolytic mixture showed high chronic toxicity and growth inhibition. The toxic effect of the photolytic mixture could therefore be caused by TP-219 as we could not indentify TP-205 and TP-162 in the photolytic mixture. Comprehensive data situation of Diuron TPs indicates that especially TP-219, TP-205, and TP-162 turned out to be more toxic than the parent compound. They are already considered in risk assessments. TP-205 and TP-219 were already declared to be relevant (EFSA, 2005). The experimental results received at tier II confirmed the results received at tier I and underline the accuracy of our approach.

### 3.3.4. Terbutryn-TPs

The results of the literature review (tier I) showed that Terbutryn and three TPs (TP-196, TP-168, and TP-140) did not inhibit luminescence of *V. fischeri* at tested concentration of about 6.5–8 mg $L^{-1}$ (Bollmann et al., 2016). Villa et al. (2012) reported an $EC_{50}$ value of 89.4 mg $L^{-1}$ in an acute toxicity test (15 Min.) with *V. fischeri* of TP-226 (also known as Terbumeton). TP-212, which is known to be a TP of the herbicide Terbutylazin as well, is classified as relevant due to the fact that its parent compound Terbutylazin is classified as carcinogen category 3 although the risk of TP-212 onto aquatic organisms was assessed to be low (EFSA, 2011). Except two TPs that were predicted to have no ecotoxic effects, all other TPs were out of domain of the *in silico* prediction model, since fragments of these compounds were not present in any of the training sets of chemicals of the model used.

Toxicity tests of Terbutryn and its photolytic mixture (tier II) showed that there was an increase in acute toxicity by the photolytic mixture (Table 2), whereas no toxic effect could be measured for Terbutryn for all three endpoints. As chronic toxicity is generally the more sensitive parameter (Backhaus et al., 1997; Menz et al., 2013), it was surprising that the photolytic mixture of Terbutryn showed solely acute toxic effects. This might be a hint that TPs are acting more specifically in *V. fischeri* in this case. Derived from the results of the study by Bollmann et al. (2017a), we can assume that these effects can be caused by other TPs than TP-196, TP-168, and TP-140, as they were tested to have no effects. The conducted single LBT at tier III for available TP-212 turned out to be negative at the tested concentration of 2.5 mg $L^{-1}$. As no TP was found causing the toxic effect as it was seen in the photolytic mixture, other TPs of the mixture (TP-184, TP-186, TP-210, TP-214, TP-256, or TP-258) or mixture effects (Villa et al., 2012) by different TPs could have caused the luminescent inhibition. However, this study showed for the first time that some TPs of Terbutryn are more ecotoxic towards luminescent bacteria than their parent compound. This fact underlines the benefits of this multimethod approach as in silico results (applicability domain of the model) and single toxicity tests (availability of analytical standard) have reached their limits.

### 3.3.5. OIT-TPs

Literature stated that $EC_{50}$ values of acute toxicity on *V. fischeri* of OIT, TP-184, and TP-214 were 0.05 mg $L^{-1}$, 4.51 mg $L^{-1}$, and 1.1 mg $L^{-1}$, respectively. $EC_{50}$ values of other five TPs, such as TP-172, could not be assessed in the tested concentration range in that study (Bollmann et al., 2017b). QSAR results in our study at tier I underlined these results as OIT and OIT-TP-214 were the only substances that were (inconclusively) positively assessed with a probability of being positive of 46.2%.

At tier II, toxicity test of OIT showed high toxic effects for all three endpoints. The photolytic mixture showed also high acute toxicity but slightly lower effects for chronic toxicity and growth inhibition. Thus, TPs of the mixture are probably less specifically acting in *V. fischeri*. Calculated $EC_{50}$ of chronic toxicity and growth inhibition values of OIT are depicted in S9. The results of tier II were in accordance with the results obtained at tier I. As no analytical standard of OIT-TP-214 was commercially available and as it was already tested previously with an synthesized standard by (Bollmann et al., 2017a) no LBT at tier III was conducted.

### 3.3.6. Mecoprop-TPs

Results of tier I revealed that no literature data of toxicity to*V. fischeri* were available for Mecoprop. Instead, an $EC_{50}$ of Mecoprop for another test species (*Pseudomonas putida*) was found to be $EC_{50}$ = 24 mg $L^{-1}$ (Strachan et al., 2001). According to other studies, *P. putida* turned out to be generally more sensitive than *V. fischeri* which in turn means that $EC_{50}$ for *V. fischeri* of Mecoprop is probably higher than 24 mg $L^{-1}$ (Schmitz et al., 1998; de Zwart and Slooff, 1983). Regarding the toxicity to *V. fischeri* of TPs, $EC_{50}$ of TP-107 was found to be 27.1 mg $L^{-1}$ (Jenning et al., 2001) which indicates that the TP is probably more toxic than Mecoprop itself. TP-141 was found to be more toxic than Mecoprop to *D. magna* (Mottier et al., 2014). Toxicity of the photolytic mixture of MCPA, another chlorophenoxy herbicide, to *V. fischeri* increased after five minutes of UV irradiation (Zertal et al., 2001). Due to the fact that Mecoprop and MCPA only differ by different length of the carbon chain of the carboxyl group they have similar TPs such as TP-107 and TP-141. The toxic effect might therefore be triggered by these TPs. *In silico* prediction revealed toxic effects of the parent compound and TP-141, TP-195, and TP-213.

Due to the positive results received at tier I, we conducted toxicity test of the photolytic mixture at tier II. The results showed that no inhibition of luminescence was present for Mecoprop. In contrast to the parent compound the photolytic mixture showed clear effects of more than 60% luminescence inhibition for all three endpoints (Fig. 4A) indicating that the TPs are more toxic than Mecoprop on *V. fischeri*.

At tier III, we tested Mecoprop-TP-141 as this TP showed positive results at Tier I and II and an analytical standard was commercially available. To the authors best knowledge no toxicity test on *V. fischeri* was done previously for TP-141. It was seen found luminescence of *V. fischeri* was inhibited by 60% at a concentration of 12.5 mg $L^{-1}$ for all three endpoints (Fig. 4B). $EC_{50}$ values of 2.28 (acute toxicity), 7.63 (chronic toxicity), and 14.46 (growth inhibition) mg $L^{-1}$ were calculated (Fig. 5).

It is thus most likely that the toxic effects observed in the photolytic mixture were caused by Mecoprop-TP-141. Nonetheless, other TPs such as TP-213 - the photoisomer of Mecoprop - might additionally cause the

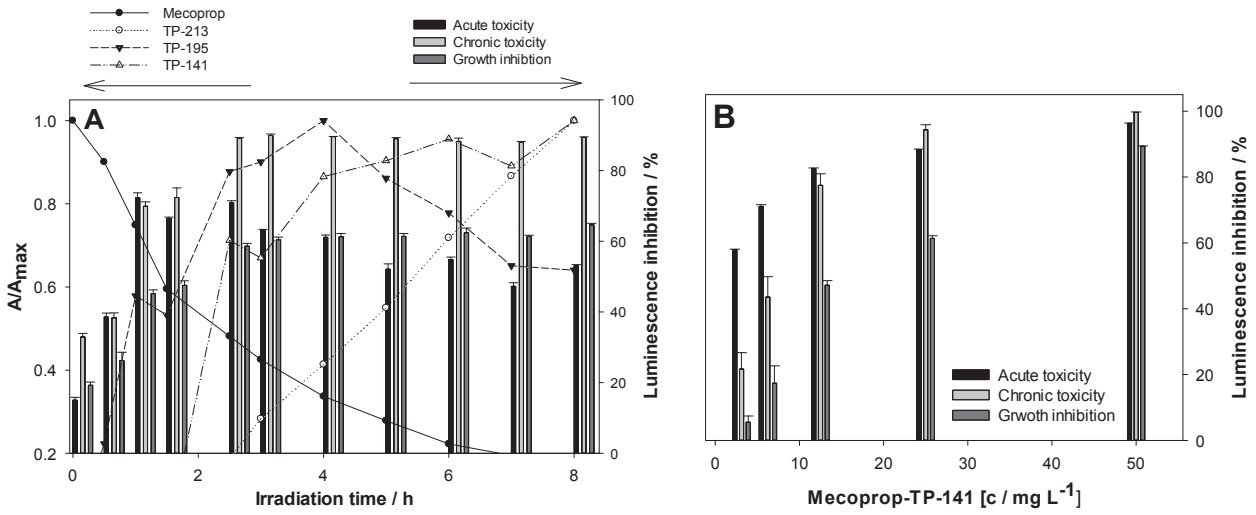


**Fig. 4.** A: Analysis of the toxicity of the photolytic mixture of Mecoprop over a time period of eight hours. Mean values of the Area of Mecoprop and its TPs (left ordinate) and luminescent inhibition of acute, chronic toxicity and growth inhibition (right ordinate). B: Single toxicity test of Mecoprop-TP-141 at a concentration range between 50 and 3.125 mg $L^{-1}$.

effect of the photolytic mixture. This was found for MCPA by (Zertal et al., 2001) as well. In that study the photoisomer of MCPA that was formed by photo-claisen-rearrangement as well as TP-213 here. It was found to be more toxic than MCPA itself. Hence, toxicity tests of the single substance of TP-213 need to be conducted in future. Besides, TP-195 should be taken into account as QSAR results showed positive results and it was present in the photolytic mixture as well.

### 3.4. Workflow and its classification of results

By the proposed workflow for the ecotoxicological assessment of TPs it was possible to initially assess 43 out of 45 TPs (96%). Here the benefits of the combined application arise. For example, in silico tests take effect for low soluble substances such as Boscalid that could not be tested by the conducted experiments. Or vice versa, experiments take effect for substances that do not fall under the applicability domain of the in silico tool such as Terbutryn and the majority of its TPs.

38% of the TPs (17 TPs) could be evaluated by more than one tier (Table 3), which resulted in a strong evidence to be toxic or non-toxic. For 58% of the TPs (26 TPs) slight evidence for their (non–) ecotoxicity was found based on the assessment at tier I. The knowledge on the potential environmental risk of TPs was therefore significantly enlarged by our approach, as the investigated ecotoxic properties were hitherto known for thirteen TPs only. Hence, the knowledge on the ecotoxic properties was more than tripled. Only 2 TPs (Penconazole-TP-130 and OIT-TP158) could not be evaluated by neither tier I nor tier II and III of the proposed workflow.

By starting at tier I (literature review and QSAR), it was possible to assess 80% of the TPs. Hence, the majority of TPs could be initially assessed here. By following the workflow at tier II (mixture toxicity testing) further 16% of TPs could be evaluated that were out of domain or not studied before at tier I. The number of TPs that could be assessed was not increased by conducting tier III (single toxicity test). But some TPs could be assessed more closely. Hence, for some TPs (Penconazole-TP-70, Terbutryn-TP-212, and Terbutryn-TP-226) single toxicity tests gave more information as their toxicities could be considered as negative in these tests. In case of Mecoprop-TP-141, the single LBT confirmed the results received by the toxicity test of the mixture at tier II as this TP was tested to be positive on *V. fischeri*. In total, 33% of the TPs could be assessed by both QSAR and mixture toxicity testings. Out of these TPs, 87% that could be assessed by QSAR could be confirmed by the toxicity testing of the reaction mixture. Hence, our procedure, which is much faster than an experimental assessment of each single TP and the high precision turned out to be beneficial.

By *in silico* prediction, 31% of the analyzed TPs were out of domain, implying that these TPs contain structural features that were not covered by the training set chemicals of the respective model. Most of such TPs were found for Terbutryn-TPs (83% of all Terbutryn-TPs). It

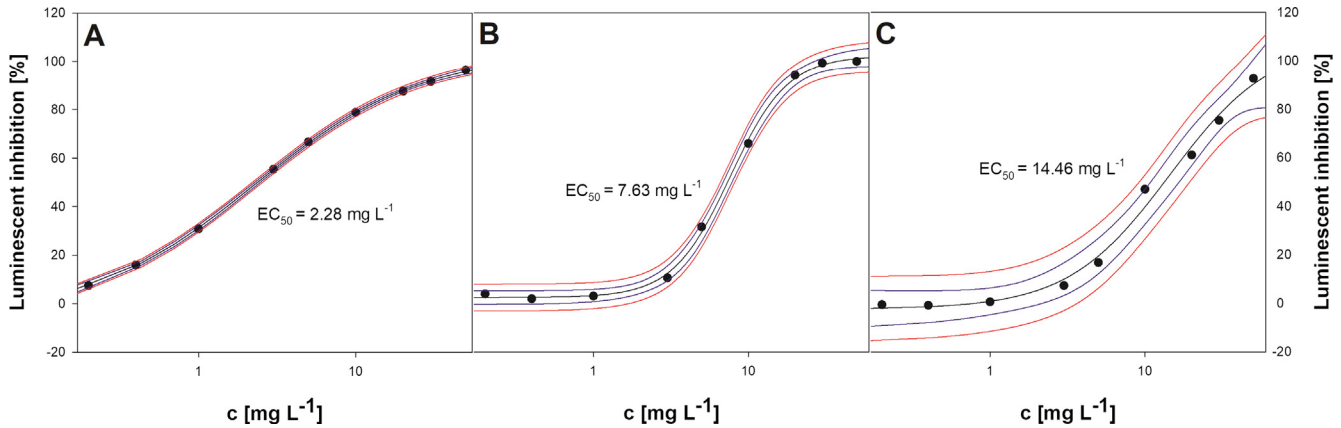


**Fig. 5.** Dose-Response-Curves of Mecoprop-TP-141. Luminescent inhibition is plotted against concentration between 60 and 0.2 mg $L^{-1}$. Luminescence inhibition was measured after 30 min (acute toxicity, A), 24 h (chronic toxicity, B), and 12 h (growth inhibition, C).

**Table 3**
Classification of TPs according to their probability to be toxic or non-toxic. No probability could be constituted if the TPs could neither be assessed by *in silico* nor experimental approaches. Categorization criteria could be received from the methodology approach depicted in Fig. 2.

| Most probably toxic | Probably toxic | No probability | Probably non-toxic | Most probably non-toxic |
|---|---|---|---|---|
| Penconazole-TP-70 | Boscalid- TP-307(a) | Penconazole-TP-130 | Boscalid- TP-157 | Terbutryn-TP-140 |
| Penconazole-TP-286 | Boscalid-TP-307(b) | OIT-TP-158 | Boscalid- TP-158 | Terbutryn-TP-168 |
| Diuron-TP-162 | Bosclaid-TP-325(a) | | Boscalid- TP-309 | Terbutryn-TP-196 |
| Diuron-TP-205 | Boscalid-TP-325(b) | | Terbutryn-TP-156 | Terbutryn-TP-212 |
| Diuron-TP-219 | Penconazole-TP-184 | | OIT-TP-202 | Terbutryn-TP-226 |
| OIT-TP-214 | Penconazole-TP-248(a) | | OIT-TP-216 | OIT-TP-172 |
| Mecoprop-TP-141 | Penconazole-TP-248(b) | | | OIT-TP-130 |
| Mecoprop-TP-195 | Penconazole-TP-264(a) | | | Mecoprop-TP-107 |
| Mecoprop-TP-213 | Penconazole-TP-264(b) | | | |
| | Penconazole-TP-266(a) | | | |
| | Penconazole-TP-266(b) | | | |
| | Diuron-TP-215(a) | | | |
| | Diuron-TP-215(a) | | | |
| | Terbutryn-TP-184 | | | |
| | Terbutryn-TP-186 | | | |
| | Terbutryn-TP-210 | | | |
| | Terbutryn-TP-214 | | | |
| | Terbutryn-TP-256 | | | |
| | Terbutryn-TP-258 | | | |
| | OIT-TP-184 | | | |

became evident that the obtained results are strongly dependent on the availability of a sufficient large quantity of experimental results of test and training substances as it could be seen in case of Terbutryn-TPs.

By the additional photolysis experiments, it was possible to elucidate 62% of the selected and already known TPs. Most of them were found for Terbutryn and Penconazole. It can be assumed that some of the TPs described in literature were present in the photolytic mixture, although they could not be identified due to limitations in mass spectrometry or chromatography. This demonstrates the possibility to draw wrong conclusion regarding the toxicity of the photolytic mixture, as TPs could cause toxic effects of the mixture that were not detected.

For 9 TPs (20%) we found strong evidence to be toxic. Out of these TPs, TP-214 of OIT and TP-141, TP-195, and TP-213 of Mecoprop are not under consideration so far by any pesticide approval report. For 20 TPs (44%) there were slight indications to be ecotoxic. None of these TP was considered within approval procedure so far. Thus, the approach presented here can be considered as a first screening to guide further analysis and assessment with respect to the identification of possibly (eco-) toxicological relevant TPs.

The contribution of in silico models to the legal framework of pesticide risk assessment could be generally beneficial as time and costs are reduced compared to solely experimental approaches. This is, however, especially important when considering the huge amount of probably occurring TPs. Until now, the use of in silico methods is not generally recommended by the legislative framework of the EU since individual models have their own strengths and weaknesses (EFSA, 2010). However, according to the regulation (EC) No.1107/2009 the authorization shall be performed in light of current scientific and technical knowledge and, thus, in silico tools could support the risk assessment of pesticides (Villaverde et al., 2017) even of recent nanoformulations (Villaverde et al., 2018a). Hence, the herein introduced combination of experiment and *in silico* method in a tiered approach might be advantageous in terms of both applicability and validity considering the current legal requirements of risk assessment.

## 4. Conclusion

Our tiered approach for the preliminary assessment of pesticide TPs exemplified by selected endpoints demonstrates the extension of the body of the knowledge on the overall relevance and impact of TPs on human health and the environment. The combined use of published data, in vitro, and *in silico* toxicity assessment within the suggested tiered approach was demonstrated as a useful starting point to handle the increasing number of substances that need to be considered within hazard-oriented assessment of TPs. This approach supports faster decision and priority setting and depending on the issue being addressed the consideration of other endpoints (e.g. toxicity to fish and algae).

The comparison of parent compounds that show toxic effects to environmental bacteria to the number of probably toxic TPs suggests that the number of substances that pose a risk onto the aquatic environment increased by a factor of > 4. This is even more notable as about 33% of the TPs have already been detected in the surface- and groundwater. It may be necessary to implement this proactive assessment of TPs more consequently into the existing regulations to prevent the occurrence and effects of TPs in the water cycle. However, the study presented was a very first one. Its applicability domain needs to be assessed further. For this purpose as a very next step the approach suggested here should be verified by applying additional endpoints and including additional parent compounds.

## CRediT authorship contribution statement

**Birte Hensen:** Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft. **Oliver Olsson:** Conceptualization, Writing - review & editing, Project administration, Funding acquisition. **Klaus Kümmerer:** Resources, Writing - review & editing, Supervision.

## Declaration of Competing Interest

We declare that we have no conflict of interest.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2020.105533.

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