IN SILICO TOOLS TO PRIORITIZE TRANSFORMATION PRODUCTS OF PESTICIDES ORIGINATING FROM DRINKING WATER TREATMENTS: S-METOLACHLOR AS A PROOF OF PRINCIPLE

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Acronyms

AOPs = advanced oxidation processes

DART = Developmental and Reproductive Toxicology

DBPs = disinfection by-products

ECHA = European Chemicals Agency, EU

ECs = Emerging Contaminants

EDs = Endocrine Disruptors

EFSA = European Food and Safety Authority

EPA = Environmental Protection Agency, US

HPLC-MS = High Performance Liquid chromatography–Mass Spectrometry

LC- QToF MS = Liquid Chromatography–hybrid Quadrupole Time-of-Flight Mass Spectrometry

MoA = mechanism (or mode) of action

NAMs = New Approach Methodologies

NTS = Non-Targeted Screening

OECD = Organization for Economic Co-operation and Development, US

PCC = physicochemical characteristics

RSF = Rapid Sand Filtration

SPE = Solid-Phase Extraction

TPs = Transformation Products

TTC = Threshold of Toxicological Concern

UV = UltraViolet

WWT = Wastewater Treatments

Layman summary

Water is an essential life resource and should be a human right (Boylan, 2019), and water quality must be preserved (UNESCO, 2020). There is an urge to assess chemicals in drinking water towards the goal of a toxic-free environment forecasted by the EU Chemical Strategy for Sustainability and the EU Green Deal for 2030. Equivalent intentions constitute the sixth of Europe's 17 Sustainable Development Goals (SDGs) adopted in 2015. The EU Water Framework Directive (WFD) adopted in 2000 requires EU member states to achieve acceptable chemical status in surface water and groundwater by 2027 (WISE, 2022). However, in the water sources, contaminants are present, either microbial or chemical ones, which may be of natural or anthropogenic origin, meaning chemicals produced by humans and released into the environment. Therefore, over time, EU member states implemented specific drinking water treatment processes to purify water sources (EU Commission, 2022). These treatments, however, may lead to the transformation of contaminants into often unknown chemicals – called transformation products (TPs). Therefore, their formation and related health risks must be assessed. Indeed, we live in a world of chemical compounds interacting to create our reality. Some of them may constitute a risk to human health, other living organisms, and ecosystems. Environmental toxicology aims to predict which chemicals may threaten living organisms and natural resources. The preliminary step in evaluating the health risks is the hazard assessment, namely the consideration of the intrinsic capacity of the newly formed TPs to cause harm to living organisms. Finding a solution starts with understanding the interconnected relations between chemicals and biological systems. The human health risks also depend on human exposure over time, which was not considered in the current research project.

Nowadays, the approach towards the toxicological assessment of chemicals is changing rapidly. This change is mainly guided by sustainable goals towards the replacement, reduction, and refinement of animal tests (*in vivo*), which have traditionally been used to assess the safety of chemicals (RIVM, 2022). The development of New Approach Methodologies (NAMs) for risk assessment – which includes microorganisms or cell tests (*in vitro*) and predictive toxicology (*in silico*) – is a necessary step for the transition towards animal-free testing. *In silico* tools can accelerate the risk assessment of chemicals and meet the goals of European policies (Kavlock et al., 2018). Therefore, the current research focused on selecting and applying *in silico* tools for evaluating TPs formed during drinking water treatments.

Abstract

Environmental contaminants are present in water sources, so drinking water treatments are applied. However, these contaminants can be transformed into new chemicals – called transformation products (TPs) – often unknown and undetected by analytical techniques (Gogoi et al., 2018; Brunner et al., 2019; Zahn et al., 2019; Menger et al., 2021). Therefore, predicting TPs' formation during drinking water treatments must be addressed (Kiefer et al., 2019). At the same time, predictive toxicology can help identify TPs of great toxicological concern and steer further analysis. However, there is an urge to assemble available methods to design and implement the next-generation risk assessment (NGRA) in regulatory frameworks (Pallocca et al., 2022). Therefore, this research focused on developing a rational scheme for predicting TPs formation because of drinking water treatments, their physicochemical characteristics (PCC), and toxicological hazards. The effectiveness of freely available *in silico* tools in predicting, prioritizing, and evaluating TPs was discussed here. S-metolachlor TPs were used as proof of the applicability of the methodology. The reliability of the methods varies depending on the specific reaction pathway, PCC, or endpoint considered. The Chemical Transformation Simulator (CTS) and enviPath were demonstrated to be the best available combination for predicting TPs originating from drinking water treatments. EpiSuite^{TM} was recommended for the PCC evaluation, and VEGA QSAR for the hazard prioritization. Whether the predicted prioritized S-metolachlor TPs could represent a human health risk via drinking water or an environmental concern for their impact on ecosystems requires further research, as well as the development of an automation workflow for the use of the applied *in silico* tools, is required.

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INTRODUCTION

Natural or anthropogenic chemicals can contaminate water. Examples of these are pharmaceuticals, personal care compounds, and pesticides, typically found in water sources at low concentrations - thus, called micropollutants. Because of contaminants in the water, drinking water companies apply water treatments to eliminate chemical contamination and ensure the European water quality standard[1] of 0.1 µg/L (WHO, 2022).

Developing and implementing adequate drinking water treatments to respond to the increasing environmental pollution (Dos Santos et al., 2022) is crucial to ensure safe drinking water for consumers. Emerging contaminants (ECs) pose growing challenges to water purification (Tang et al., 2019); therefore, water companies constantly adapt and implement drinking water treatment processes according to contaminants' presence in the water sources. Indeed, the pollution level of water sources determines the specific demand for purification, and consequently, different sequences and conditions of drinking water treatment processes are applied by water companies. The most common water treatment processes include the use of ultraviolet (UV) radiation, chlorine, ozone, or other disinfectants, flocculation, filtration, and advanced oxidation (AOPs) (WHO, 2022).

The abovementioned drinking water treatments are of "unquestionable importance in the supply of safe drinking water" (WHO, 2022, p. 5). However, increasing scientific evidence demonstrates the transformation of contaminants in the water into new and often unknown transformation products (TPs)[2] (Brunner et al., 2019; Yang et al., 2022). A wide range of organic contaminants can undergo transformation reactions during drinking water treatments since drinking water treatment processes employ disinfectants or biological metabolism (WHO, 2022). The lack of information about TPs of environmental contaminants, as reviewed by Gogoi et al. (2018), reveals the concern they cause for water quality. The transformation processes contaminants encounter might indirectly increase their impact on drinking water quality, especially considering that they are often structurally and toxicologically unknown compounds (Brunner et al., 2019; Menger et al., 2021). Even when these have been identified in the water, they remain toxicological data-poor chemicals for which further research is necessary (Gogoi et al., 2018, Zahn et al., 2019).

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[1] The World Health Organization (WHO) periodically updates the Guideline for Drinking Water Quality, making standard levels of contaminants available for human consumption (WHO, 2022).

[2] When the precursor of the newly formed substances is the natural organic matter in the water, the newly formed chemicals are referred to as disinfection by-products (DBPs). However, this research focused on anthropogenic precursors, which products are generally called TPs (Yang et al., 2022).

Among anthropogenic contaminants in the environment, critical are the active substances – the ingredients that interact with biological systems and therefore exert the desired pharmacological or toxicological effect (EU Commission, 2022). For their inherent biological activity, active substances appear as suitable micropollutants candidates for the formation of TPs (Bura et al., 2019). Between the various active substances found in the environment, pesticides are relevant because of their diffused application, which is expected to contaminate surface water and groundwater (Syafrudin et al., 2021). In Europe, 448 active substances are authorized as pesticides – which include plant protection products used in agriculture and biocides found in numerous applications – and 258 are approved in the Netherlands (EU Commission, 2022).

The formation of TPs from pesticides can derive from reaction processes occurring in the environment (Pico' et al., 2015; Cormier et al., 2015) or during drinking water treatment processes (Petrie et al., 2015; Tang et al., 2019; Brunner et al., 2019). Therefore, there is the urge to prioritize research on pesticides TPs, as the quantity of pesticide products released into the environment has constantly been increasing over the years (Sharma et al., 2019) and the reactions possibly involved in TPs formation are diverse (Kotthoff et al., 2019; Suman et al., 2022).

The EU regulations that apply to pesticides in the European context are Regulation (EC) n° 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and Regulation (EU) n° 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products, which state that active substances "shall have no immediate or delayed harmful effect [...] directly or through drinking water" (EC Regulation n° 1107/2009 article 4, 3b; EU n° 528/2012, article 19, 1b iii). Moreover, the EU Directive n° 2020/2184 of the European Parliament and of the Council of 16 December 2020 on the quality of water intended for human consumption specifies that it must be considered whether "transformation products generate a health risk for consumers" (EU Directive n° 2020/2184, Annex I, part B, p.37). Therefore, active substance degradation and TPs should be investigated according to the EU regulatory framework for active substances of pesticides (Bura et al., 2019) to evaluate the indirect health effects they could generate, because – as pointed out by Ji et al. (2020) – TPs could be the blind spot of pesticides risk assessment.

Knowing the chemical structure of newly formed TPs aids their detection in the water by targeted screening, which uses reference standards to identify and quantify specific structures (Hinnenkamp et al., 2021). However, most newly formed TPs are unknown chemical structures, and unknown compounds are not revealed by targeted analysis. Therefore, the only possible alternative is non-targeted screening (NTS), which can detect known and unknown chemicals in water matrices. Innovative NTS approaches allow the identification of previously unknown TPs (Brunner et al., 2020; Lai et al., 2021). Lowering the detection limits of analytical methods allows the detection of an increasing number of micropollutants (Muter & Bartkevics, 2020).

A technique to detect TPs in the water is liquid chromatography (LC) combined with high-resolution tandem mass spectrometry (HRMS/MS) (Schollée et al., 2017; Lai et al., 2021). The first applied chromatography technique is used to separate the compounds present in a sample, and the second, the mass spectrometry, analyses the mass of the separated compounds to identify them. These techniques can identify various pesticide TPs in water sources (Hollender et al., 2018; Kiefer et al., 2019) and water samples after drinking water treatment processes (Glassmeyer et al., 2017; Brunner et al., 2019; Tröger et al., 2021). Sometimes (Hladik et al., 2008*;* Kiefer et al., 2019; Rousis et al., 2022), the reported levels were estimated to be higher than the EU drinking water standards of 0.1 µg/L (WHO, 2022). However, this analysis's time and costs are higher than the targeted analysis, due to the detection of multiple signals (Hinnenkamp et al., 2021). Therefore*,* TPs are not currently regularly detected in drinking water quality analysis unless added in a risk-based monitoring framework and, moreover, they are not included in the risk assessment framework for approval of active substances (European Commission, 2022a).

The toxicological activity of the TPs must be assessed, together with the exposure to humans, to calculate the derived human health risks. Even though an increasing number of papers are being published regarding TPs of pesticides in Europe – as reviewed by Anagnostopoulou et al. (2022) – TPs formation must be further analysed since the related human health risks still remain uncertain (Hayes et al., 2006; Petrie et al., 2015; Skanes et al., 2021). On the one hand, the toxicological profile of an active substance put on the market is generally widely characterized following the EU legislation. On the other hand, limited information is available about their TPs (Worth et al., 2011; Bura et al., 2019). Therefore, TPs represent a global environmental and potential health concern to be addressed (Anagnostopoulou et al., 2022).

The risk assessment requires the hazard identification, namely the intrinsic capacity of molecules to cause harm. Traditionally, the hazards have been evaluated by exposing animals to chemicals and observing the consequent effect, translating the results to humans using safety factors to consider the difference between species (animals and humans) and the personal susceptibility of individuals (higher, for example, for children and elderly). However, what happens in animals is defined as a 'black box' and the reliability of the extrapolation animal-human is uncertain (Paparella et al., 2020). Alternative testing methods to animal testing for the hazard assessment of chemicals include *in silico* tools – software able to predict the effect of chemicals on biological systems based on available experimental information – and *in vitro* tools – bioassays performed on biological systems (cells, tissues, or other biological components), possibly target of the chemical of interest.

The latter New Approach Methodologies (NAMs) can clarify how toxicants act towards living organisms. For instance, for metolachlor, *in vivo* research demonstrated changes in the reproductive endocrinology of male rats without pointing out the mechanisms of action (MoA) involved (Mathias et al., 2012). However, an *in vitro* study revealed the activity of metolachlor towards the mRNA expression of human aromatase, the enzyme responsible for the biosynthesis of estrogen, clarifying a possible mechanism of action (Laville et al., 2006).

Predictive toxicology is time and cost-effective, but is strictly linked to the availability, quality, and concordance between experimental data. The understanding of the mechanisms of action for specific toxicological endpoints influences the predictivity of the models. Nevertheless, it can help identify and prioritize compounds of toxicological concern, direct further analysis, and speed up the evaluation of contaminants in the environment. Indeed, *in silico* methods have been increasingly used under regulations EC n° 1107/2009 and EU n° 528/2012 concerning plant protection and biocides (Berggren et al., 2017; Khan et al., 2020; Klutzny et al., 2022; Anagnostopoulou et al., 2022). However, *in silico* tools still need to be assembled in a rational scheme to guarantee their application in risk assessment frameworks (Pallocca et al., 2022).

In addition to the hazard assessment, the exposure to humans and other living organisms must be evaluated to characterize potential health risks. However, this research focused on the preliminary steps for the risk assessment of TPs: their prediction and hazard identification. Therefore, further research was warranted.

The presented research proposes an efficient predictive framework for prioritizing TPs of active substances originating during drinking water treatments. The drinking water treatment processes involving bio metabolism or disinfectants - such as rapid sand filtration, UV treatments, ozonation, and chlorination - were considered. Literature data mining and *in silico* tools were combined to assess TPs formation because these treatments and predictive toxicology was also used to define their potential related hazards.

The scheme was applied to S-metolachlor as a proof of principle for applying *in silico* methods to prioritize TPs research in drinking water. While the racemic mixture metolachlor is no longer authorized in Europe as an active substance, the use of S-metolachlor is still approved in agriculture (European Commission, 2022e), and S-metolachlor has been widely used in Europe (O'Connell et al., 1998; Jursík & Holec, 2019). The approval was extended even though the S-enantiomer is the active portion of the racemic mixture (Shaner et al., 2006) and research suggested that metolachlor and S-metolachlor have similar toxicological profiles (EFSA, 2012). S-metolachlor was first characterized to confirm that it was of high toxicological concern and likely to be found in water sources. S-metolachlor TPs were then predicted and prioritized based on the likelihood of being created, structural reasoning, and hazard structural alert identification. Ultimately, those were tentatively identified by non-target HPLC-HRMS screening. The present research demonstrated that the formation of TPs from S-metolachlor during drinking water treatment might happen and be relevant from a toxicological point of view.

METHODS

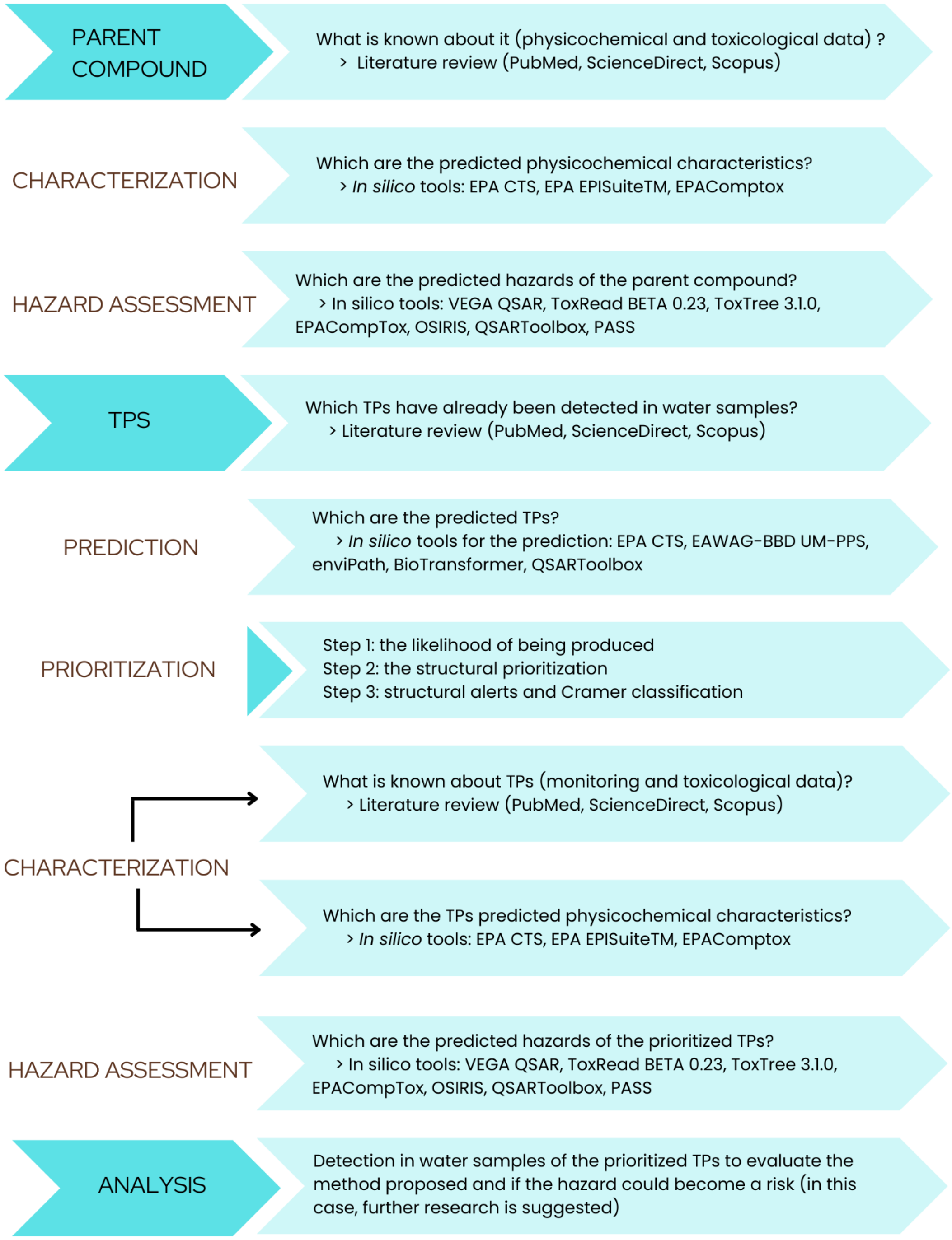
The present research proposed a rational scheme for predicting TPs and their related hazards due to the reaction of active substances of pesticides during drinking water treatment processes, based on a combination of literature research and *in silico* approaches (Figure 1).

A preliminary characterization of the parent compound was done here to confirm that the selected active substance – S-metolachlor – was possibly present in water samples and represented relevant toxicological concerns (see paragraph 1.3). Firstly, S-metolachlor was characterized for water solubility and partitioning coefficients between environmental compartments, indicative of its presence in the water, and, secondly, for five toxicological endpoints. Both steps combined literature information and *in silico* tools (see chapter 1).

Thirdly, the TPs possibly formed from S-metolachlor were collected using a systematic literature review and predictive tools. A comparison between the collected literature information (i.e., monitoring data, databases) and the predictions' results was also discussed (see chapter 2).

The predicted TPs were then prioritized based on their previous identification in water samples and their toxicological characteristics. The reasons for prioritizing specific TPs over others were reported here (see chapter 3). *In silico* tools were additionally used to assess the hazard of prioritized TPs (see chapter 4).

Lastly, the prioritized TPs were tentatively identified using non-target HPLC-HRMS screening data made available by Brunner et al. (2019) (see chapter 5). The relevance, preference, and limitations of the applied scheme were reported in the Discussion section (see chapter 3).



PARENT COMPOUND

What is known about it (physicochemical and toxicological data) ?
› Literature review (PubMed, ScienceDirect, Scopus)

CHARACTERIZATION

Which are the predicted physicochemical characteristics?
› *In silico* tools: EPA CTS, EPA EPISuiteTM, EPAComptox

HAZARD ASSESSMENT

Which are the predicted hazards of the parent compound?
› In silico tools: VEGA QSAR, ToxRead BETA 0.23, ToxTree 3.1.0, EPACompTox, OSIRIS, QSARToolbox, PASS

TPS

Which TPs have already been detected in water samples?
› Literature review (PubMed, ScienceDirect, Scopus)

PREDICTION

Which are the predicted TPs?
› *In silico* tools for the prediction: EPA CTS, EAWAG-BBD UM-PPS, enviPath, BioTransformer, QSARToolbox

PRIORITIZATION

Step 1: the likelihood of being produced
Step 2: the structural prioritization
Step 3: structural alerts and Cramer classification

CHARACTERIZATION

What is known about TPs (monitoring and toxicological data)?
› Literature review (PubMed, ScienceDirect, Scopus)

Which are the TPs predicted physicochemical characteristics?
› *In silico* tools: EPA CTS, EPA EPISuiteTM, EPAComptox

HAZARD ASSESSMENT

Which are the predicted hazards of the prioritized TPs?
› In silico tools: VEGA QSAR, ToxRead BETA 0.23, ToxTree 3.1.0, EPACompTox, OSIRIS, QSARToolbox, PASS

ANALYSIS

Detection in water samples of the prioritized TPs to evaluate the method proposed and if the hazard could become a risk (in this case, further research is suggested)

Figure 1. Framework to evaluate transformation products (TPs) formation derived from active substances during drinking water treatments combining literature data and prediction tools.

1 Characterization of the parent compound

This research used systematic literature reviews and data mining approaches to collect relevant monitoring and toxicological data. Systematic literature reviews were performed by adopting the standard process defined by Egger et al. (2022). It consists of an *a priori* definition of inclusion and exclusion criteria, the location of studies, the extraction of the data, and the assessment of their quality fixed on pre-defined schemes. The list of actions to perform a literature review is listed here:

1. Formulate the review question.

2. Define inclusion and exclusion criteria to ensure that biases are excluded, and comparable data are collected.

3. Locate studies using research engines, specifically PubMed, Science Direct, and Scopus.

4. Select studies based on the defined inclusion criteria; a reason is given for excluding studies.

5. Extract data using a pre-defined form to make the information comparable.

6. Analyze and present results using a pre-defined method to synthesize the information.

Physicochemical characterization

The environmental availability of S-metolachlor in water sources entering drinking water treatment plans depends on the physicochemical characteristics (PCC), such as hydrophobicity/phylicity, lipophilicity and water solubility. It is crucial to determine whether S-metolachlor is soluble in water to understand whether it is likely found in water sources. Hydrophobicity was characterized via water solubility, the octanol-water partition coefficient (Kow), and the organic carbon-water partition coefficient (Koc).

Water solubility, generally expressed in mg/L or ppm, represents the likelihood of a chemical dissolving in water. A low solubility is associated with water solubility valued below 10 mg/L or 10 ppm, a high solubility is represented by water solubility values higher than 1000 mg/L or 1000 ppm, while values in between are considered a moderate solubility (NPIC, 2022).

The octanol-water partition coefficient (Kow) is a parameter to define the lipophilicity and hydrophilicity of a chemical. If higher than 1, the chemical tends to stay more in the lipophilic phase, while for values lower than 1, the affinity with the aquatic phase is higher (Speight, 2016).

The organic carbon-water partition coefficient (Koc) defines the likelihood of finding a chemical in the organic phase rather than the water phase. For values higher than 1, the chemical is more likely to be adsorbed onto the organic phase (the soil or suspended in the water) instead of being found in the water. Therefore, the Kow can predict the migration of hydrophobic organic compounds dissolved in soil and groundwater (Speight, 2016).

1.1 *In silico* tools for the prediction of physicochemical properties

Three freely available *in silico* tools were selected to predict the PCC of S-metolachlor. These tools were used to assess the reliability of the predictive tools when available measured data in the literature. The reliability of the predicted tools is discussed in the Discussion section (see paragraph 2.1).

Chemical Transformation Simulator (CTS)

The Chemical Transformation Simulator version 1.2 is a web-based tool that predicts the transformation pathways of organic chemicals using reaction libraries. The PCC Module in CTS was used to predict water solubility as the main PCC influencing the persistence of a chemical in the water (Covaci, 2014). Further information is available in the supplementary documents (see Annex 2).

US EPA EPI (Estimation Programs Interface) Suite™

EpiSuite ™ is a Windows®-based tool that collects physical and chemical properties and estimations on the environmental fate of chemicals. The different models can be run simultaneously or be specifically selected. EpiSuite has models for partition coefficients in different environmental departments. The different models can be run simultaneously or precisely selected. Further information on the models and other models available in the software is reported in the supplementary documents (see Annex 2).

The models adopted to predict the partition coefficients relevant to water solubility were:

- KOWWIN™ (version 1.68) estimates the log octanol-water partitioning coefficient (Log Kow or Log pOW), which measures its lipophilicity/hydrophilicity. If the ratio is >1, the compound will likely stay in the lipophilic phase. If it is <1, the chemical prefers water.
- KOCWIN™: the program estimates the organic carbon-normalized sorption coefficient for soil and sediment KOC = Kd*100 / % organic carbon. If the ratio is >1, the compound tends to be adsorbed to the solid phase. If it is <1, the chemical is more likely to be found in the water.
- LEV3EPI™ was used to predict the partitioning of chemicals among air, soil, sediment, and water under steady-state conditions for a default environmental model.

The models adopted to predict water solubility were:

- WSKOWWIN™, which predicts water solubility applying corrections when needed.
- WATERNT™ (version 1.1) estimates water solubility directly using a “fragment constant” method.

The models used to predict degradation processes were:

- BIOWIN™ which predicts the aerobic and anaerobic biodegradability of organic chemicals using seven different models. The model was evaluated to predict rapid sand filtration/biodegradation during water disinfection treatments.
- STPWIN™, which predicts the removal of a chemical in an activated sludge-based sewage treatment plant.

US EPA CompTox

The CompTox Chemicals Dashboard (epa.gov) is a freely available tool to predict water solubility. Moreover, the tool was adopted for the hazard assessment of the parent compounds and their TPs (see paragraph 1.3.2). Further information is available in the supplementary documents (see Annex 2).

1.2 *In silico* hazard assessment

This research is focused on developing a work frame for using *in silico* approaches to prioritize TPs of toxicological concern. When applying predictive toxicology, it is always preferable to use at least two models (ECHA, 2008; 2016; 2017). Marzo et al. (2016) suggested integrating multiple models to reduce the uncertainty of *in silico* predictions. The combination of different tools has been demonstrated to increase the reliability of the predictions (Rallo et al., 2005; Basant et al., 2016). Furthermore, it is recommended that at least an expert rule-based and statistical-based model (QSAR) and a *read-across* approach are applied in parallel to enhance the reliability of the prediction.

Various freely available *in silico* models were used to allow the validation of the results and make comparisons between different schemes. This research did not consider commercial tools to guarantee more extensive access to the proposed methodology. Moreover, the prediction must be justified by reasonings and mechanistic interpretations by expert judgment to increase the reliability of the results.

The *read-across* and the Quantitative Structure-Activity Relationship (QSAR) are the main computer-based methods used in predictive toxicology. Both were considered in this research to evaluate the hazard related to TPs. Further information on the *in silico* hazard assessment is available in the supplementary documents (see Annex 3).

1.2.1 Considered endpoints

A literature review was used to define relevant endpoints for drinking water quality assessment and identify existing *in silico* models capable of providing reliable predictions for the evaluated endpoints. The endpoints were selected based on the relevance of drinking water exposure. For instance, neurotoxicity was not included as the contaminants in the water – and therefore their TPs – are usually found at low-level concentrations. The blood-brain barrier is a highly selective membrane. The open-source availability of *in silico* tools for their assessment was considered a strength for selecting the endpoints analysis of this research.

Five relevant endpoints for drinking water quality are discussed here, and further endpoint-specific information is available in the supplementary documents (see Annex 3).

Genotoxicity

Genotoxicity is the ability of a chemical to damage the genetic information in cells, which can lead to a series of health consequences, including tumor initiation (More et al., 2019). Therefore, genotoxicity is a crucial endpoint in water quality assessment, considering the severe effects that would be caused on human health after chronic exposure to low concentrations of genotoxic chemicals. The information on predictive toxicology for the genotoxicity endpoint available in the literature is vast, and the reliability of QSARs models has already been proven in several studies (Benigni & Bossa, 2019). As the reliability of *in silico* approaches relies on the availability of literature data used to build the model, the more the related mechanisms of action are understood, the more solid the prediction for the specific endpoint.

Direct genotoxicity involves mutagenic effects that result in a permanent transmissible variation in the structure of genetic material. As regards the mutagenicity endpoint, many models have been created to predict the results of the Ames Mutagenicity test. A vast amount of *in vitro* data was available to build the models. Moreover, the standardized testing methodologies applied for genotoxicity have provided a homogenous and reliable understanding of the chemicals' characteristics of genotoxicity, allowing the creation of reliable algorithms to predict the genotoxicity potential of chemicals (Benigni & Bossa, 2019) (see Discussion section, paragraph 2.2). A discussion on the reliability of QSAR models for genotoxicity is available in the supplementary documents (see Annex 4).

Carcinogenicity

The cancerogenic health effect is related to two mechanisms of action (MoA) direct genotoxicity, when the chemical interacts directly, linking the DNA and altering its structure. Indirect genotoxicity, also named non-genotoxic carcinogenicity, comprehends mechanisms such as stimulating cell proliferation or inhibiting the physiological mechanism of apoptosis (EPA, 2005). Apart from the health relevance of this endpoint, it is crucial for the hazard assessment of TPs in drinking water because the latter involves chronic exposure, with which the processes mentioned above of carcinogenicity are related. Different software is available to predict the carcinogenetic effect of chemicals. Usually, endpoints are evaluated as binary output (active or non-active). However, for carcinogenicity, it is possible to assess the potency of the toxic activity, thus expressing it as a dose, using the well-known dataset built available from the Carcinogenic Potency Database (National Library of Medicine, 2022). However, the purpose of this work was qualitative and not quantitative. Therefore, only a binary output was investigated.

Reproductive and developmental toxicology

The endpoint of reproductive toxicology includes developmental toxicology, and these aspects are usually overall evaluated in developmental and reproductive toxicology (DART) studies aimed at assessing the reproductive performance of animals and the consequences on the development of the offspring after repeated or chronic exposure. However, they are different endpoints, but in this research were considered together. On one side, reproductive toxicology entails any adverse effect on the fertility of the exposed generation and the development of the progeny. On the other side, developmental toxicity, also referred to as teratogenicity, is a different endpoint than reproductive toxicology, even if one can lead to the other (Faqi et al., 2013).

While for genotoxicity, the mechanisms of action are well known (EPA, 2005), reproductive toxicology is more composite, as demonstrated by the number of endpoints considered in the multigeneration studies: more than 100 as regards varying life stages and generations are usually integrated (Martin et al., 2011). That makes reproductivity toxicology a complex endpoint (Jensen et al., 2008) and developmental toxicology (Cassano et al., 2010). Once again, computational toxicology is a helpful tool (Martin et al., 2011). The US EPA Toxicity Reference Database (ToxRefDB) is an available online database of high-quality mammal toxicity data that covers effects such as reproductive performances and measures, male and female reproductive tract effects, and sexual development landmarks, and it is a valuable resource for retrospective analysis for the development of predictive models. It includes data obtained by *in vivo* experiments on environmental chemicals, including pesticides, regarding acute, chronic, reproductive, and developmental toxicity (Martin & Judson, 2010). A discussion on predictive toxicology for the evaluation of reproductive toxicology is reported in the supplementary documents (see Annex 4).

Endocrine disruption

The endocrine system is a complex of glands, hormones, and receptors that influence a wide variety of essential mechanisms, such as the differentiation, growth, and function of reproductive organs, body development, energy production, and the levels of sugar in the circulatory system (Zou, 2020). Alterations of the endocrine system could lead to a series of effects on different physiological mechanisms in humans, including those involved in reproduction and development. Every chemical that interferes with the endocrine system is defined as an endocrine disruptor (ED). It is generally agreed that it is a relevant endpoint for the variety and complexity of the mechanisms (Zou, 2020). For predictive toxicology, the more the endpoint is defined, the more predictions could be reliable. Endocrine system alterations could modify the reproductive system and other health effects. Jensen et al. (2008) investigated the development of models to predict ED effects based on *in vitro* tests. Different QSAR tools have been developed to evaluate this endpoint, such as QSAR Toolbox, VEGA HUB, CAESAR project, or DeepTox.

Skin sensitization/irritation

Skin sensitization is the capacity of a molecule to exert an allergic reaction in susceptible individuals (European Commission, 2022b), while skin irritation is the reaction due to topical exposure to a chemical which can lead to skin corrosion, an irreversible health effect (European Commission, 2022c). Skin sensitization and irritation are endpoints widely assessed *in silico* because of the relevance of cosmetic products for which market trading was forbidden in Europe in 2011. Therefore, alternative methods become relevant and characterized (Kleinstreuer et al., 2018).

1.2.2 *In silico* tools for the hazard assessment

Freely available *in silico* tools for endpoints relevant to drinking water quality were used to perform the hazard assessment of S-metolachlor and its predicted TPs (Table 1). Only the freely available *in silico* tools were considered to guarantee extended application in academic research. All models accept the SMILES (simplified molecular-input line-entry system) as input. SMILES are unique and, therefore, the preferred input because chemicals can have multiple names and CAS numbers. However, the offered reliability of the models for the assessment was not equivalent for the different relevant endpoints considered, and the relative reliability was discussed in the Discussion section (see paragraph 2.2).

Table 1. Freely available in silico tools for the hazard assessment of genotoxicity, carcinogenicity, reproductive/developmental toxicology, endocrine disruption, and skin sensitization (see paragraph 1.2.1).

Software	GENOTOXICITY	CHROMOSOMAL ABERRATION	CARCINOGENICITY	REPRODUCTIVE/ DEVELOPMENTAL TOXICOLOGY	ENDOCRINE DISRUPTION	SKIN SENSITIZATION
VEGA	CONSENSUS v1.0.3	CORAL v1.0.0	CEASAR model	CAESAR model	NRMEA Thyroid Receptor Alpha effect v1.0.0	CAESAR model v2.1.6
	CEASAR v2.1.13	IRFMN *In vitro* micronucleus v1.0.0	CEASAR v2.1.9	CEASAR v2.1.7	NRMEA Thyroid Receptor Beta effect v1.0.0	IRFMN/JRC v1.0.0
	SarPy/IRFMN v1.0.7	IRFMN *In vivo* micronucleus v1.0.1	ISS v1.0.2	Developmental/Reproductive Tox library v.1.1.0	IRFMN Aromatase activity v1.0.0	
	ISS v1.0.2		IRFMN/Antares v1.0.0	IRFMN/CORAL Zebrafish embryo AC50 v1.0.0	IRFMN Estrogen Receptor Relative Binding Affinity v1.0.1	
	KNN/*Read-Across* v1.0.0		IRFMN/ISSCAN-CGX v1.0.0		IRFMN/CERAPP Estrogen Receptor v1.0.0	
			IRFMN carcinogenicity oral v1.0.0		IRFMN/COMPARA Androgen Receptor v1.0.0	
			IRFMN carcinogenicity inhalation v1.0.0			
ToxRead	*read-across* assessment					
	QSAR consensus					
ToxTree	Ames alerts		Cramer rules			
	Benigni/Bossa rulebase		Benigni/Bossa rulebase			
CompTox	Consensus Ames mutagenicity			Developmental toxicity	Estrogen Receptor binding	
OSIRIS PROPERTY EXPLORER	Mutagenic alerts		Tumorigenic alerts	Reproductive effects alert		Irritant alerts
QSARToolbox	Mutagenicity profiling	Chromosomal aberration profiling	Carcinogenicity profiling	Developmental tox profiling	OECD estrogen binding profiling	OECD protein binding profiling

VEGA HUB

VEGA HUB is a project conducted by The Mario Negri Institute for Pharmacological Research, Italy, and offers a variety of models to predict the hazard of chemicals. The VEGA, ToxRead, ToxWeight, ToxDelta, and JANUS models are included in VEGA HUB. This research used VEGA QSAR and ToxRead to assess the endpoints of genotoxicity, carcinogenicity, developmental and reproductive toxicity, and endocrine disruptors.

VEGA QSAR

VEGA QSAR provides predictions on the activity for specific endpoints for a target molecule, relying on QSARs. Version 1.1.5 was initially used during the preliminary research, and the new version 1.2.0 was adopted in June 2022. For genotoxicity, eight models were available, based on different algorithms, and developed to assess different mechanisms of genotoxicity. Five models predict the mutagenicity in Salmonella Typhimurium (Ames test), but there are also models available for predicting chromosomal aberration mechanism and micronucleus test results. For skin sensitization, three models were available. Regarding complex endpoints, eight models were available for carcinogenicity based on different databases. In contrast, three models were used for reproductive and developmental toxicology, based either on the Developmental/Reproductive Tox library or the prediction of the AC50 in Zebrafish.[3]. Six models were selected on specific mechanisms of action to evaluate endocrine disruption. The VEGA models for endocrine disruption are based on hormone receptor bunding or aromatase activity. The aromatase, also called estrogen synthetase, is a key enzyme (P450)[4] in the formation of estrogens from androgens through aromatization, and alteration in its activity can lead to hormone imbalances that may result in sexual and skeletal development (Zorn et al., 2020). As S-metolachlor showed in vitro activity towards the aromatase activity (Laville et al., 2006), the endpoint was relevant to this research.

The prediction output is reported along with the reliability of the prediction, the reasoning relative to the six molecules selected in the training set, the structural alerts detected, and, eventually, critical aspects. An in-built algorithm assesses the model's applicability for the chemical, and expert reasoning was used to determine the uncertainty level associated with the final prediction. The model provides the six chemicals more similar to the target chemicals included in the training set. The inclusion in the applicability domain depends on the recognition of structural features or molecular descriptors. The applicability Domain Index (ADI) has values from 0 (worst case) to 1 (best case) (Benfanti et al., 2013). A detailed explanation of the software model and the scores of reliabilities provided by an independent algorithm included in the model is available in the supplementary documents (see Annex 5).

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[3] AC50 is a toxicological threshold representing the concentration at which 50% of the maximum activity towards a specific endpoint is observed.

[4] The enzymes of the P450 family are proteins responsible for the synthesis and metabolism of internal and external cell components

Here are reported Applicability Domains scores which are the internal statistical validation of the model. All range from 0 to 1 and analyze different aspects of the prediction:

- Applicability Domains scores, which are the internal statistical validation of the model. All range from 0 to 1 and analyze different aspects of the prediction.
- Similarity index = how much the training set molecules resemble the chemical.
- Accuracy index = how much the experimental values of the training set molecules agree with their predicted value by the model.
- Concordance Index = how much the experimental values of the training set molecules agree with the predicted value for the molecule in analysis.
- Atom Centered Fragment (ACF) = how many atom-centered fragments have been found in the molecules of the training set.
- Global Applicability Domain Index (ADI), the overall score calculated from the other parameters.

The prediction was considered positive if there were an indication of activity towards the selected endpoint and negative if inactivity was indicated. The results were considered inconsistent if suspected to be not included in the model's applicability domain (AD). In other words, if the most similar compound has not had enough overlapping characteristics compared to the target (similarity below 0.5) or the experimental values of the two most similar chemicals disagree with the predicted output value. If experimental values were identified, the ADI equals 1.

During the proposed *in silico* hazard assessment, a consensus score higher than 0.5 was considered. For the single models, if the ADI was inferior to 0.75, the prediction was considered inconsistent as the compound analyzed fell outside the AD of the model, which means the prediction could not be reliable for that compound. The reliability of predictions was independently assessed for each model. In some cases, some parameters were considered more relevant to justify the prediction. For instance, the similarity index was considered more relevant than the ACF index because the atom-centered fragment can be found in molecules notably different from the target compound. In contrast, the similarity index better represents the target's overall similarity with the molecules selected in the training set. Similarly, the concordance index was seen to be more relevant than other parameters for the reliability assessment of the prediction because a high number of contrasting experimental data with the model's prediction invalidated the prediction.

The applicability domain assessment can improve the interpretation and reliability of the predictions (Marzo et al., 2016; Benfanti et al., 2013). However, expert judgment reasoning was applied to justify the selected threshold of 0.75 and assess each model's reliability, followed by an overall evaluation collecting all the results on a specific endpoint.

ToxRead

ToxRead 0.23 Beta is a Java application that allowed the execution of reproducible *read-across* evaluations for the mutagenicity endpoint. It revolves around structural alert detection in the target molecule and the location of those on structurally similar molecules present in its database, for which experimental data are available. It provides the most similar compounds and the grade of similarity detected for each structural alert. The tool provides both a *read-across* assessment and a QSAR consensus assessment based on CAESAR, ISS, SarPy, and KNN mutagenicity models. Further information is available in the supplementary documents (see Annex 5).

ToxTree

ToxTree version 3.1.0 is freely available and predicts the hazards of compounds using a decision tree approach, meaning applying a series of rules to associate it with the result. This research used software for Cramer class classification[5], mainly to prioritize predicted TPs (see chapter 3). The tool includes 18 plugins, for which more information is available in the supplementary documents (see Annex 5).

CompTox

The CompTox tool was previously mentioned as a prediction tool for the PCC (see chapter 1.2.1). For toxicological endpoints, the model was adopted to evaluate developmental toxicity, Ames mutagenicity, and estrogen receptor binding (endocrine disruption).

OSIRIS PROPERTY EXPLORER

The Osiris Property Explorer tool is a JAVA app freely available software that evaluates mutagenicity, tumorigenicity, irritant, and reproductive effects. The results are not downloadable and therefore not sharable.

OECD QSAR Toolbox

The OECD QSARToolbox tool is a freely available application that supports reproducible hazard assessment for chemicals. It allows the profile of a target and obtains experimental data from its database concerning the target analogs, guaranteeing a *read-across* and trend analysis approach to fill data gaps. This research used the OECD QSAR Toolbox version 4.4 for the preliminary assessment of the tool, while later version 4.5, released in March 2022, was used. The software was used to predict genotoxicity, carcinogenicity, and developmental toxicology. Only the profiling step was considered; in total, 21 models were selected for profiling the chemicals analyzed in this research. A detailed list is present in the supplementary documents (see Annex 5).

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[5] The Cramer classification is used to estimate the TTC for a chemical based on its structure, which guarantees a qualitative assessment of the related hazard of chemicals. The Threshold of Toxicological Concern (TTC) values for Cramer Classes I, II and III are 30 µg/kg bw per day, 9 µg/kg bw per day and 1.5 µg/kg bw per day, respectively. For substances with exposures below the TTC values, the probability that they would cause adverse health effects is low (EFSA, 2019).

2 Prediction of transformation products

Predicting which TPs can be formed during drinking water treatments is challenging because of the various reactions occurring during different treatment processes – and before, in the environment. However, literature research on detected compounds in drinking water could provide indications of TPs formed during water disinfection treatments. Furthermore, the simulation provided by computerized methods was considered in parallel to existing monitoring data to understand if there is any correspondence between predicted TPs and compounds found in the water.

A list of suspect TPs possibly formed from the parent compound due to drinking water treatments were provided after comparing the different results. Moreover, three steps of prioritization were applied to select the TPs of more serious concern for prioritizing further research on the hazard assessment of predicted TPs. The combination of predictive toxicology and literature data was used to create a prioritized list of the possible TPs based on the likelihood of production and the documented detection in the water.

2.1 Collection of detected transformation products

Concerning the case study S-metolachlor, to answer the question 'Which are the identified TPs of metolachlor, and which are the reactions involved?' systematic literature research has been performed using the research engines PubMed, Science Direct, and Scopus. The research terms were: (transformation products) OR (DBP) AND (drinking water treatments) OR (water) AND (metolachlor). That means TPs formed during drinking water treatments and in the environment are possibly found. This information is collected to analyze the type of reactions involved and evaluate if the reported transformation reaction could be representative of processes occurring during water treatments. The research has been restricted to the last ten years (2012-2022), as most of the papers available have been published in this period, as recent analytical development has consent to identify TPs better. The availability of the full text was a prerequisite of the research.

2.2 *In silico* prediction of transformation products

The present research was focused on the identification of freely available in silico tools for predicting transformation products possibly formed from the active substance of pesticides during drinking water treatments. Different tools were considered as various drinking water treatment processes entail biotic and abiotic reactions (WHO, 2022).

2.2.1 Considered reactions libraries

Relevant reactions occurring during drinking water treatment are hydrolysis, photolysis, oxidation, reductive transformation, elimination, and substitution (Brunner et al., 2019). Hydrolysis and photolysis occur during advanced oxidation processes and UV treatments, while reduction occurs during advanced reduction processes. Abiotic hydrolysis can occur during (advanced) oxidation processes and ozonation and chlorination (Bletsou et al., 2015). Abiotic photolysis is a typical reaction occurring during UV treatments and other (advanced) oxidation processes, ozonation, and chlorination. Conversely, the abiotic reduction is relevant for (advanced) reduction processes. Biodegradation can be representative of rapid sand filtration (RSF) (Di Marcantonio et al., 2020) and wastewater treatments (WWT) (Nolte et al., 2020).

Freely available *in silico* tools were found to predict abiotic hydrolysis, abiotic photolysis, abiotic reduction, and biodegradation. However, no tools were available for other reactions that might occur during drinking water treatment processes.

2.2.2 *In silico* tools for predicting transformation products

Free software provides models that predict reactions relevant to specific reactions of drinking water treatments (Table 2). On one side, abiotic reactions, such as hydrolysis or photolysis, can occur during water treatment processes such as advanced oxidation/reduction processes, UV treatments, ozonation, and chlorination. On the other side, biotic reactions can occur during – and are here used as a model for – RSF and WWT. The selected models, threfore, help assessing different reactions occurring during water treatment processes. However, some reactions were assessed by different models, which partially shared common databases; therefore, overlapping results were expected. A discussion of the compatibility of results offered by UM-PPS, enviPath, and BioTransformer, which are based on the same database (BBD-EAWAG), is presented in the Discussed section (see chapter 1).

Table 2. In silico tools to model specific reactions occurring during drinking water treatments.

Drinking water treatments → Software ↓	Advanced oxidation processes Ozonation Chlorination UV treatments	Advanced reduction processes	Rapid sand filtration (biodegradation)
US EPA CTS	Abiotic hydrolysis library Photolysis library	Abiotic reduction library	
UM-PPS			EAWAG-BBD
enviPath			EAWAG-BBD
BioTransformer			ENVIMICRO

Chemical Transformation Simulator (CTS)

The US EPA Chemical Transformation Simulator tools also offer the Reaction Pathway Simulator (RPS) Module that predicts TPs. The tool calculates the TPs possibly formed due to specific reactions and given a parent compound. It works using a series of libraries built on experimental data, and it recognizes reactive functional groups that are susceptible to be processed through, for example, reduction and hydrolysis. Different pathways are included or excluded for the specific chemical based on the available experimental data. For the included reaction schemes, a relative reaction rate (rank) is assigned, leading to a prediction of the percentage production of each TP. Thus, the tool already evaluates the likelihood of being produced. Once the TPs are predicted, it is possible to see the calculated PCC of the parent compound and its TPs.

The RPS allows for predicting different types of reactions possibly occurring during drinking water treatment processes. It allows the prediction of potential TPs based on specific reaction libraries that are pre-defined by the user. The relevant libraries for the scope of this study were: 1) Abiotic hydrolysis; 2) Abiotic photolysis; 3) the combined libraries (which may provide different results than the libraries only (see Discussion chapter 1). The tool works for organic chemicals, while the program cannot process organometallics, non-dissociating salts of organic chemicals, and polymers. Thus, this must be considered while analyzing an active substance included in these three chemical classes. Furthermore, this module allows predicting TPs and characterizing their PCC (see chapter 1.2.1).

EAWAG-BBD UM Pathway Prediction System (UM-PPS)

The EAWAG-BBD Pathway Prediction System (ethz.ch) includes rules derived from the Biocatalysis/Biodegradation Database (BBD), developed by the University of Minnesota and now maintained by the Swiss Federal Institute of Aquatic Science and Technology (EAWAG). It is a computational metabolic pathway predictor based on metabolic rules related to organic functional groups, which allow the prediction of the microbial metabolism for chemicals that have not been studied yet, based on biotransformation rules. The tool was used to evaluate the RSF and WWT, but it only provides a visual representation of the pathway and does not consent to download the results in .cvs format.

enviPath

The enviPath tool is a freely available database and prediction system that predicts the microbial transformation of organic chemicals, showing the experimental biotransformation pathways involved and the relative rule-based reasonings. Information about the enzyme-catalyzed reactions of environmental xenobiotics allows for predicting TP formation in the environment. It is adopted as a model to determine the formation of TPs and the biodegradation pathways occurring in rapid sand filtration. While the tool was adopted to assess biotic processes, it is not predictive of abiotic water treatments such as advanced oxidation/reduction processes, UV treatments, ozonation, and chlorination. Further information on the tool is available in the supplementary documents (see Annex 6).

BioTransformer

The BioTransformer tool is an open-access software tool for *in silico* metabolism prediction and metabolite identification. It was adopted as a model for biodegradation occurring in RSF and WWT. The BMPT tool was used in this research, and the library 'Environmental Microbial Transformation' is a model for biotransformation that can occur during rapid sand filtration (Brunner et al., 2019). It contains EAWAG rules, such as UM-PPS and enviPath. Further information on the tool is available in the supplementary documents (see Annex 6).

2.3 Identification of predicted transformation products

The research engines PubChem PubChem (nih.gov), RMG: Molecule Search RMG: Molecule Search (mit.edu), and ChemSpider ChemSpider | Search and share chemistry were used to associate the SMILES strings output of the prediction tools to a name (chemical, IUPAC, or commercial name). They were collected if the compound was found in these tools, and different names were reported. The CAS name and the information available in these chemical research engines were also collected if found.

3. Prioritization of predicted transformation products

The predicted TPs were prioritized based on three steps of prioritization. The combination of predictive toxicology and literature data was used to create a prioritized list of the possible TPs based on the likelihood of being produced or being found in the water – confirmed by literature data mining (step 1), the structural relevance compared with other similar structures present in the list (step 2), and any association with relevant toxicological concern (step3).

Step 1: the likelihood of being produced provided by the models

It is assessed by the tools and considered higher if different tools confirm the prediction. The likelihood of being produced provided by the tools was reported while collecting the TPs and was already considered. The prediction by different tools was used to prioritize the TPs. In this step, only the TPs predicted by at least two models or found in the literature research previously conducted were selected, thus, combining literature information with the *in silico* predictions.

Step 2: structural prioritization

Similar chemical structures were collected, and the prioritization was done using ToxRead BETA 0.23 to recognize the structural alerts for mutagenicity. The latter is one of the endpoints better predicted *in silico* because of the understanding of the specific interactions occurring with the DNA and the availability of standardized *in vivo* and *in vitro* experimental data (like in the Ames test). Structurally similar S-metolachlor TPs prioritized in step 1 were gathered. Using the predictions of ToxRead BETA 0.23, S-metolachlor TPs with the higher number of structural alerts and the higher *Read-Across* and QSAR assessment scores were prioritized over structurally similar ones.

Step 3: toxicity prioritization

The last step of prioritization was assessed using ToxTree v3.1.0 Cramer Class classification and QSARToolbox Cramer classification. Only the TPs predicted in the High concern Cramer class III by at least one model and found in the literature were prioritized. In practice, a priority point was assigned for each prediction of high toxicological concern (Cramer class III) and the detection of information in the literature. Only compounds associated with at least two priority points out of three were prioritized.

4. Characterization of prioritized transformation products

Water solubility was collected from the literature as well as predicted using *in silico* tools to fill data gaps for the prioritized TPs. Only water solubility was considered for S-metolachlor TPs – conversely to a more extensive characterization performed for S-metolachlor (see chapter 1.2) – as it is one of the essential properties affecting chemical substances' bioavailability and environmental fate (Covaci, 2014).

A systematic literature review was done to collect information on the prioritized predicted TPs as regards toxicological data or their detection in the water. A systematic literature review on S-metolachlor TPs was previously performed in this research (see paragraph 2.1), but this research was done specifically on the prioritized TPs to check for available toxicological information in the literature. The performance of a systematic literature review (defined by Egger et al., 2008) is based on an *a priori* definition of inclusion and exclusion criteria, the location of studies, the extraction of the data, and the assessment of their quality fixed on pre-defined schemes - see paragraph 2.1 for a detailed explanation.

The research engines used were PubMed, Science Direct, and Scopus. As research terms were used, the names (chemical name, IUPAC name, commercial name) were previously identified (see chapter 2.3) or the SMILES string if it was not identified any name.

Moreover, the hazards of the prioritized transformation products were assessed using the same models used for the hazard assessment of the parent compound (see paragraph 1.3.2) were applied for the prioritized TPs to assess whether the transformation likely leads to detoxification (the transformation into less critical TPs) or toxification (increment of the activity of the TPs in comparison to the parent compound).

5. Detection of prioritized transformation products in water samples

The prioritized TPs were tentatively identified using HPLC-HRMS data earlier collected by Brunner et al. (2019). The purpose of the data screening was to check whether the prioritized S-metolachlor TPs were identified in water samples, which were experimentally spiked with the racemic mixture metolachlor and treated with rapid sand filtration (RSF) and ozonation – as a model for biotic and abiotic drinking water treatments respectively - by Brunner et al. (2019). The researchers proposed an *in-house* suspect list based on literature mining for known TPs and metabolites, entered in NORMAN SusDat, STOFF-IDENT, and predicted using enviPath. A comparison with the suspect metolachlor TPs list documented by Brunner et al. (2019) was discussed here (see Results chapter 5).

Brunner et al. (2019) used metolachlor as a parent compound, while the present research considered only the enantiomer S-metolachlor as a parent compound. However, the results are expected to be comparable because HPLC cannot discern between isomers. Moreover, S-metolachlor is the active portion of the racemic mixture (Shaner, 2006), thus was expected to exert similar effects. Within the scope of this research, only the RSF experiment data were considered a model for the biotic treatmen*t* processes of drinking water. The reason is that the predicted S-metolachlor TPs prioritized by the present research were all at least once predicted by biotransformation in silico model. Therefore, the chances of finding them in water samples were higher than in the ozonation experiments. RSF is a process extensively implemented in drinking water treatment plants to remove particles and facilitate the biodegradation of organic compounds.

The treatment was experimentally simulated by Brunner et al. (2019) using sand disposed of by Waternet (NL). As Brunner et al. (2019) reported, water samples were spiked with metolachlor and treated with RSF, and both influent and effluent samples were taken 8h and 96h after the experiment. The water samplings before and after treatment were analyzed with a Tribrid Orbitrap Fusion mass spectrometer (ThermoFisher Scientific) coupled to a Vanquish HPLC system (ThermoFisher Scientific).

During the reversed-phase LC, compounds with a high retention time (RT) (the time needed to exit the column and be seen in the chromatogram) are hydrophobic. Conversely, the compounds with a lower RT (which elute in fewer minutes) are less hydrophobic (more hydrophilic). It is crucial to exclude compounds eluted before the solvent as they are not diluted in the analyzed matrix (water, in this case).

After that, high-resolution tandem mass spectrometry, known as HRMS/MS and involving two steps of ionization, was applied since it is a known approach to facilitate the identification of unknown compounds (Schollée et al., 2017). When the molecules enter the mass spectrometer, they are ionized using electrospray ionization (ESI) and therefore separated based on their mass-to-charge ratio (m/z) and detected. The resulting MS1 spectrum contains the masses of the ionized compounds, respectively protonated masses [M+H]+ when operated in positive mode or deprotonated masses [M-H]- when operated in the negative mode. Next, specific ions are selected and fragmented using higher energy collisional dissociation (HCD). These fragments are again separated on their mass-to-charge ratio (m/z), and the resulting MS2 spectrum contains the m/z values of the formed fragments.

The spike-in of metolachlor during the RSF experiment by Brunner et al. (2019) was 10 µg/L, which is one order of magnitude higher than the average environmental concentrations of the compound in surface water.

High concentrations of the spiked-in parent compound were chosen to allow checking for minor TPs (produced at lower rates). Blank samples (without the parent compound) were spiked every 5-10 samples to check for possible contaminations.

The ratio between the compounds detected in the samples before and after the application of RSF was considered: being a transformation reaction, the scope of this analysis, when the intensity of the peak of the parent compound is reduced, and other peaks are increased, transformation processes are pointed out. Therefore, a fold change filter of log 2-fold change (log2FC) > 2 between samples spiked with the parent compound and the blank samples was selected by Brunner et al. (2019). Moreover, a log2FC > 1 filter was applied between samples before and after treatment to exclude compounds whose presence in the water treatment did not intensify. The signals decreased during the treatment were likely to be the parent compound, while an increment of the signal after RSF likely represented TPs.

The software Compound Discoverer 2.1 (ThermoFisher Scientific) was used for data analysis by Brunner et al. (2019). The software can extract features from the dataset, perform suspect screening and calculate several statistical parameters. The results are given in a table with features, their *bruto* formula, expected molecular weight (subtracting or adding the hydrogen value), attempted identification, peak areas, and other information. The present research used the new versions of Compound Discoverer 3.1 for processing the data and the later version of Compound Discoverer 3.3 for collecting relevant results. The software analyzes the HPLC-HRMS data providing a table with peaks and potential candidates. The provided table already calculates the compounds' molecular weight by subtracting or adding the molecular mass of the hydrogen. Two filters were applied to exclude irrelevant information. First, the background signal was excluded, and second, peaks with RT inferior to 2.4 minutes (equal to the RT of the solvent) were excluded. A screening based on the molecular mass was done to search for the prioritized S-metolachlor TPs.

For the compounds matching the prioritized S-metolachlor TPs, the MS data were used to confirm the attempted identification of TPs. For the suspect TPs, which presented molecular weight compatible with the prioritized S-metolachlor TPs, a check of the other features present in the MS1 spectrum (m/z – expressed as +H or -H – peak and RT) was done. If the mass spectrum was likely of the same molecule seen in the chromatogram, the MS2 fragmentation data were used to identify the predicted prioritized S-metolachlor TPs.

CFM-ID v4.0.0 (Wang et al., 2021) was used to predict the fragmentation spectrum of the suspect metolachlor TPs identified in the first screening. The CFM-ID is a freely available tool that predicts the ESI-MS/MS spectrum for a given molecular structure and metabolite identification from tandem mass spectra. Both the positive and negative ionization were evaluated. The input can be the SMILES string or the InChl, and the SMILES option was adopted. The fragmentation is given for three energies, leading to different spectra.

RESULTS

1. Characterization of S-metolachlor

Firstly, introduced in 1998, S-metolachlor (Figure 2) is a synthetic organic compound widely used in Europe as an active substance for herbicides, such as the commercial product Dual Gold®, mainly used in cereal cropping (such as corn, soybeans, potatoes, and sunflower – Global data, 2019). Its applications are extended to various formulations associated with other active substances, and it is among the ten most used herbicides in Europe (O'Connell et al., 1998; Jursík & Holec, 2019). S-metolachlor is a chloroacetanilide, thus derived from aniline. Aniline is a class of molecules moderately soluble in water; therefore, relevant for water, including drinking water. The available molecular information is collected in Table 3.

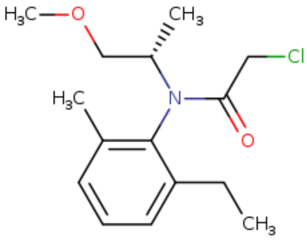

Figure 2. S-metolachlor (PubChem, 2022)

Table 3. Molecular information of S-metolachlor collected in PubChem (NIH, 2022).

chemical name	S-metolachlor
IUPAC	2-chloro-N-(2-ethyl-6-methylphenyl)-N-[(2S)-1-methoxypropan-2-yl]acetamide
Canonical SMILES	CCC1=CC=CC(C)=C1N([C@@H](C)COC)C(=O)CCl
Isomeric SMILES	CCC1=CC=CC(=C1N([C@@H](C)COC)C(=O)CCl)C
formula	$C_{15}H_{22}ClNO_2$
mass	283.79 g/mol
CAS number	87392-12-9
EC number	257-060-8

1.1 Information available in the literature for S-metolachlor

Traditionally, the most diffused formulation, including S-metolachlor as an active substance, was the racemic mixture 1:1 of S- and R- metolachlor. However, commercial formulations nowadays contain above 80% of the enantiomer S-metolachlor since it is the active compound inhibiting cell division and growth by interfering with protein synthesis. Therefore, racemic formulations were substituted with formulations at higher concentrations of S-metolachlor to achieve higher effectiveness with lower application rates (Shaner et al., 2006), and therefore could be associated with lower environmental and health risks (Liu et al., 2006; Cao et al., 2008). Moreover, the use of S-metolachlor combined with other pesticides (such as glyphosate) has increased the applicability of S-metolachlor and, therefore, enhanced its use in agriculture (Mesnage & Antoniou, 2021). Moreover, exposure to mixtures of pesticides containing S-metolachlor revealed enhanced toxicity compared to S-metolachlor exposure alone (Hayes et al., 2006; Liu et al., 2022).

The active substance of the racemic mixture is S-metolachlor, while R-metolachlor is primarily inactive (Shaner et al., 2006). However, while Metolachlor is no more authorized in Europe as an active substance, S-metolachlor is still widely used. Even though S-metolachlor is not approved anymore by EC Regulation 1107/2009, it is still approved for use under EC 1107/2009 in 27 EU Countries, including the Netherlands. Its approval was extended to July 2022, then modified to July 2023 (European Commission, 2022e), and might be renewed again as, in the UK, it was already approved until 2024 (University of Hertfordshire, 2022). According to the EU Commission, the extension is due to a temporal delay of S-metolachlor risk assessment for reasons “beyond the control of the applicants” (European Commission, 2022e, p.2).

Monitoring data

The drinking water standard for pesticides is 0,10 µg/l (EU Directive n° 2020/2184). However, the racemic mixture metolachlor was often identified at higher concentrations of water standards (Tröger et al., 2021). Metolachlor has been repeatedly identified in water sources for drinking water production (Glassmeyer et al., 2017; Zambito Marsala et al., 2020; Halbach et al., 2021; Tröger et al., 2021; la Cecilia et al., 2021; Koroša et al., 2022). A review (de Souza et al., 2020) identified metolachlor as the second most frequently detected herbicide in water sources. It is primarily found in surface water but also contaminates groundwater (WHO, 2022). Furthermore, metolachlor was found in untreated wastewater, with some derived TPs (Rousis et al., 2021), and in the treated wastewater used in agriculture (Murrell et al., 2021).

A review over seven years (Rousis et al., 2021) investigated human exposure to metolachlor by analysis of untreated influent wastewater. Metolachlor was associated with a high mean concentration (4.8 µg/L), and its TP metolachlor morpholinone was also found at relatively high mean concentrations (2.0 µg/L). Even though the review refers to the racemic mixture metolachlor, the information is relevant to understand the fate of S-metolachlor in water.

Zambito Marsala et al. (2020) reported a Groundwater Ubiquity Score (GUS)[6] of 1.91, soil adsorption coefficient (Koc) of 226.1 mL/g, DT50 of 21 days, and water solubility of 480 mg/L. Based on the PCC, metolachlor seemed not persistent in the soil and unlikely to be found in groundwater. Nevertheless, the study detected metolachlor levels higher than Environmental Quality Standard (EQS) for groundwater, equal to 0.1 ng/L. However, metolachlor concentration decreased significantly over time: from 0.01 µg/L in November 2017 to 0.007 µg/L in July 2018 and 0.0008 µg/L in September 2018.

Glassmeyer et al. (2017) detected metolachlor in water sources and drinking water samples at maximum concentrations of 130 and 100 µg/L, with a frequency of detection, of one sample out of three. Hladik et al. (2008) had already shown the detection of Metolachlor in 100% of drinking water samples produced with conventional treatment processes at levels that varied between 49 ng/L in the fall (2003) and 310 ng/L in the spring (2004). A recent review study (Tröger, 2021) analyzed the presence of Metolachlor in raw water and drinking water collected from drinking water plants in different countries of Europe and outside Europe. The highest level was found in Italy, equal to 245.92 ng/L, followed by China (12.53 ng/L) and Belgium (6.86 ng/L). Metolachlor was also found in seawater (Sicily, Italy) at low-levels of 0.11 ng/L (Brumovský et al., 2017). Nonetheless, de Souza et al. (2020) reported the complete removal of metolachlor using advanced oxidation processes (EAOPs) verified by Guelfi et al. (2018).

Specifically, the enantiomer S-metolachlor was detected at levels three orders of magniture higher than the water stardards in surface water (Otto et al., 2016) and groundwater (Zambito Marsala et al., 2020). Also, Michel et al. (2021) confirmed the presence of S-metolachlor in groundwater. Indeed, S-metolachlor is known to disperse in surface water by agricultural runoff (Zemolin et al., 2014) and can contaminate groundwater after its application, especially when the soil has a low organic carbon (OC) content (Marín-Benito et al., 2021).

Table 4 reports the selected research resulting from the systematic literature review, while details on the selection process is available in the supplementary documents (see Annex 7, Table 1).

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[6] The Groundwater Ubiquity Score (GUS) is a value used to define the likelihood of pesticides migrating to groundwater. It is calculated to combine the pesticide's half-life ($t_{1/2}$), and the Koc obtained experimentally (Pfeiffer, 2010). Values above 2 are associated with pesticides having a moderate potential to move towards groundwater, while values higher than 3 with ones having a high potential (Muendo et al., 2021).

Table 4. Systematic literature review on metolachlor and S-metolachlor detection in water samples. The higher concentrations detected were collected here. In red are the values that exceeded the water standards for pesticides (0.1 µg/L, which equals 100 ng/L).

reference		water samples	location	analysis	concentration max (µg/L)
Glassmeyer et al., 2017		water sources drinking water	-	-	0.049
Brumovský et al., 2017		seawater	Italy		0.00011
de Souza, 2020 (review)	*Proia et al., 2013*	surface water	Spain	various	1.30
	Glinski et al, 2018		USA		1.5
	Peng et al., 2018		China		0.316
	Sun et al., 2018		China		0.08
	Battaglin et al., 2018		USA		0.0447
	Xie et al., 2019		China		0.0055
	Kapsi et al., 2019		Greece		0.077
Tröger et al., 2021		raw water & drinking water	Europe Asia		0.246 (Italy)
Rousis et al., 2021		untreated influent wastewater (IWW)	Greece	LC- QToF MS[7]	4.48
S-metolachlor					
Otto et al., 2016		surface water	Italy	-	99
Zambito Marsala et al., 2020		groundwater	Italy	SPE[8] + HPLC/MS[9]	1.2
Suciu et al., 2020		groundwater	Italy	HPLC/MS	0.08
Michel et al., 2021		groundwater	-	-	2

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[7] LC- QToF MS: Liquid Chromatography–hybrid Quadrupole Time-of-Flight Mass Spectrometry

[8] SPE: Solid-Phase Extraction

[9] HPLC/MS: High Performance Liquid Chromatography-Mass Spectrometry

Toxicological data

Toxicological information on S-metolachlor is available in a series of databases, such as the ToxRefDB 2.0 Toxicity Reference Database[10], the ToxCast database[11], and NORMAN Substance Database[12], registered in the S60 SWISSPEST 19 watchlist. Moreover, in the Pesticide Properties DataBase (PPDB)[13], S-metolachlor is associated with high alerts for human health regarding endocrine disruption and reproductive and developmental effects (Lewis et al., 2016). The racemic mixture metolachlor is associated with relevant evidence of toxicity, therefore, is included in class III - the second lowest of four categories for toxicological concern (EPA, 1995).

By the Global Harmonized System (GHS) of Classification, Labelling, and Packaging (CLP) regulation, S-metolachlor is associated the Hazard Statements H317, meaning it may cause an allergic skin reaction and H410, underlying the high toxicity towards aquatic life with acute and chronic effects. Moreover, since positive *in vivo* results demonstrated the sensitization effects, it is associated with Category 1 for Skin Sensitization[14]. The racemic mixture of metolachlor is non-genotoxic (EFSA, 2018; WHO, 2022). However, US EPA classified the racemic mixture metolachlor as a possible human carcinogen (EPA, 1995) and was related to reproductive and endocrine disruption effects. Metolachlor was indeed recognized to impact aromatase activity related to endocrine disruption (Laville et al., 2006).

No data on genotoxicity nor carcinogenicity for the enantiomer S-metolachlor are available for S-metolachlor. Nonetheless, available research on S-metolachlor confirmed the reproductive and developmental toxicity. For instance, *in vivo* testing in male rats treated in the prepubertal period with 5 or 50 mg/kg/day (Mathias et al., 2012). Moreover, human liver cell growth was already inhibited at doses of 50 ppb, and the reduction of cell division was induced at higher doses (Hartnett et al., 2013). Moreover, the following research proved that S-metolachlor affects the swim bladder's morphology in zebrafish at concentrations of 0.5-5 uM in the water (Yang et al., 2021), revealing the ecotoxicity potential of the molecule.

The toxicological profile of S-metolachlor was evaluated in the framework of Directive 91/414/EEC. The Acceptable Daily Intake (ADI) was established at 0.1 mg/kg bw/day based on studies performed on metolachlor

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[10] The Toxicity Reference Database (ToxRefDB), developed by guidelines of the US Environmental Protection Agency and the National Toxicology Program, includes chemical structures from over 5000 *in vivo* toxicity studies (Watford et al., 2019).

[11] The ToxCast Chemical Database contains around 4400 unique chemicals lacking data that may have toxicological relevance for humans and ecosystems (*ToxCast Chemicals*, 2022).

[12] The NORMAN Substance Database is formed by various contributions of laboratories, research centers, and organizations to monitor emerging environmental substances (NORMAN, 2022).

[13] The Pesticide Properties DataBase was developed by the Agriculture & Environment Research Unit (AERU) at the University of Hertfordshire to support the risk assessment of chemicals used in agriculture

[14] Category I for skin sensitization indicates that a chemical has a sensitizer potential, either because there is evidence in humans of skin contact sensitization or positive results from *in vivo* testing.

on oral exposure to dogs for one year (European Commission, 2004). A safety factor (SF) of 100 was applied. However, it was not specified by the European Commission the origin of the applied factor (European Commission, 2004), even though it is presumable that factor 100 applied considered the translation of experimental data to humans and the interindividual variation between humans. EFSA reviewed the maximum residual level (MRL) in food products at 0.05 mg/kg for plant products and 0.01 mg/kg for meat products (EFSA, 2012).

As regards drinking water, metolachlor has also been incorporated in the Guidelines for drinking-water quality GDWQ (last update March 2022). For drinking water, the guideline value for the racemic mixture metolachlor is 0.01 mg/L (10 µg/L) (WHO, 2022). The Tolerable Daily Intake (TDI) is reported as 3.5 µg/kg body weight, derived from the Non-Observed Adverse Effect Level (NOAEL) of 3.5 mg/kg calculated from the experimental exposure to high dose levels to dogs for 1 year, which showed a decrease in kidney weight. The translation to values for humans was done using an SF of 1000, accounting for both interspecies (factor 100) and intraspecies uncertainties (factor 10) (WHO, 2022).

1.2 *In silico* physicochemical characterization of S-metolachlor

Relevant PCC of S-metolachlor were collected to understand the chemical fate in the water and, therefore, justify, from a physicochemical point of view, its detection in drinking water. To be found in the water, it must be at least partially soluble in water, and it has not to be entirely absorbed by the soil (considering the herbicide is distributed in a field and slowly spreads around, arriving at water sources for drinking water production). S-metolachlor is moderately soluble in water and miscible in organic solvents such as acetone, ethyl acetate, toluene, and xylene (Wołejko et al., 2017).

In silico tools for evaluating the environmental fate of chemicals (see Method section pargraph 1.1) were used to justify the affinity of S-metolachlor for the water phase and therefore validate the selection of the compound as a parent compound in this research study. The results are available in the supplementary documents (see Annex 8).

S-metolachlor was predicted to be more lipophilic than hydrophilic, as the estimated log Know by EPI Suite^{TM} was 3.24. Accordingly, the experimental Kow is 3.13 (Martin, 1996). Indeed, the model LEV3EPI™ predicted soil as the primary environmental department for S-metolachlor, with water collecting almost 12% of the chemical in the environment. The experimentally reported water solubility at 20° was 480 mg/L, while the experimental value was almost ten-fold higher than the predicted water solubility by CTS (74.9 mg/L), while double the predicted value by the WATERNT ™ (v1.1) model (227.63 mg/L). However, all the values fall into moderate/ low water solubility. Therefore, S-metolachlor is expected to be partially dissolved in water but found in water samples at low concentrations.

The applied preliminary models for degradation within EpiSuite^{TM} suggested that biodegradation processes, either in the environment or during water treatment processes such as RSF will not metabolize and transform S-metolachlor. Further information is available in the supplementary documents (see Annex 8).

The overall consideration of the different parameters suggests that S-metolachlor could be found in water sources when subjected to treatment processes. Indeed, the research studies described in paragraph 1.1 demonstrated the presence of S-metolachlor in various water samples. In line with the studies abovementioned, S-metolachlor is partially soluble in water and not absorbed enough by the soil to exclude its presence in the water. Nevertheless, S-metolachlor can be adsorbed by suspended organic matter being transported in the water, which could also explain its detection in water sources.

1.3 *In silico* hazard assessment of S-metolachlor

The S-metolachlor *in silico* hazard assessment for relevant endpoints was summarized here for genotoxicity, carcinogenicity, developmental and reproductive toxicology, endocrine disruption, and skin sensitization. Other endpoints were not included here based on the availability of freely available *in silico* tools. The selection of the endpoints was discussed in the methods section (see Annex 4). The overall *in silico* results are summarized in Table 5. Details on the specific models' results are available in the supplementary documents (see Annex 9).

Genotoxicity

Various models have identified structural alerts in S-metolachlor and mechanistic pathways responsible for genotoxicity. S-metolachlor was predicted positive both to mutagenicity and chromosomal aberration endpoints. The different predictions offered by the considered software are here combined and summarized.

Structural alerts for genotoxicity were identified regarding mutagenicity and chromosomal aberration, and reliable predictions were available to support the assessment. The structural alert non-tertiary aliphatic halogens were recognized (Figure 3), which is linked to possible mutagenic effects.

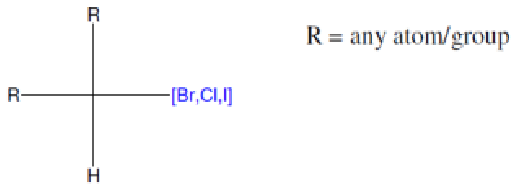


Figure 3. Aliphatic halogens structural alert for mutagenicity (VEGA, 2022)

ToxRead BETA 0.23 also confirmed the activity prediction towards the mutagenicity endpoint, recognizing the non-tertiary aliphatic halogens' structural alert. Additionally, the profiling performed in QSARToolbox recognized the aliphatic halogens' structural alert as indicating a possible mutagenic effect. Moreover, other structural alerts were identified by VEGA as responsible for mutagenicity (Figure 4). Also, OSIRIS recognized similar structural alerts defined as high-risk and medium-risk fragments for mutagenicity.

Fragment found: SM45

Fragment found: SM73

Sarpy alert n. 45 for Mutagenicity, defined by the SMARTS: c1cc(ccc1NCCCl)

Sarpy alert n. 73 for Mutagenicity, defined by the SMARTS: CCNCCCl

Fragment found: SM161

Fragment found: SM106

Sarpy alert n. 161 for NON-Mutagenicity, defined by the SMARTS: c1ccc(NC(C)C)cc1

Sarpy alert n. 106 for Mutagenicity, defined by the SMARTS: CCCl

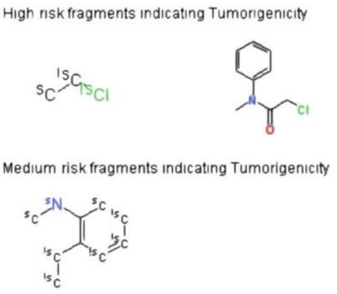
Figure 4. Structural alerts of S-metolachlor founded by the SarPy/IRFMN mutagenicity model by VEGA, 2022

Also, one model in CompTox confirmed the positive prediction for mutagenicity, even though the overall Consensus Ames mutagenicity was negative for the endpoint.

As regards chromosomal aberration, which is also a mechanism of genotoxicity, VEGA predicted S-metolachlor to be active for this endpoint, with high reliability. QSAR Toolbox also profiled S-metolachlor as possible genotoxic through the chromosomal aberration MoA, based on the recognition of alpha-activated carbon due to the presence of the halogen.

Carcinogenicity

S-metolachlor was predicted as cancerogenic by four models in VEGA. The aliphatic halogens structural alert (Figure 3) already associated with mutagenicity was pointed out by the software VEGA and QSARToolbox. In addition, the software OSIRIS recognized two high-risk fragments and a medium-risk fragment, indicating tumorigenicity (Figure 5).


High risk fragments indicating Tumorigenicity

Medium risk fragments indicating Tumorigenicity

Figure 5. Risks fragments identified by the OSIRIS carcinogenicity model for S-metolachlor (OSIRIS, 2022)

On the contrary, the IRFMN models for predicting the oral and inhalation classification of carcinogenicity reported experimental data on the S-metolachlor of inactivity. Moreover, the most similar molecule in the training set presenting the same structural alert was associated with a non-carcinogenic experimental value. Therefore, the overall results are insufficient to draw a conclusion on the complex endpoint carcinogenicity.

Reproductive and developmental toxicology

The applied models indicated structural alerts in S-metolachlor and similarities with compounds responsible for the activity towards the endpoint. The IRFMN/CORAL Zebrafish embryo AC50 (v.1.0.0) reported an experimental value of 9536.43 µg/L, while the model prediction was 5372.73 µg/L (good reliability defined by the model). The difference between the experimental value and the predicted one was relevant – with the first almost two-fold the second one. However, the values are of the same order of magnitude. Being AC50, the concentration at which 50% of the activity was shown, these values indicate that S-metolachlor was toxic at least one order of magnitude higher than the concentrations detected in water (see Result section pargraph 1.1). In CompTox software, the consensus result was positive for developmental toxicity, with all the models in agreement. Moreover, The model predicted reproductive effects due to recognizing a high-risk fragment and three medium-risk fragments indicating reproductive effects (Figure 6).

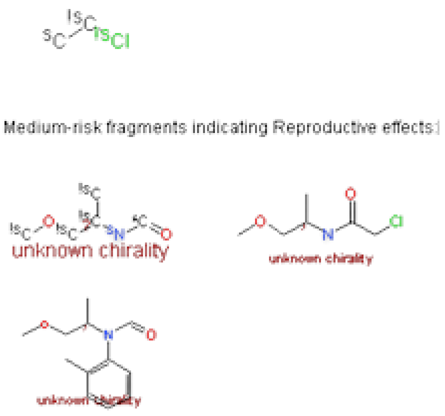


Figure 6. Risks fragments identified by OSIRIS reproductive toxicology model for S-metolachlor (OSIRIS, 2022)

Therefore, concerns about its ability to interfere with reproduction and developmental processes are raised. However, some predictions conversely predicted S-metolachlor as non-active. QSAR Toolbox reported an experimental value for developmental toxicity/teratogenicity as Low Observed Adverse Effect Level (LOAEL) = 500 mg/kg bw/day (Knudsen et al., 2009). The value indicates that relatively high amounts of S-metolachlor are needed to exert the toxicological effect, thus presumably not reached via drinking water exposure since S-metolachlor has been detected at lower levels (see Table 4). Moreover, various models did not provide reliable predictions for these complex endpoints. Therefore, further research is needed.

Endocrine disruption

The reported results for the endocrine disruption endpoint suggested the activity of S-metolachlor towards the aromatase, the enzyme responsible for the conversion of androgens into estrogens. The VEGA IRFMN Aromatase activity model (v 1.0.0) reported experimental data for S-metolachlor of active antagonism. Notably, the activity of S-metolachlor towards the aromatase activity had already been demonstrated *in vitro* (Laville et al. 2006) and confirmed by the prediction tools here. Therefore the disturbance of the aromatase activity could be the MoA of endocrine disruption of S-metolachlor.

On the other hand, reliable prediction assessed S-metolachlor as inactive towards the estrogen and androgen receptors both in the VEGA and CompTox software. Moreover, no experimental data pointed out the activity towards the endpoint. The QSARToolbox profiling defined S-metolachlor as a non-binder of the estrogen receptor, as chemicals with molecular weight inferior to 500 and have a cyclic structure without a -OH or -NH2 functional groups.

Skin sensitization/irritation

S-metolachlor appeared to be a possible irritant and sensitizer. Indeed, the software OSIRIS predicted irritant effects due to the recognition of six high-risk fragments indicating irritating effects (Figure 7).

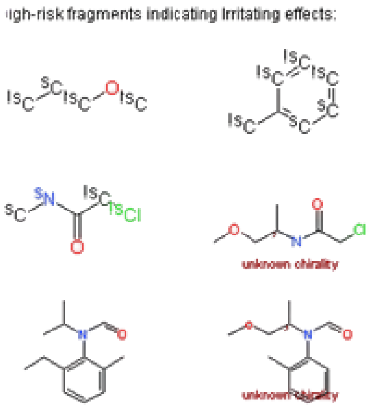


Figure 7. Risks fragments identified by OSIRIS irritating model for S-metolachlor (OSIRIS, 2022)

QSARToolbox also identified two protein-binding alerts for skin sensitization. Protein binding was predicted to undergo a nucleophilic substitution (SN2) by the OASIS model, with a direct acylation involving a leaving group by the OECD model. According to the GHS database, S-metolachlor was categorized as 1B for skin sensitization. However, an exclusion rule for skin sensitization defined by BfR was found, particularly in the group CNHal with aqueous solubility < 0.1 g/L. Nevertheless, no inclusion rules were detected. Moreover, using the automatized workflow Skin sensitization from the GPMT assay and EC3 LLNA assay to fill data gaps, the S-metolachlor was predicted to be a sensitizer.

Table 5. In silico hazard assessment of S-metolachlor for genotoxicity, carcinogenicity, developmental and reproductive toxicology, endocrine disruption, skin sensitization, and Cramer class evaluation. The prediction was = positive, 🟨 = intermediate, 🟩 = negative, or ⬜ = inconclusive. The Applicability Domain Index (ADI) scores, thus the internal validation of the models, are reported (see Methods section paragraph 1.2.2). exp = the experimental value was the experimental value was reported by the model

endpoint		software	model	prediction & score
genotoxicity	mutagenicity	VEGA	CONSENSUS v1.0.3	🟥 0.525
			CEASAR v2.1.13	🟥 0.781
			SarPy/IRFMN v1.0.7	🟥 0.781
			ISS v1.0.2	🟥 0.916
			KNN/*Read-Across* v1.0.0	⬜ 0
		ToxRead	*Read-across*	🟥 0.8
			QSAR consensus	🟥 0.525
		CompTox	Consensus Ames mutagenicity	⬜ -
		OSIRIS	Mutagenic	🟥 -
		QSARToolbox	Mutagenicity	🟥 -
	chromosomal aberration	VEGA	CORAL v1.0.0	🟥 0.936
			IRFMN *In vitro* micronucleus v1.0.0	⬜ 0.754
			IRFMN *In vivo* micronucleus v1.0.1	⬜ 0.658
		QSARToolbox	Chromosomal aberration	🟥 -
carcinogenicity		VEGA	CEASAR v2.1.9	⬜ 0.775
			ISS v1.0.2	🟥 0.916
			IRFMN/Antares v1.0.0	⬜ 0.748
			IRFMN/ISSCAN-CGX v1.0.0	⬜ 0.748
			IRFMN carcinogenicity oral v1.0.0	🟩 exp
			IRFMN carcinogenicity inhalation v1.0.0	🟩 exp
		OSIRIS	Tumorigenic	🟥 -
		QSARToolbox	Carcinogenicity	🟥 -

developmental/reproductive toxicology	VEGA	CEASAR v2.1.7	0.771
		Developmental/Reproductive Tox library v.1.1.0	0
		IRFMN/CORAL Zebrafish embryo AC50 v1.0.0	exp
	CompTox	Developmental Toxicity	-
	OSIRIS	Reproductive effective	-
	QSARToolbox	DART scheme	exp
endocrine disruption	VEGA	NRMEA Thyroid Receptor Alpha effect v1.0.0	exp
		NRMEA Thyroid Receptor Beta effect v1.0.0	exp
		IRFMN Aromatase activity v1.0.0	exp
		IRFMN Estrogen Receptor Relative Binding Affinity v1.0.1	0.965
		IRFMN/CERAPP Estrogen Receptor-mediated effect v1.0.0	exp
		IRFMN/COMPARA Androgen Receptor-mediated effect v1.0.0	exp
	CompTox	Estrogen Receptor Binding	exp
	QSARToolbox	OECD Estrogen Binding	🟥
skin sensitization	VEGA	CEASAR v2.1.6	0.75
		IRFMN/JRC v1.0.0	0
	OSIRIS	Irritant	-
	QSARToolbox	OECD protein binding	-

1.4 Conclusions on S-metolachlor characterization

The qualitative hazard assessment of S-metolachlor pointed out structural alerts and *read-across* evaluations linked to genotoxicity - mutagenicity and chromosomal aberration - carcinogenicity, and skin sensitization. In contrast, the assessment of developmental and reproductive toxicology and endocrine disruption was inconclusive, even though the experimentally demonstrated activity towards the aromatase was pointed out (Laville et al., 2006). Indeed, different models have predicted S-metolachlor as a genotoxic, carcinogenic, and skin sensitizer. The higher reliability, however, was linked to genotoxicity and skin sensitization, as most of the models agreed. On the contrary, regarding reproductive and developmental toxicology, the model's reliability was insufficient to conclude on the endpoint. Even though the models suggested the inactivity of S-metolachlor for these endpoints, the information obtained was insufficient to validate the prediction (the output of the *in silico* hazard assessment is summarized in Table 5). Therefore, further research is still needed.

Overall, S-metolachlor can be considered a predicted hazard, as it was identified as a possible toxicant for different relevant endpoints. The priority code assigned was 3/3 according to the following predefined assessments:

- Is there any prediction positive for relevant toxicological endpoints? Yes. (1/3)
- Is there more than one model agreeing with that prediction? Yes. (2/3)
- Is there any model that predicts positivity with high reliability? Yes. (3/3)

Together with the associated toxicological concern, the presence of S-metolachlor in drinking water sources detected in the literature and confirmed by the physicochemical characterization of the compound justifies the selection as parent compounds of possibly formed TPs in drinking water. Indeed, since it is known to be present in water sources and related to toxicological concerns, evaluation is required to understand the possible indirect impact on drinking water quality.

2. Collection of S-metolachlor transformation products

A systematic literature review was performed to collect S-metolachlor TPs known to be formed from S-metolachlor in water. The research terms inserted in the research engines were: (transformation products) AND (drinking water treatments) OR (water) AND (Metolachlor). The review was extended to all the TPs possibly formed from S-metolachlor in the environment because environmental biotic processes could represent the microbial biotransformation processes that can occur during RSF (Brunner et al., 2019). Metolachlor was used instead of S-metolachlor based on the assumption that Metolachlor can represent S-metolachlor. A suggestion to assess the change of toxicity between R and S-metolachlor is discussed in this report (see Discussion section paragraph 2.2). However, the activity of the racemic mixture with the S-metolachlor is predicted to be comparable, as S-metolachlor is recognized to be the active portion of the enantiomer mixture.

The main S-metolachlor TPs found in the literature were: metolachlor oxanilic acid (OA), metolachlor ethane sulfonic acid (ESA), metolachlor-OXA, metolachlor NOA 413173, metolachlor CGA357704, metolachlor CGA368208 (acetochlor sulfonic acid), metolachlor morpholinone, metolachlor mercapturate, metolachlor_TP250, metolachlor_TP266, TP SYN542490 (Table 6). Furthermore, metolachlor ethane sulfonic acid, metolachlor oxanilic acid, and O-Desmethylmetolachlor were found in the soil. The concentration at which they were detected is generally lower than 0.1 µg/L, but in some cases, exceeded 1 µg/L (metolachlor ESA, metolachlor OA, metolachlor NOA, metolachlor morpholinone). The details of the systematic literature review are available in the supplementary documents (Annex 7, Table 2).

Table 6. Systematic literature review on S-metolachlor transformation products (TPs) detection in water samples. The higher concentrations detected were collected here. In red are the values that exceeded the water standards for pesticides (0.1 µg/L, which equals 100 ng/L).

reference	**water samples**	**analysis**	**identified S-metolachlor TPs**	**concentration max (ng/L)**
Hladik et al., 2008	Treated drinking water samples	SPE GC/MS[15] HPLC-DAD	Metolachlor-2-hydroxy	61
			Metolachlor deschloro	30
			Metolachlor morpholinone	37
			Metolachlor propanol	73
			Deschloroacetylmetolachlor	35
			Deschloroacetyl metolachlor propanol	22
			Metolachlor OA	220
			Metolachlor ESA	1500
Soulier & Togola, 2016		POCIS HPLC-HRMS	Metolachlor deschloro 2-hydroxymetholachlor Deschloroacetylmetolachlor Metolachlor morpholinone	na
Guelfi et al., 2018	AOP treated water	GC-MS	Metolachlor deschloro	na
Hollender et al., 2018	groundwater	LC-HRMS/MS	Metolachlor-ESA	0.007
Farlin et al., 2018	water springs	LC-ESI-MS/MS	Metolachlor ESA Metolachlor OXA	na
Kiefer et al., 2019	groundwater	LC-HRMS/MS	Metolachlor-ESA	970
Brunner et al., 2019	drinking water after sand filtration and ozonation	HRMS/MS	Deschlormetolachlor	na
Gago-Ferrero et al., 2020	influent wastewater effluent watewater	UPLC-Q-ToF-HRMS/MS	Metolachlor-ESA	0.003 0.005
Rousis et al., 2021	untreated influent wastewater (IWW)	LC- QToF MS	Metolachlor morpholinone	3.304 (2016)
			Dimethachlor-ESA	2500
			Metolachlor mercarpturate	2000

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[15] GC-MS: Gas Chromatography–Mass Spectrometry

Kiefer et al., 2021	groundwater	LC-HRMS/MS	Metolachlor TP SYN542490	estimated up to 100–500
			Metolachlor TP SYN547969/SYN542488	100-500
			Metolachlor TPSYN547977	< 100
			Metolachlor TP SYN542489	<100
			Metolachlor TP SYN542607	<100
			Metolachlor TP SYN542491	<100
			Metolachlor TP CGA357704	< 100
Halbach et al., 2021	surface water	LC-HRMS/MS	metolachlor-ESA	< 0.02
			Metolachlor OA	< 0.02
			Metolachlor NOA 413173	up to 0.08
			Metolachlor CGA 368208	< 0.02
			Metolachlor CGA 357704	< 0.01
Finckh et al., 2022	wastewater treatment plants	LC-HRMS/MS	Metolachlor ESA	2500
			Metolachlor OA	1900
			Metolachlor-NOA 413173	2400
			Metolachlor CGA 357704	< 0.01
			Metolachlor CGA 368208	< 0.01
Eysseric et al., 2022	surface water and wastewater-treated water	top-down NTS UHPLC + MetFrag and Similar Partition Algorithm (SPS)	metolachlor-ESA	na
			metolachlor-OA	na
			metolachlor morpholinone	na
			metolachlor_TP250	na
			metolachlor_TP266	na

2.1 *In silico* prediction of S-metolachlor transformation products

Various software tools available were used to predict the formation of TPs from S-metolachlor during drinking water treatments. An explanation of the predictivity of different tools for reactions occurring during water treatments was discussed in the Methods chapter (see paragraph 2.2). All the tools accept the SMILES as input, which is a unique identifier of the chemical structure and, therefore, preferable (as discussed in the methods session). To discriminate S-metolachlor from its enantiomer R-metolachlor, the isomeric SMILES CCC1=CC=CC(=C1N([C@@H](C)COC)C(=O)CCl)C (NIH, 2022) was used. As an output, the CTS tool provided the IUPAC name of the predicted compounds, while the other tools only provided the SMILES and chemical formula. Therefore, identification in the literature was attempted. For every predicted TP, mining research was done to identify the compound's names. After checking for duplicates (S-metolachlor TPs predicted by different tools), the molecules were identified using PubChem, ChemSpider, and RMG: Molecule Identifier.

In total, 115 S-metolachlor TPs were predicted, of which 29 were predicted by more than one tool; thus, 83 unique structures were collected. Different *in silico* tools represent some reactions occurring during drinking water treatment processes (see Methods section paragraph 2.2.1). The number of predicted S-metolachlor TPs is reported in Table 7. Among these, 35 unique S-metolachlor TPs were identified, which may be formed due to drinking water treatments and the environment. Further information is available in the supplementary documents (see Annex 10).

Table 7. Summary of S-metolachlor predicted TPs using freely available in silico tools.

in silico tools	reactions considered	water treatment relevance	S-metolachlor TPs	
			predicted	identified
CTS	abiotic hydrolysis	advanced oxidation processes (AOPs), ozonation, chlorination	3	3
	photolysis	UV treatments, AOPs, ozonation, chlorination	3	3
	combined hydrolysis/photolysis	UV treatments, AOPs, ozonation, chlorination	3	3
	abiotic reduction	advanced reduction processes	1	1
UM-PPS	biotransformation	rapid sand filtration, wastewater treatments	34	25
enviPath	biotransformation	rapid sand filtration, wastewater treatments	60	23
BioTransformer	biotransformation	rapid sand filtration, wastewater treatments	11	9
		(Tot)	(115)	(67)
		Tot unique products	83	35

Chemical Transformation Simulator (CTS)

Accessing the Reaction Pathway Simulator (RPS) Module, it was possible to run S-metolachlor using the SMILES string. Different libraries are available, and the abiotic hydrolysis, photolysis, and abiotic reduction libraries were considered relevant for drinking water treatments (see Methods paragraph 2.2.1). The combined hydrolysis and photolysis library was also used to predict S-metolachlor TPs to evaluate the correspondence with the single libraries adopted alone. Further information on the predicted TPs is available in the supplementary documents (see Annex 10, Table 3).

The CTS abiotic hydrolysis library was used to predict some reactions occurring during (advanced) oxidation processes. The model predicted that it was unlikely that the S-metolachlor is not transformed. Three unique products were predicted: the main product of 1st generation (expected production of 96.08% and an accumulation of 92.16%) metolachlor-2-hydroxy, formed by nucleophilic substitution, and the minor products (production of 1.96%) metolachlor des(chloroacetyl) and 2-hydroxyacetic acid, formed by amide hydrolysis reaction. The amide hydrolysis reaction pathways were reported as one of the mechanisms possibly occurring in the biological WWT in the review conducted by Beretsou et al. (2016).

The CTS direct photolysis library was adopted in this research as a model for reactions occurring during UV treatments and (advanced) oxidation products. The model predicted S-metolachlor as likely to react and forecasted three unique products in the 1st generation: the main product, metolachlor morpholinone, was predicted as a consequence of an acetanilide O-dialkyl dehalogenative photocyclization reaction. The closure of a second ring with the loss of a -Cl and -CH_3 groups from S-metolachlor and the minor products 2-chloroacetic acid and 2-ethyl-N-(1-methoxypropan-2-yl)-6-methylaniline, the latter, already predicted by the CTS abiotic hydrolysis.

The CTS combined abiotic hydrolysis and direct photolysis library was used to assess TPs formed during (advanced) oxidation processes, which can involve these reactions simultaneously. The software assessed that it was unlikely that the S-metolachlor was not transformed by one of the reactions included in the combined libraries. The six compounds predicted by the single libraries were found, but two different S-metolachlor TPs not previously predicted by the single libraries were predicted. However, only three unique compounds were evaluated from the software likely to be produced. The main product (69.72%) was 2-{2-[(2-ethyl-6-methylphenyl)amino]propoxy}acetic acid, a chemical of the 3rd generation, was not predicted by the single libraries (even though in the single libraries, the highest number of generation was selected). However, it was predicted to be the central TP using the combined library. The minor products were metolachlor des(chloroacetyl) and hydroxy acetic (glycolic) acid, representing 14.84% of the predicted S-metolachlor TPs, respectively. Both were already predicted using single libraries, and the reaction pathways overlapped.

The abiotic reduction library represented reactions occurring during (advanced) reduction processes. S-metolachlor was predicted to be fully transformed by hydrogenolysis into metolachlor deschloro.

Overall in CTS, seven unique S-metolachlor TPs were identified. The more likely S-metolachlor TPs predicted by CTS were four: metolachlor-2-hydroxy, metolachlor morpholinone, 2-{2-[(2-ethyl-6-methylphenyl)amino]propoxy}acetic acid, and metolachlor deschloro (Table 8).

Metolachlor des(chloroacetyl) was repeatedly predicted, either for amide hydrolysis or amide photohydrolysis. However, the percentage of production and accumulation was relatively low compared to the other predicted S-metolachlor TPs.

Table 8. Summary of the likely predicted S-metolachlor TPs from CTS using the libraries of abiotic hydrolysis (blue), photolysis (yellow), the two combined (green), and the reduction library (orange).

model	SMILES	name	reaction pathway	production and accumulation (%)
CTS_abiotic hydrolysis	CCC1=CC=CC(C)=C1N(C(C)COC)C(=O)CO	N-(2-ethyl-6-methylphenyl)-2-hydroxy-N-(1-methoxypropan-2-yl)acetamide OR metolachlor-2-hydroxy	Nucleophilic Substitution	92.16
CTS_abiotic hydrolysis	CCC1=CC=CC(C)=C1NC(C)COC	2-ethyl-N-(1-methoxypropan-2-yl)-6-methylaniline OR metolachlor des(chloroacetyl) OR Deschloroacetylmetolachlor	Amide Hydrolysis	1.96
CTS_abiotic hydrolysis	OCC(O)=O	2-hydroxyacetic acid OR glycolic acid	Amide Hydrolysis	1.96
CTS_photolysis	CCC1=CC=CC(C)=C1N1C(C)COCC1=O	4-(2-ethyl-6-methylphenyl)-5-methylmorpholin-3-one OR metolachlor morpholinone	Acetanilide O-dialkyl Dehalogenative Photocyclization	77.78
CTS_photolysis	OC(=O)CCl	2-chloroacetic acid	N-aryl Amide Photohydrolysis	11.11
CTS_photolysis	CCC1=CC=CC(C)=C1NC(C)COC	2-ethyl-N-(1-methoxypropan-2-yl)-6-methylaniline OR metolachlor des(chloroacetyl) OR Deschloroacetylmetolachlor	N-aryl Amide Photohydrolysis	11.11
CTS_hydrolisis_photolysis	CCC1=CC=CC(C)=C1NC(C)COCC(O)=O	2-{2-[(2-ethyl-6-methylphenyl)amino]propoxy}acetic acid	Lactam Hydrolysis	69.72
CTS_hydrolisis_photolysis	CCC1=CC=CC(C)=C1NC(C)COC	2-ethyl-N-(1-methoxypropan-2-yl)-6-methylaniline OR metolachlor des(chloroacetyl) OR Deschloroacetylmetolachlor	N-aryl Amide Photohydrolysis, after nucleophilic substitution	14.84
CTS_hydrolisis_photolysis	OCC(O)=O	2-hydroxyacetic acid OR glycolic acid	Nucleophilic Substitution + N-aryl Amide Photohydrolysis	14.84
CTS_reduction	CCC1=CC=CC(C)=C1N(C(C)COC)C(C)=O	N-(2-ethyl-6-methylphenyl)-N-(1-methoxypropan-2-yl)acetamide OR metolachlor deschloro	Hydrogenolysis	100

EAWAG-BBD UM Pathway Prediction System (UM-PPS)

S-metolachlor TPs were evaluated by selecting all the transformations and all possible levels of biotransformation (7), allowing the maximum number per level (20), and let showing all the products possible (all numbers of C). A total of 12 S-metolachlor TPs were predicted, among which nine were associated with a neutral likelihood of being produced and three probably unlikely to be produced. Amongst these, six compounds contained the halogen group of the parent compound. Three compounds had less than three carbon atoms. A detailed list is available in the supplementary documents (Annex 10, Table 4).

Secondly, S-metolachlor TPs were investigated by selecting only aerobic biotransformations pathways and maintaining the other parameters such as the levels of biotransformation allowed (7), the maximum number per level (20), and the number of C allowed in the TPs prediction (all number of C). In total, 22 unique structures were predicted. The schematic representation is illustrated in Figure 8 and listed in the supplementary documents (see Annex 10). Surprisingly, by selecting aerobic transformations, more S-metolachlor TPs were predicted rather than selecting all types of transformations. This could be explained since other types of transformation prevent aerobic transformations, as modifying the conditions with oxygen could intervene in the reaction. However, the likelihood of production was different for the predicted S-metolachlor TPs, being in yellow the medium likely and in green, the most luckily to be produced (Figure 8). A detailed list is available in the supplementary documents (Annex 10, Table 5).

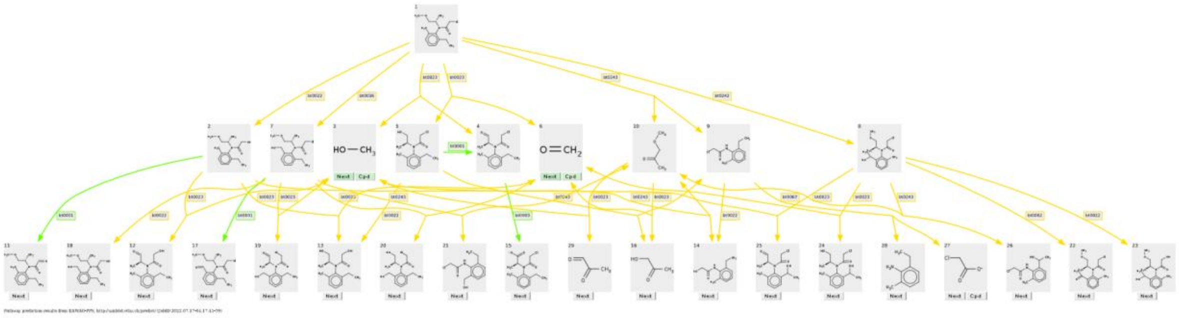


Figure 8. Prediction pathway for S-metolachlor provided by UM-PPS aerobic biotransformation predictor (UM-PPS, 2022).

enviPath

The tool predicts TPs formed due to biotranformation reactions, which can occur in the environment or during rapid sand filtration or wastewater treatments. The results were downloadable in a .cvs file. In total, 60 S-metolachlor TPs were predicted, among which ten were already predicted by other applied tools. Only 23 chemical structures were identified using PubChem, ChemSpider, and RMG Molecule Identifier. The other 37 were not identified, among which some SMILES were not recognized by the RMG tool (invalid adjacent list in RMG). Therefore it was not possible to proceed with the identification research. A summary of the results is available in the supplementary documents (see Annex 10, Table 6).

BioTransformer 3.0

The BioTransformer tool allows metabolism prediction, which is relevant for RSF and WWT since involving biodegradation. It was possible to select up to three generations of TPs. The tool predicted 11 S-metolachlor TPs, and six were already predicted from other tools. The results are available in the supplementary documents (see Annex 10, Table 7).

2.2 Conclusions on S-metolachlor transformation products

In total, 83 unique S-metolachlor TPs were predicted by different tools, among which the majority were products of biotransformation. Only 35 chemical structures were identified in the literature, therefore associated with the chemical or IUPAC name. The next step was to prioritize the more likely to be produced – and more potentially concerning from a toxicological point of view – S-metolachlor TPs.

3. Prioritization of S-metolachlor transformation products

Due to the feasibility and aims of this research project, the 83 predicted S-metolachlor TPs were prioritized following three steps accounting for the probability of being produced, the structural similarity between different predicted S-metolachlor TPs (selecting one TP for each group of structurally similar compounds), and structural alerts for genotoxicity and Cramer class classification. This step was done to choose the predicted S-metolachlor TPs of potentially more significant concern and direct further analysis.

Likelihood of being produced

S-metolachlor TPs were prioritized for the likelihood of being produced and the correspondence with the literature research (Table 9). Even though it was predicted by only one tool, metolachlor morpholinone was included in the list as it was the main product predicted for hydrolysis by CTS and was found in the literature (see Result section paragraph 2.1). In total, 12 S-metolachlor TPs were prioritized for the likelihood of being produced: if found in the literature research or predicted at least by two *in silico* tools.

Table 9. S-metolachlor predicted TPs prioritized for the likelihood of being produced.

		CTS				**UM-PPS**	**envipath**	**BioTransformer**	Literature
	Mass	hydrolysis	photolysis	combined	reduction				
metolachlor-2-hydroxy	265.35	√				√	√	√	√
metolachlor des(chloroacetyl)	207.1623	√	√	√			√	√	√
metolachlor deschloro	249.17				√		√	√	√
2-{2-[(2-ethyl-6-methylphenyl)amino]propoxy}acetic acid	251.33			√		√	√		
2-chloro-N-(2-ethyl-6-methylphenyl)-N-(1-hydroxypropan-2-yl)acetamide	269.77					√	√	√	√
2-chloro-N-(2-ethyl-6-methylphenyl)-N-(1-methoxypropan-2-yl)acetamide	283.13					√	√	√	√
2-chloro-N-(2-ethyl-6-methylphenyl)acetamide	211.08					√		√	√
2-Oxopropanal	72.02					√	√		
1-methoxypropan-2-one	88.05					√	√		
1-hydroxypropan-2-one	74.04					√	√		
2-ethyl-6-methyl alanine	135.10					√	√		
metolachlor morpholinone	233.31		√						√

Structural prioritization

The chemical structure of the 12 prioritized S-metolachlor TPs in step 1 was considered to proceed with the second prioritization step. Structurally similar molecules were manually gathered based on the research judgment, and one S-metolachlor TP for each group was prioritized. Two groups of similar structures were recognized, and two compounds were prioritized over the other structurally similar.

Group1. 2-chloro-N-(2-ethyl-6-methylphenyl)acetamide, 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(1-hydroxypropan-2-yl)acetamide, and 2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-methoxypropan-2-yl)acetamide (Figure 9).

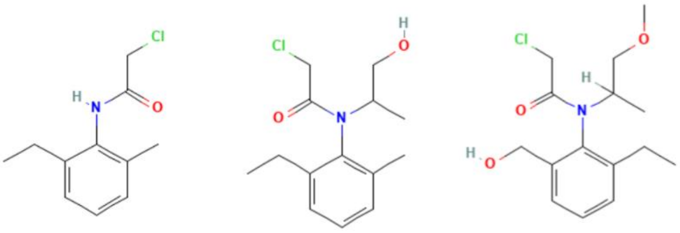
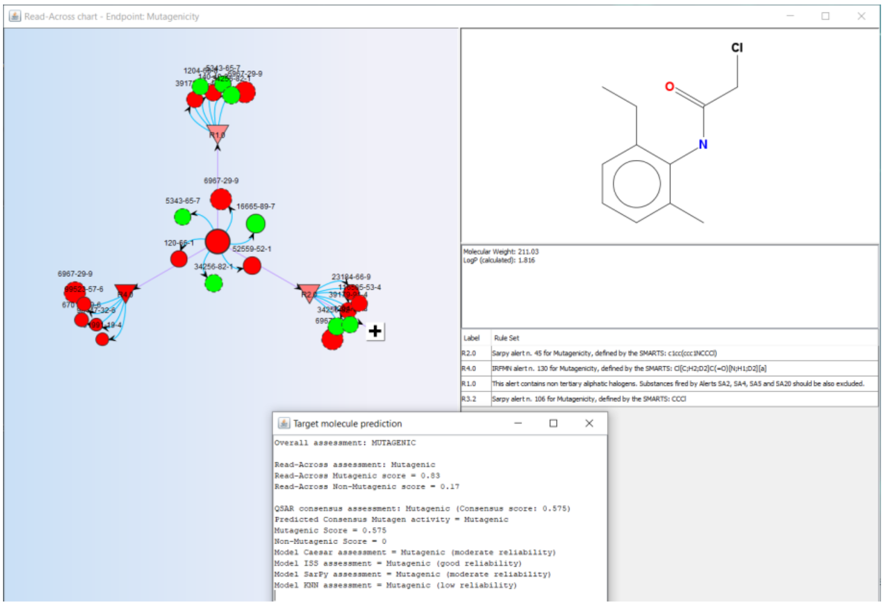


Figure 9. S-metolachlor TPs presenting similar structures (group 1): A) 2-chloro-N-(2-ethyl-6-methylphenyl)acetamide B) 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(1-hydroxypropan-2-yl)acetamide C) 2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-methoxypropan-2-yl)acetamide

Amongst the structures shown in Figure 9, the latter two were predicted by the majority of the models (three out of four), while 2-chloro-N-(2-ethyl-6-methylphenyl)acetamide was predicted by two models out of four (see Table 9). In order to prioritize the predicted S-metolachlor TPs, ToxRead BETA 0.23 was used to verify that the *read-across* and QSAR assessments for mutagenicity were comparable, thus, to justify that it was reasonable to perform the *in silico* hazard assessment only for one of them. Six molecules in the model training set were selected. The results of the ToxRead mutagenicity assessment for the three structurally similar S-metolachlor TPs are illustrated in Figure 10.



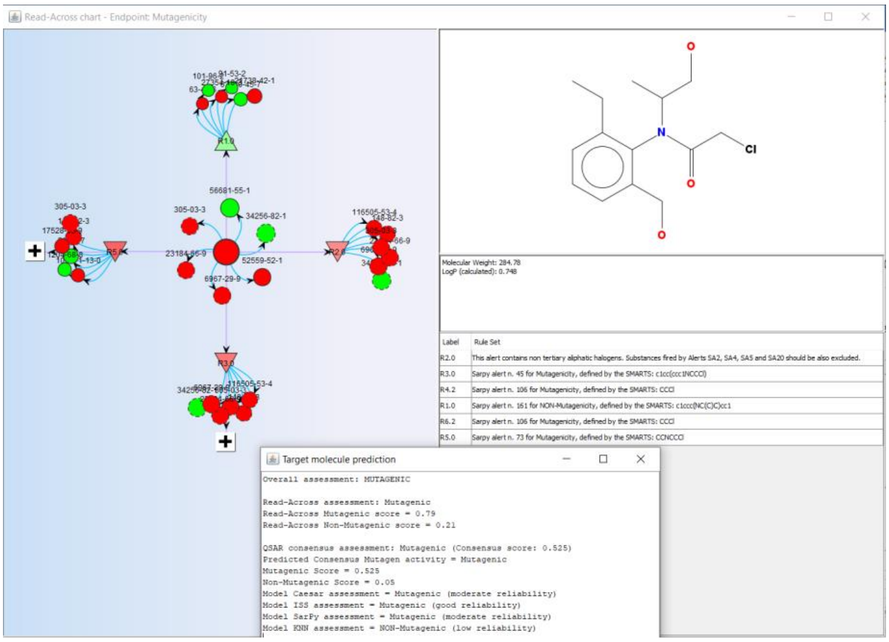
Molecular Weight: 211.03
LogP (calculated): 1.816

Label	Rule Set
R2.0	Sarpy alert n. 45 for Mutagenicity, defined by the SMARTS: c1cc(ccc1NCCCl)
R4.0	IRFMN alert n. 130 for Mutagenicity, defined by the SMARTS: Cl[C;H2;D2]C(=O)[N;H1;D2][a]
R1.0	This alert contains non tertiary aliphatic halogens. Substances fired by Alerts SA2, SA4, SA5 and SA20 should be also excluded.
R3.2	Sarpy alert n. 106 for Mutagenicity, defined by the SMARTS: CCCl

```
Overall assessment: MUTAGENIC

Read-Across assessment: Mutagenic
Read-Across Mutagenic score = 0.83
Read-Across Non-Mutagenic score = 0.17

QSAR consensus assessment: Mutagenic (Consensus score: 0.575)
Predicted Consensus Mutagen activity = Mutagenic
Mutagenic Score = 0.575
Non-Mutagenic Score = 0
Model Caesar assessment = Mutagenic (moderate reliability)
Model ISS assessment = Mutagenic (good reliability)
Model SarPy assessment = Mutagenic (moderate reliability)
Model KNN assessment = Mutagenic (low reliability)
```



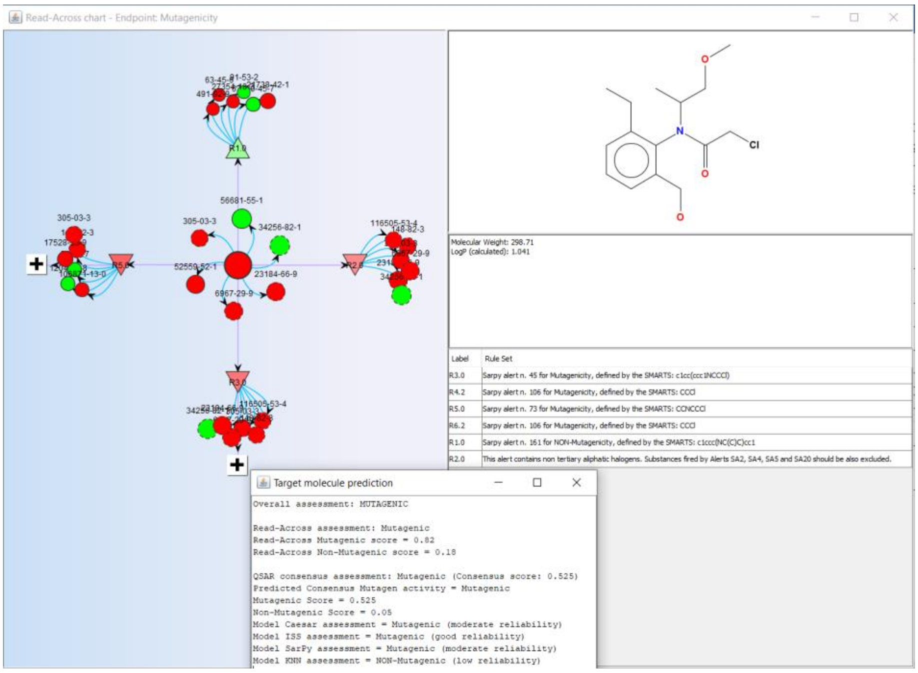
Molecular Weight: 284.78
LogP (calculated): 0.748

Label	Rule Set
R2.0	This alert contains non tertiary aliphatic halogens. Substances fired by Alerts SA2, SA4, SA5 and SA20 should be also excluded.
R3.0	Sarpy alert n. 45 for Mutagenicity, defined by the SMARTS: c1cc(ccc1NCCCl)
R4.2	Sarpy alert n. 106 for Mutagenicity, defined by the SMARTS: CCCl
R1.0	Sarpy alert n. 161 for NON-Mutagenicity, defined by the SMARTS: c1ccc(NC(C)C)cc1
R6.2	Sarpy alert n. 106 for Mutagenicity, defined by the SMARTS: CCCl
R5.0	Sarpy alert n. 73 for Mutagenicity, defined by the SMARTS: CCNCCCl

```
Overall assessment: MUTAGENIC

Read-Across assessment: Mutagenic
Read-Across Mutagenic score = 0.79
Read-Across Non-Mutagenic score = 0.21

QSAR consensus assessment: Mutagenic (Consensus score: 0.525)
Predicted Consensus Mutagen activity = Mutagenic
Mutagenic Score = 0.525
Non-Mutagenic Score = 0.05
Model Caesar assessment = Mutagenic (moderate reliability)
Model ISS assessment = Mutagenic (good reliability)
Model SarPy assessment = Mutagenic (moderate reliability)
Model KNN assessment = NON-Mutagenic (low reliability)
```



Molecular Weight: 298.71
LogP (calculated): 1.041

Label	Rule Set
R3.0	Sarpy alert n. 45 for Mutagenicity, defined by the SMARTS: c1cc(ccc1NCCCl)
R4.2	Sarpy alert n. 106 for Mutagenicity, defined by the SMARTS: CCCl
R5.0	Sarpy alert n. 73 for Mutagenicity, defined by the SMARTS: CCNCCCl
R6.2	Sarpy alert n. 106 for Mutagenicity, defined by the SMARTS: CCCl
R1.0	Sarpy alert n. 161 for NON-Mutagenicity, defined by the SMARTS: c1ccc(NC(C)C)cc1
R2.0	This alert contains non tertiary aliphatic halogens. Substances fired by Alerts SA2, SA4, SA5 and SA20 should be also excluded.

Target molecule prediction

```
Overall assessment: MUTAGENIC

Read-Across assessment: Mutagenic
Read-Across Mutagenic score = 0.82
Read-Across Non-Mutagenic score = 0.18

QSAR consensus assessment: Mutagenic (Consensus score: 0.525)
Predicted Consensus Mutagen activity = Mutagenic
Mutagenic Score = 0.525
Non-Mutagenic Score = 0.05
Model Caesar assessment = Mutagenic (moderate reliability)
Model ISS assessment = Mutagenic (good reliability)
Model SarPy assessment = Mutagenic (moderate reliability)
Model KNN assessment = NON-Mutagenic (low reliability)
```

*Figure 10. Read-across chart for mutagenicity provided by ToxRead BETA 0.23 for group 1: a) 2-chloro-N-(2-ethyl-6-methylphenyl)acetamide, b) 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(1-hydroxypropan-2-yl)acetamide, c) 2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-methoxypropan-2-yl)acetamide**.** Circles represent the molecules of the training set, with mutagenic (red) or non-mutagenic (green) experimental values, while triangles indicate the structural alerts for mutagenicity (red) or non-mutagenicity (green) recognized in the target molecule – (ToxRead, 2022).*

Using ToxTree v3.1.0, all the predicted S-metolachlor TPs discussed here were recognized as belonging to the Cramer Class III, associated with the lower Threshold of Toxicological Concern (TTC). The results obtained for genotoxicity and TTC were comparable for the three molecules considered; therefore, the analysis of only one was justified for this academic research. The genotoxicity alert identification provided by ToxRead was evaluated to prioritize one over the others. It recognized a higher number of structural alerts in the latter two S-metolachlor TPs than the smaller S-metolachlor TP 2-chloro-N-(2-ethyl-6-methylphenyl)acetamide. Between the latter two S-metolachlor TPs, the highest mutagenicity score was associated with 2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-methoxypropan-2-yl)acetamide (*Read-across* mutagenic score 0.92), which was thus prioritized amongst the predicted S-metolachlor TPs within the identified group of structurally similar molecules. When molecular size increases, toxicity decreases because bioavailability also decreases (Fishbein et al., 2016). However, as the changes were relatively small, in this research were prioritized the bigger molecules because they were associated with greater preliminary indagated structural alerts for toxicity. As the structure was similar between molecules B and C of group 1 since they differ only for a methyl group, 2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-methoxypropan-2-yl)acetamide was prioritized, because the non-mutagenic score was inferior (therefore, the chances it is mutagenic were considered higher). However, assuming that the presence of the methyl group does not modify the toxicity of the molecule significantly was not supported by evidence.

Group 2. 1-hydroxypropan-2-one and 1-methoxypropan-2-one (Figure 11).

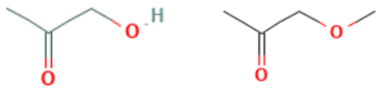
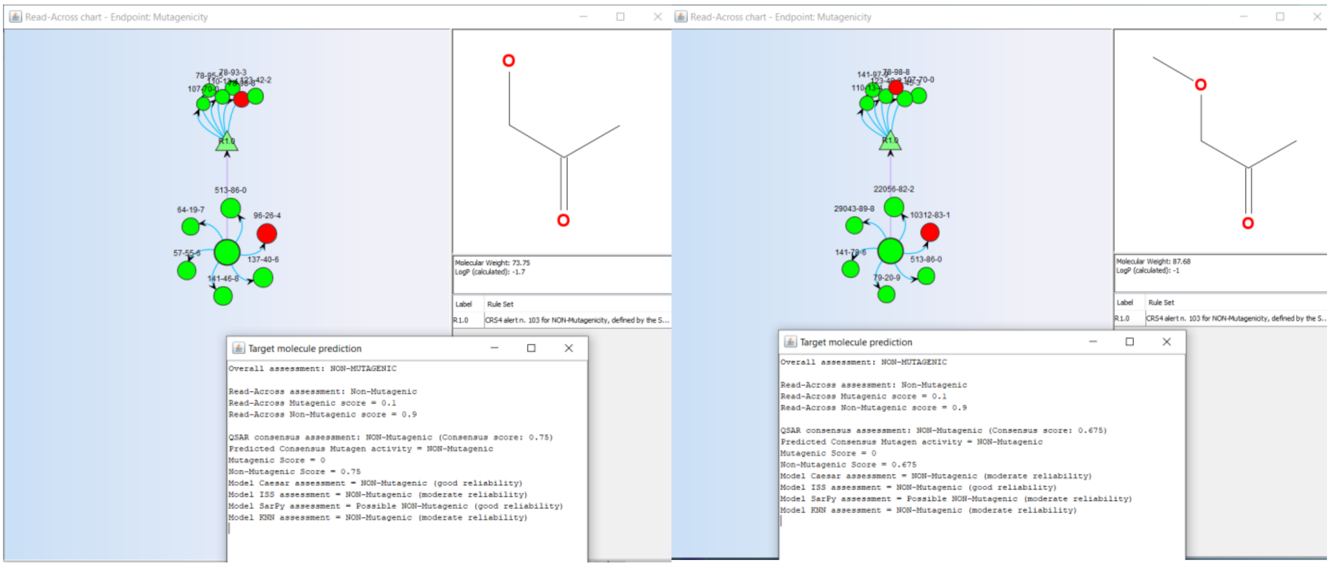


Figure 11. S-metolachlor TPs presenting similar structures (group 2): A) 1-hydroxypropan-2-one and B) 1-methoxypropan-2-one.

ToxRead BETA 0.23 generated the same prediction for both chemical structures. To prioritize the S-metolachlor TPs, the Cramer classes of both were predicted using ToxTree v3.1.0, and the results (Figure 12) showed that the 1-methoxypropan-2-one could be more critical (Cramer class III, associated with the lower TTC) then 1-hydroxypropan-2-one (Cramer class I). The difference in the prediction of the Cramer class depends on the presence of the -CH3 group in 1-methoxypropan-2-one. This characteristic makes the predicted S-metolachlor TP potentially more critical than the structurally similar S-metolachlor TP 1-hydroxypropan-2-one. Therefore, the bigger molecule was prioritized, in line with what was concluded for the prioritization of 2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-methoxypropan-2-yl)acetamide.



```
Overall assessment: NON-MUTAGENIC

Read-Across assessment: Non-Mutagenic
Read-Across Mutagenic score = 0.1
Read-Across Non-Mutagenic score = 0.9

QSAR consensus assessment: NON-Mutagenic (Consensus score: 0.75)
Predicted Consensus Mutagen activity = NON-Mutagenic
Mutagenic Score = 0
Non-Mutagenic Score = 0.75
Model Caesar assessment = NON-Mutagenic (good reliability)
Model ISS assessment = NON-Mutagenic (moderate reliability)
Model SarPy assessment = Possible NON-Mutagenic (good reliability)
Model KNN assessment = NON-Mutagenic (moderate reliability)
```

```
Overall assessment: NON-MUTAGENIC

Read-Across assessment: Non-Mutagenic
Read-Across Mutagenic score = 0.1
Read-Across Non-Mutagenic score = 0.9

QSAR consensus assessment: NON-Mutagenic (Consensus score: 0.675)
Predicted Consensus Mutagen activity = NON-Mutagenic
Mutagenic Score = 0
Non-Mutagenic Score = 0.675
Model Caesar assessment = NON-Mutagenic (moderate reliability)
Model ISS assessment = NON-Mutagenic (good reliability)
Model SarPy assessment = Possible NON-Mutagenic (moderate reliability)
Model KNN assessment = NON-Mutagenic (moderate reliability)
```

Figure 12. Read-across chart for mutagenicity provided by ToxRead BETA 0. 23 for the S-metolachlor TPs 1-hydroxypropan-2-one (left) and 1-methoxypropan-2-one (right). Circles represent the molecules of the training set, with mutagenic (red) or non-mutagenic (green) experimental values; while triangles indicate the structural alerts for mutagenicity (red) or non-mutagenicity (green) recognized in the target molecule – (ToxRead, 2022)

Toxicity prioritization

The relevance of the nine prioritized predicted S-metolachlor TPs was evaluated using ToxTree v3.1.0 Cramer Class classification and QSARToolbox Cramer classification. Only the compounds predicted in the High concern Cramer class III by at least one model and found in the literature were prioritized. A priority point was assigned for each prediction of high toxicological concern (Cramer class III) and the detection of information in the literature. Only compounds associated with at least two points were prioritized. Three S-metolachlor TPs were therefore prioritized as predicted to belong to the Cramer class III by both the applied models: 2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-methoxypropan-2-yl)acetamide, 1-methoxypropan-2-one, and metolachlor morpholinone. The results are summarized in Table 10.

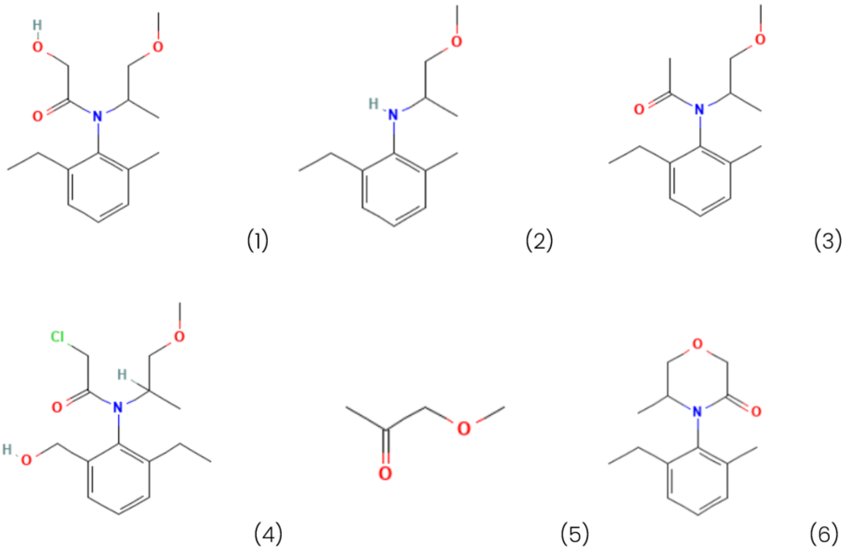
Table 10. S-metolachlor prioritized TPs Cramer classification provided by ToxTree (v3.1.0), showing whether they were found in the literature (√) or not (-). The prioritization points were used to select the S-metolachlor TPs of more significant toxicological concern. In orange are underlined the selected S-metolachlor TPs in this prioritization step.

	Cramer class		Literature	Prioritization points
	ToxTree	QSARToolbox		
2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-methoxypropan-2-yl)acetamide	III	III	√	3
1-methoxypropan-2-one	III	III	-	2
metolachlor morpholinone	III	III	√	3
metolachlor-2-hydroxy	I	III	√	2
metolachlor des(chloroacetyl)	I	III	√	2
metolachlor deschloro	I	III	√	2
2-{2-[(2-ethyl-6-methylphenyl)amino]propoxy}acetic acid	I	III	-	1
2-Oxopropanal	I	I	-	0
2-ethyl-6-methyl alanine	I	III	-	1

The methodology applied led to the exclusion of 2-oxopropanal, as both models predicted it as following in the Cramer class I associated with low toxicological concern. Furthermore, a second cross-research with the literature data was done, after which 2-ethyl-6-methyl alanine and 2-{2-[(2-ethyl-6-methylphenyl)amino]propoxy}acetic acid were also excluded because there was no correspondence in the literature and only one model predicted them as chemicals belonging the Cramer class III. Even though a direct correlation between 1-methoxypropan-2-one and S-metolachlor degradation was not found in the literature, it was included in the prioritized list because predicted by two *in silico* models as belonging to the Cramer Class III associated with the more toxicological severe concern. However, through a risk assessment for regulatory purposes, even compounds predicted of Cramer class III by only one model but not found in the literature should be considered.

Conclusions prioritization S-metolachlor transformation products

Conclusively, six S-metolachlor TPs were prioritized for the likelihood of being produced, or the toxicological predicted concern: metolachlor-2-hydroxy, metolachlor des(chloroacetyl), metolachlor deschloro, 2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-methoxypropan-2-yl)acetamide, 1-methoxypropan-2-one, and metolachlor morpholinone, visible in Figure 13.


(1) (2) (3)

(4) (5) (6)

Figure 13. Prioritized predicted S-metolachlor TPs: metolachlor-2-hydroxy (1), metolachlor deschloroacetyl (2), metolachlor deschloro (3), 2-chloro-N- (2-ethyl-6-(hydroxymethyl)phenyl)-N-(1-methoxypropan-2-yl)acetamide (4), 1-methoxypropan-2- one (5), and metolachlor morpholinone (6).

Table 11 summarizes the prioritized predicted TPs, the associated SMILES string, the exact mass, and the collected CAS number.

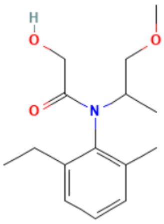
Table 11. Prioritized S-metolachlor TPs.

name	SMILES	formula	exact mass	CAS
metolachlor-2-hydroxy	CCC1=CC=CC(C)=C1N(C(C)COC)C(=O)CO	$C_{15}H_{23}NO_3$	265.1678	131068-72-9
metolachlor des(chloroacetyl)	CCC1=CC=CC(=C1NC(C)COC)C	$C_{13}H_{21}NO$	207.1623	51219-00-2
metolachlor deschloro	CCC1=CC=CC(C)=C1N(C(C)COC)C(C)=O	$C_{15}H_{23}NO_2$	249.1729	126605-22-9
2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-methoxypropan-2-yl)acetamide	CCC1=C(N(C(C)COC)C(=O)CCl)C(CO)=CC=C1	$C_{15}H_{22}ClNO_3$	299.1288	96394-97-7
1-methoxypropan-2-one	COCC(C)=O	$C_4H_8O_2$	88.05244	107-98-2
metolachlor morpholinone	CCC1=CC=CC(C)=C1N1C(C)COCC1=O	$C_{14}H_{19}NO_2$	233.1416	120375-14-6

4 Characterization S-metolachlor prioritized transformation products

Here are reported the in silico hazard assessment for the prioritized predicted S-metolachlor TPs. A summary of the most relevant prediction is here reported, while further information is reported in the supplementary documents (see Annex 11). In Table 18 (page 92) is available a summary of the *in silico* hazard assessment of S-metolachlor TPs.

4.1 Metolachlor-2-hydroxy



Molecular information metolachlor-2-hydroxy	
name	Metolachlor-2-hydroxy
IUPAC	N-(2-ethyl-6-methylphenyl)-2-hydroxy-N-(1-methoxypropan-2-yl)acetamide
routes	Halogenated Aliphatics: Nucleophilic Substitution (no adjacent X) (CTS_hydrolisis library) Biotransformation
likelihood	High* Predicted by all the TPs prediction tools Main product CTS_hydrolisis library (96.08%, accumulation 92.16%)
SMILES	CCC1=CC=CC(C)=C1N(C(C)COC)C(=O)CO
canonical SMILES	CCC1=CC=CC(=C1N([C@@H](C)COC)C(=O)CO)C
formula	$C_{15}H_{23}NO_3$
mass	265.353
CAS	131068-72-9

All in silico tools predicted Metolachlor-2-hydroxy. Moreover, it was the main product predicted by the CTS hydrolysis library. It was the product of hydrogenolysis, therefore, relevant for advanced oxidation processes

(AOPs), ozonation, and chlorination. Moreover, it was also predicted by all the models of biotransformation considered, therefore relevant for rapid sand filtration and wastewater treatments.

Metolachlor-2-hydroxy is reported with a GHS Hazard Statement H412: harmful to aquatic life with long-lasting effects, and the Precautionary statements to avoid environmental release (P273) (ECHA, 2022a). It was found in groundwater (Soulier et al., 2016).

Two tools confirmed the predicted moderate water solubility. The prediction of CompTox and CTS were found to be of the same order of magnitude: for CompTox, it was 746.481 mg/L, while for CTS, it was 985 mg/L. These values are associated with moderate solubility in water. Therefore metolachlor-2-hydroxy can be expected to be found in the water after its formation. Moreover, metolachlor-2-hydroxy seemed to be more soluble than S-metolachlor. This is justified by substituting the halogen group -Cl with an -OH group that is more hydrophilic.

In silico hazard assessment

The mutagenicity endpoint assessment was inconclusive, but some indications of possible genotoxicity via chromosomal aberration were identified. Results on carcinogenicity were inconclusive as well. Overall, the results indicated negativity for the endocrine disruption endpoint, suggesting a reduction of toxicological potential compared to the parent compound. On the other hand, some models predicted metolachlor-2-hydroxy to be active for developmental and reproductive toxicology. Moreover, an overall strongly reliable prediction for the skin sensitization endpoint was also observed. The results of the specific *in silico* predictions were here discussed in detail. The selection of the endpoints is available in the Methods section (see paragraph 1.2.1), and the results are summarized in Table 12.

Genotoxicity

Metolachlor-2-hydroxy appeared to be non-genotoxic either through mutagenicity or chromosomal aberration. In particular, the ISS models for mutagenicity and CORAL for chromosomal aberration offered by VEGA have provided reliable predictions. However, the CONSENSUS model for mutagenicity (v 1.0.3) predicted metolachlor-2-hydroxy non-mutagenic; thus, the prediction was considered inconclusive. The SarPy structural alert for non-mutagenicity was found, which was already found in S-metolachlor (SM161, see Figure 4). However, the prediction presented some criticism since some molecules of the training set presented experimental values that disagreed with the prediction. The *read-across* assessment was non-mutagenic with (a non-mutagenic score of 0.77), to support the prediction of the inactivity of S-metolachlor towards the endpoint. The CompTox model for the Ames mutagenicity model also predicted negativity to the endpoint for metolachlor-2-hydroxy, as none of the models recognized positivity to the endpoint. In agreement, no alerts were found for *in vitro* mutagenicity (Ames test) in QSARToolbox.

However, one alert indicated interaction with the DNA via non-covalent binding, an *in vivo* mutagenicity (micronucleus) alert defined by ISS. Also, OSIRIS recognized a medium-risk fragment indicating mutagenicity, already found in S-metolachlor. Nevertheless, were absent the high-risk and medium-risk fragments associated with the halogen group in the parent compound; therefore, the *in silico* assessment was not sufficient to conclude on the mutagenicity endpoint.

Carcinogenicity

The results did not sufficiently define the carcinogenic potential of metolachlor-2-hydroxy, as the predictions did not offer concordant results.

Two out of six models in the VEGA software provided a reliable prediction for metolachlor-2-hydroxy, and they disagreed with the result. The CAESAR (v 2.1.9), ISS (v.1.0.0), IRFMN/Antares (v1.0.0), and IRFMN Carcinogenicity inhalation classification models were inconclusive for metolachlor-2-hydroxy (ADI < 0.75). On the other hand, the IRFMN/ISSCAN-CGX (v1.0.0) predicted metolachlor-2-hydroxy to be a carcinogen with high reliability (3/3) for the recognition of two structural alerts defined by the SMART: N(CCO)CCO and Nc1ccccc1 (the latter, already found in S-metolachlor). Nevertheless, the IRFMN carcinogenicity oral classification model (v 1.0.0) predicted metolachlor-2-hydroxy to be non-carcinogenicity, with high reliability (3/3).

OSIRIS predicted tumorigenic effects due to the recognition of a medium-risk fragment indicating tumorigenicity, the same fragment recognized as an alert for mutagenicity for metolachlor-2-hydroxy and already found in S-metolachlor (see Figure 5). However, no structural alerts were found by the QSARToolbox profiling for carcinogenicity, both for a genotoxic and non-genotoxic MoA.

Reproductive and developmental toxicology

Various models predicted metolachlor-2-hydroxy to be active for the endpoints.

In VEGA, two models gave moderate reliable predictions, assessing metolachlor-2-hydroxy as possibly active for the selected endpoints. The CAESAR developmental Toxicity model (v 2.1.7) predicted metolachlor-2-hydroxy to be active for the developmental toxicity endpoint, with moderate reliability (2/3). Nevertheless, the concordance index was 0.49; therefore, the prediction was considered inconclusive. The IRFMN/CORAL Zebrafish embryo AC50 (v.1.0.0) predicted metolachlor-2-hydroxy AC50 as 3712.45 μg/L, notably inferior to the experimental AC50 collected for S-metolachlor (9536.43 μg/L). However, the predicted AC50 for metolachlor-2-hydroxy was closer to the one predicted for S-metolachlor. The predicted value AC50 represented an intermediate risk factor for reproductive and developmental toxicology. Accordingly, the CompTox consensus result was positive for developmental toxicity as all models have predicted positivity for the endpoint and recognized two medium-risk fragments for reproductive effects, already found in S-metolachlor (see Figure 6). However, as the halogen group was absent, no high-risk fragments were identified.

Conclusively, QSARToolbox reported a precedent known reproductive and developmental toxic potential associated with toluene and alkyl toluene derivates (8a).

Endocrine disruption

The reported experimental values by the models available for the endpoints suggested the inactivity of S-metolachlor for this endpoint.

Most of the VEGA models agreed with the prediction of the inactivity of metolachlor-2-hydroxy towards endocrine disruption toxicological effects with high reliability. Only a model predicted the possible interaction of metolachlor-2-hydroxy with the androgen receptor, but with moderate reliability, as data in the training set disagreed with the positive prediction. Moreover, four models predicted metolachlor-2-hydroxy to be inactive towards the receptor, with high reliability (3/3). The IRFMN/COMPARA Androgen Receptor-mediated effect (v.1.0.0) predicted activity instead towards the androgen receptor with moderate reliability (2/3), as similar molecules found in the training set had experimental values that disagreed with the prediction. Also, QSARToolbox recognized metolachlor-2-hydroxy as a non-binder of the Estrogen Receptor, confirming the prediction provided by VEGA. Only CompTox predicted positivity for the Estrogen Receptor Binding for metolachlor-2-hydroxy.

Skin sensitization/irritation

Metolachlor-2-hydroxy appeared to be a possible irritant and sensitizer.

In VEGA, one model predicted activity for skin sensitization, while the other did not provide a reliable prediction, leaving the assessment of the potential skin sensitization uncertain. The CAESAR Skin sensitization model (v 2.1.6) assessed metolachlor-2-hydroxy as a possible sensitizer, with moderate reliability (2/3). Even though the similarity and accuracy indexes were optimal, some atom-centered fragments are rarely found in the training set. Nevertheless, the prediction was validated because the molecules of the training had experimental values in agreement with the prediction.

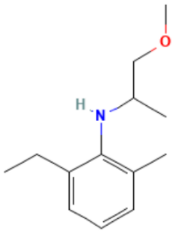
OSIRIS predicted irritant effects due to recognizing four high-risk fragments indicating irritating effects out of the six detected for S-metolachlor (see Figure 7). Accordingly, the profiling applied in QSARToolbox predicted protein binding (OECD rules) by direct acylation involving a leaving group. On the other hand, the OASIS rules for protein binding did not identify possible structural alerts. Protein binding could be responsible for skin protein binding and, therefore, for the sensitizing/irritating effect. However, no inclusion criteria for skin irritation/corrosion were found by the BfR rules, while exclusion rules were met: group -CN.

Table 12. In silico hazard assessment of metolachlor-2-hydroxy for genotoxicity, carcinogenicity, developmental and reproductive toxicology, endocrine disruption, skin sensitization, and Cramer class evaluation. The prediction was 🟥 *= positive,* 🟨 *= intermediate,* 🟩 *= negative, or* ⬜ *= inconclusive. The Applicability Domain Index (ADI) scores, thus the internal validation of the models, are reported (see Methods section paragraph 1.2.2).*

endpoint		software	model	prediction & score
genotoxicity	mutagenicity	VEGA	CONSENSUS v1.0.3	⬜
			CEASAR v2.1.13	⬜ 0.643
			SarPy/IRFMN v1.0.7	⬜ 0.76
			ISS v1.0.2	⬜ 0
			KNN/*Read-Across* v1.0.0	⬜ 0
		ToxRead	*Read-across*	🟩 0.77
			QSAR consensus	⬜ 0.25
		CompTox	Consensus Ames mutagenicity	🟩
		OSIRIS	Mutagenic	🟨
		QSARToolbox	Mutagenicity	🟩
	chromosomal aberration	VEGA	CORAL v1.0.0	⬜ 0
			IRFMN *In vitro* micronucleus v1.0.0	🟥 0.759
			IRFMN *In vivo* micronucleus v1.0.1	🟩 0.772
		QSARToolbox	Chromosomal aberration	🟥
carcinogenicity		VEGA	CEASAR v2.1.9	⬜ 0
			ISS v1.0.2	⬜ 0
			IRFMN/Antares v1.0.0	⬜ 0.527
			IRFMN/ISSCAN-CGX v1.0.0	🟥 0.82
			IRFMN carcinogenicity oral classification v1.0.0	🟩 0.967
			IRFMN carcinogenicity inhalation classification v1.0.0	⬜ 0
		OSIRIS	Tumorigenic	🟨
		QSARToolbox	Carcinogenicity	🟩

developmental/reproductive toxicology	VEGA	CEASAR v2.1.7	0.765
		Developmental/Reproductive Tox library v.1.1.0	0
		IRFMN/CORAL Zebrafish embryo AC50 v1.0.0	3712.45 µg/L
	CompTox	Developmental toxicity	🟥
	OSIRIS	Reproductive effective	🟨
	QSARToolbox	DART scheme	🟥
endocrine disruption	VEGA	NRMEA Thyroid Receptor Alpha effect v1.0.0	0.951
		NRMEA Thyroid Receptor Beta effect v1.0.0	0.951
		IRFMN Aromatase activity v1.0.0	0
		IRFMN Estrogen Receptor Relative Binding Affinity v1.0.1	0.939
		IRFMN/CERAPP Estrogen Receptor-mediated effect v1.0.0	0.956
		IRFMN/COMPARA Androgen Receptor-mediated effect v1.0.0	0.795
	CompTox	Estrogen Receptor Binding	🟥
	QSARToolbox	OECD Estrogen binding	🟩
skin sensitization	VEGA	CEASAR v2.1.6	0.758
		IRFMN/JRC v1.0.0	0
	OSIRIS	Irritant	🟥
	QSARToolbox	OECD protein binding	🟥

4.2 Metolachlor deschloroacetyl



name	Metolachlor des(chloroacetyl) Deschloroacetylmetolachlor
IUPAC	2-ethyl-N-(1-methoxypropan-2-yl)-6-methylaniline
routes	Amide hydrolysis (CTS_hydrolysis library) N-aryl Amide Photohydrolysis (CTS_direct photolysis library) Biotransformation
likelihood	High Predicted by more tools Minor product CTS_hydrolysis library (1.96%), CTS_direct photolysis library (11.11%), and CTS_combined library (14.84%)
Canonical SMILES	CCC1=CC=CC(C)=C1NC(C)COC
Isomeric SMILES	CCC1=CC=CC(=C1N[C@@H](C)COC)C
formula	$C_{13}H_{21}NO$
mass	207.317
CAS	51219-00-2

Metolachlor deschloroacetyl was reported by most TPs models, and different reaction pathways were found. However, the percentage of production and accumulation assessed by the CTS tool was relatively low compared to the other predicted S-metolachlor TPs. It may be relevant for AOPs, UV treatments, ozonation, chlorination, RSF, and WWT.

Metolachlor deschloroacetyl may cause skin, eye, and respiratory irritation, respectively, the Hazard (H) Statements H315, H319, and H335. It is also considered toxic (H411) and harmful (H412) to aquatic life, with long-lasting effects (ECHA, 2022b). A Precautionary (P) Statement is also associated with the chemical, indicating to avoid breathing dust, fumes, gas, mist, vapors, and spray (P261). Toxicological data was absent in PubMed, Science Direct, and Scopus. It was detected by a monitoring study in drinking water at concentrations up to 35 ng/L (Hladik et al., 2008) and in groundwater (Soulier et al., 2016). Groundwater concentrations were similar to surface water in the spring but lower in the fall season (Hladik et al., 2008). The study of Hladik et al. (2008) suggested that it was not efficiently removed with conventional water treatment practices such as coagulation (removal efficiencies 10% defined by Hladik et al., 2008), flocculation, filtration, and chlorination. However, a previous study conducted by the same research group (Hladik et al., 2005) showed up to 100% of removal of metolachlor deschloroacetyl, as well as the chloroacetanilide herbicides transformation products that lack the acetanilide functional group, during ozonation at specific rates (respectively, chlorination with 6mg/L applied free chlorine and 3mg/L of ozone), and powdered activated carbons (PAC). Adsorption capacities over PAC were correlated to K_{ow} values. Nevertheless, the study pointed out that the possible resulting products are unknown; thus, further research is needed to understand whether the elimination of metolachlor deschloroacetyl leads to the formation of other TPs of concern.

Water solubility was predicted by CompTox as 252.039 mg/L, while by CTS as 3.35 mg/L, which was two orders of magnitude difference. The solubility in the water was predicted to decrease in comparison to S-metolachlor. This decrease was expected, as the hydroxy functional group (-OH) was present in S-metolachlor but not in metolachlor deschloroacetyl.

In silico hazard assessment

The overall assessment of metolachlor deschloroacetyl suggested inactivity towards the genotoxicity, even though a prediction of mutagenic potential with high reliability and optimal parameters of the molecules of the training set was available. Moreover, QSARToolbox profiled S-metolachlor as possible genotoxic through chromosomal aberration mode of action.

The inactivity of metolachlor deschloroacety towards endocrine disruption was also predicted. However, the Aromatase model (which appeared to be the mechanism of action of the parent compound S-metolachlor) provided an inconclusive prediction. Moreover, regarding carcinogenicity, the predictions are inconclusive, possibly justifiable because no data on the carcinogenic potential of S-metolachlor was found in the literature. Therefore the negative predictions still present uncertainties that need to be clarified by further research. On the other side, the reproductive and development and skin sensitization/irritation *in silico* hazard assessment suggested possible activity towards the endpoints. The predicted effect on membrane integrity could explain the possible irritating effect. The *in silico* results are in the supplementary material (see Annex 11) and summarized in Table 13.

Genotoxicity

Metolachlor deschloroacetyl was predicted to be non-genotoxic by the majority of the models, even though one model for mutagenicity and one for chromosomal aberration gave a positive prediction for the genotoxicity endpoint. The VEGA CONSENSUS model for mutagenicity (v 1.0.3) predicted metolachlor deschloroacetyl non-mutagenic, with a consensus score of 0.25, even though the CAESAR mutagenicity model (v 2.1.13) predicted the compound to be mutagenic with good reliability (3/3). On one side, a SarPy structural alert for non-mutagenicity was found, which was already identified in S-metolachlor. Nevertheless, the SarPy mutagenicity model did not provide a reliable prediction. Therefore, the VEGA CONSENSUS score could not be considered reliable as the results of the different models were discordant. Regarding chromosomal aberration, the IRFMN/VERMEER *in vitro* micronucleus model (v1.0.0) found four structural alerts for activity in the micronucleus assay. In comparison, the VEGA IRFMN *in vivo* micronucleus model (v1.0.0) recognized two structural alerts for inactivity towards the assay. Also, the results were in contrast; therefore, the overall prediction was not considered reliable.

ToxRead BETA 0.23 recognized two SarPy alerts for non-mutagenicity. The *read-across* assessment was non-mutagenic with a non-mutagenic score of 0.77, and the QSAR consensus assessment was non-mutagenic with a mutagenic score of 0.25. The most similar molecule in the training set (similarity index = 0.922) was associated with an experimental value of non-mutagenic.

CompTox predicted negativity to the mutagenicity endpoint, and all the models agreed. Moreover, no alerts were found for mutagenicity for metolachlor deschloroacetyl by OSIRIS. Accordingly, no alerts were found for *in vitro* mutagenicity (Ames test). One alert was found for interaction with the DNA via non-covalent binding, an *in vivo* mutagenicity (micronucleus) alert defined by ISS.

Carcinogenicity

None of the adopted models of VEGA provided a reliable prediction of the carcinogenicity potential of metolachlor deschloroacetyl. Moreover, no structural alerts were found by OSIRIS and QSARToolbox. However, the collected information was insufficient to exclude S-metolachlor's carcinogenic potential.

Reproductive and developmental toxicology

The VEGA CAESAR developmental Toxicity model (v 2.1.7) predicted metolachlor deschloroacetyl to be active for the developmental toxicity endpoint. Nevertheless, the concordance index was 0.49, which could mean the prediction was inconclusive. High reliability was associated with the prediction offered by the IRFMN/CORAL Zebrafish embryo AC50 (v1.0.0), which predicted metolachlor deschloroacetyl to be active for the endpoint in the 50% of the population of Zebrafish at 1642.65 µg/L. The prediction was of an activity increment when S-metolachlor was transformed into metolachlor deschloroacetyl. On the other hand, the developmental/reproductive Toxicity library (PG) v1.1.0 gave a negative prediction for the endpoint. In that case, the concordance index was low (0.511); therefore, the prediction was considered inconsistent.

CompTox predicted positivity to the developmental toxicity endpoint. The hierarchic clustering and the single model agree with the prediction, while the nearest neighbor model showed similar compounds disagreeing with the prediction. Therefore, the prediction was considered inconsistent. However, in OSIRIS, no alerts were found for the reproductive effects of metolachlor deschloroacetyl. While in QSARToolbox, it was recognized the reproductive and developmental toxic potential associated with toluene and alkyl toluene derivates.

Endocrine disruption

All the models selected in VEGA except one provided a prediction of inactivity towards specific endocrine receptors; however, the endpoint needs to be further evaluated as the mechanisms here evaluated are limited for the complexity of the mechanisms involved. Also, in this case, the aromatase model did not provide a reliable prediction, even though it is a relevant endpoint considering that the parent compound was proved to impact the aromatase activity *in vivo*.

The IRFMN Estrogen Receptor Relative Binding Affinity model (v 1.0.1), the IRFMN/CERAPP Estrogen Receptor-mediated effect (v 1.0.0), and the IRFMN/COMPARA Androgen Receptor-mediated effect (v.1.0.0) predicted metolachlor deschloroacetyl to be inactive towards the receptors with high (3/3) to moderate (2/3) reliability. Also, CompTox predicted negativity for the estrogen receptor binding, even though the nearest neighbor disagreed with the prediction. Since the *read-across* provided evidence that the most similar compound was active for the endpoint, the negative prediction was considered inconsistent. In line with it, the QSARToolbox profiled metolachlor deschloroacetyl as a non-binder of the estrogen receptor.

Skin sensitization/irritation

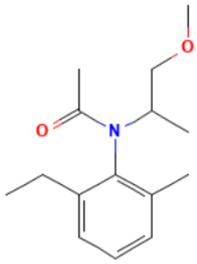
Structural alerts were found for metolachlor deschloroacetyl, but no QSAR models confirmed the sensitizer/irritating effects. Even though two high-risk fragments for irritating effects were recognized (and already present in S-metolachlor, see Figure 7), none of the models in VEGA provided a reliable prediction for metolachlor deschloroacetyl as regards skin sensitization/irritation. Moreover, no inclusion rules were found for skin irritation/corrosion in QSARToolbox.

Table 13. In silico hazard assessment of metolachlor des(chloroacetyl) for genotoxicity, carcinogenicity, developmental and reproductive toxicology, endocrine disruption, skin sensitization, and Cramer class evaluation. The prediction was 🟥 = positive, 🟨 = intermediate, 🟩 = negative, or ⬜ = inconclusive. The Applicability Domain Index (ADI) scores, thus the internal validation of the models, are reported (see Methods section paragraph 1.2.2).

endpoint		software	model	prediction & score
genotoxicity	mutagenicity	VEGA	CONSENSUS v1.0.3	⬜
			CEASAR v2.1.13	🟥 0.93
			SarPy/IRFMN v1.0.7	⬜ 0
			ISS v1.0.2	🟩 0.897
			KNN/*Read-Across* v1.0.0	⬜ 0
		ToxRead	*Read-across*	🟩 0.77
			QSAR consensus	🟩 0.25
		CompTox	Consensus Ames mutagenicity	🟩
		OSIRIS	Mutagenic	🟩
		QSARToolbox	Mutagenicity	🟩
	chromosomal aberration	VEGA	CORAL v1.0.0	🟩 0.762
			IRFMN *In vitro* micronucleus v1.0.0	🟥 0.756
			IRFMN *In vivo* micronucleus v1.0.1	🟩 0.924
		QSARToolbox	Chromosomal aberration	🟥
carcinogenicity		VEGA	CEASAR v2.1.9	⬜ 0.455
			ISS v1.0.2	⬜ 0.654
			IRFMN/Antares v1.0.0	⬜ 0
			IRFMN/ISSCAN-CGX v1.0.0	⬜ 0.617
			IRFMN carcinogenicity oral classification v1.0.0	⬜ 0.655
			IRFMN carcinogenicity inhalation classification v1.0.0	⬜ 0
		OSIRIS	Tumorigenic	⬜
		QSARToolbox	Carcinogenicity	🟩

developmental/reproductive toxicology	VEGA	CEASAR v2.1.7	0.764
		Developmental/Reproductive Tox library v.1.1.0	0.76
		IRFMN/CORAL Zebrafish embryo AC50 v1.0.0	1642.65 µg/L
	CompTox	Developmental toxicity	
	OSIRIS	Reproductive effective	🟩
	QSARToolbox	DART scheme	🟥
endocrine disruption	VEGA	NRMEA Thyroid Receptor Alpha effect v1.0.0	0.951
		NRMEA Thyroid Receptor Beta effect v1.0.0	0.951
		IRFMN Aromatase activity v1.0.0	0
		IRFMN Estrogen Receptor Relative Binding Affinity v1.0.1	0.916
		IRFMN/CERAPP Estrogen Receptor-mediated effect v1.0.0	0.925
		IRFMN/COMPARA Androgen Receptor-mediated effect v1.0.0	0.784
	CompTox	Estrogen Receptor Binding	
	QSARToolbox	OECD Estrogen binding	🟩
skin sensitization	VEGA	CEASAR v2.1.6	0.646
		IRFMN/JRC v1.0.0	0
	OSIRIS	Irritant	🟥
	QSARToolbox	OECD protein binding	🟩

4.3 Metolachlor deschloro



name	Metolachlor deschloro
IUPAC	N-(2-ethyl-6-methylphenyl)-N-(1-methoxypropan-2-yl)acetamide
route	Hydrogenolysis Biotransformation
likelihood	High Main product CTS_abiotic reduction library (production and accumulation 100%)
SMILES	CCC1=CC=CC(C)=C1N(C(C)COC)C(C)=O
Canonical SMILES	CCC1=CC=CC(=C1N([C@@H](C)COC)C(=O)C)C
formula	$C_{15}H_{23}NO_2$
mass	249.354
CAS	126605-22-9

Metolachlor deschloro, a dehalogenated predicted S-metolachlor TP, was indicated by most TP prediction tools and was the main product predicted by the CTS_abiotic reduction library. It was predicted to be formed due to hydrogenolysis or biotransformation; therefore, it may be relevant for advanced reduction processes, RFS and WWT.

CTS predicted water solubility for metolachlor deschloro was 301 mg/L; therefore, it was considered moderately soluble in water and can be found in drinking water (as the study of Hladik et al., 2008, confirmed). Accordingly, metolachlor deschloro was found in drinking water at concentrations up to 30 ng/L (Hladik et al., 2008), but it was suggested high removal (up to 100%) by chlorination, ozonation, and powdered activated carbons (PAC). Nevertheless, the study pointed out that the possible resulting products are unknown; thus, further research is needed to understand whether the elimination of metolachlor deschloro could lead to the formation of other TPs of concern. Guelfi et al. (2018) detected metolachlor deschloro after advanced oxidation processes (AOPs), in particular EO-H2O2, EF, and PEF, contiguously with the reduction of S-metolachlor. Also, metolachlor deschloro was identified in groundwater (Soulier et al., 2016).

In silico hazard assessment

Metolachlor deschloro was predicted to be possibly genotoxic only through a mutagenicity mode of action, while the carcinogenic assessment was inconclusive. Three *in silico* tools predicted developmental/reproductive potential while excluding the endocrine disruption activity. Metolachlor deschloro was recognized as a possible irritant. The *in silico* results are summarized in the supplementary documentary (Annex 11) and Table 14.

Genotoxicity

The VEGA CONSENSUS model for mutagenicity (v 1.0.3) predicted metolachlor deschloro to be mutagenic, with a consensus score of 0.15, as only the CAESAR model (v2.1.13) gave a moderate reliable (2/3) prediction. On the other hand, as regards chromosomal aberration, the IRFMN *In vivo* Micronucleus activity (v 1.0.0) gave a negative prediction to the endpoint with moderate reliability (2/3). However, the CONSENSUS assessment was considered inconsistent as justified by only one model.

In ToxRead BETA 0.23, the consensus mutagenic and non-mutagenic scores were equivalent, while the QSAR consensus assessment was mutagenic. However, the most similar molecule in the training set (similarity index = 0.9210) was associated with an experimental value of mutagenicity; therefore, the prediction was considered relevant. Also, in CompTox, the results were contradictory. Indeed, the software predicted metolachlor deschloro to be inactive for the Ames mutagenicity endpoint. However, the hierarchical clustering model contradicted the consensus prediction. In contrast, OSIRIS recognized a medium-risk fragment in metolachlor deschloro, indicating mutagenicity, already found in metolachlor-2-hydroxy and S-metolachlor. Moreover, QSARToolbox recognized an alert for *in vivo* mutagenicity (Ames test) for metolachlor deschloro.

Carcinogenicity

The overall assessment provided by VEGA was not satisfactory, as only the IRFMN/ISSCAN-CGX carcinogenicity model (v1.0.0) provided a highly reliable (3/3) prediction of carcinogenicity for the endpoint for metolachlor deschloro. In contrast, the other models did not provide a reliable prediction. In most models, the output was associated with an ADI of 0 or inferior to the defined threshold of 0.75. In agreement with the positive prediction, OSIRIS recognized a medium-risk fragment in metolachlor deschloro, indicating carcinogenicity, already found in metolachlor-2-hydroxy and S-metolachlor (see Figure 5). Conversely, the profiling in QSARToolbox did not recognize any alert for carcinogenicity.

Reproductive and developmental toxicology

The overall VEGA prediction was non-active for reproductive and developmental toxicology. However, the parameters did not justify the predictions, therefore, were considered inconsistent. The IRFMN/CORAL Zebrafish embryo AC50 (v.1.0.0) predicted a value of 3000.06 µg/L. with high reliability (3/3), which represents an indication of an intermediate concern. However, CompTox predicted positivity for the developmental toxicity endpoint with high reliability, as all the models agreed with the prediction. Also, in OSIRIS, two medium-risk fragments in metolachlor deschloro indicated reproductive effects (already found in metolachlor-2-hydroxy and

S-metolachlor, see Figure 6) were recognized. Moreover, the profiling in QSARToolbox recognized the reproductive and developmental toxic potential associated with toluene and alkyl toluene derivates.

Endocrine disruption

Metolachlor deschloro was predicted as non-active for the endocrine disruption endpoint. Only the VEGA IRFMN/COMPARA Androgen Receptor-mediated effect (v1.0.0) predicted positivity to the endpoint. However, the concordance index was below 0.5, meaning that a consistent number of molecules in the training set was associated with experimental values that disagreed with the prediction. Moreover, in this case, the IRFMN Aromatase activity v1.0.0 model did not provide a reliable prediction. In line with the inactivity predicted by VEGA, CompTox predicted negativity for the Estrogen Receptor Binding for metolachlor deschloro and, accordingly, QSARToolbox recognized metolachlor deschloro as a non-binder of the estrogen receptor. The results are available in the supplementary documents.

Skin sensitization/irritation

The VEGA results for metolachlor deschloro as regards skin sensitization were inconclusive, as none of the models could provide a reliable prediction. The model CEASAR v2.1.6 predicted the compound as active; however, some molecules of the training set were structurally dissimilar from metolachlor deschloro (similarity index was below 0.8), and not all the atom-centered fragments present in the compound were identified in the molecules of the training set. Therefore, the accuracy and concordance indexes were optimal (equal 1). However, the most similar molecule (similarity index 0.809) was associated with an experimental value of sensitizer; therefore, the prediction was considered. The fact that the similarity index was relatively low (0.798) explained why not all the atom-centered fragments were found in the training set molecules, justifying the prediction's acceptance even though the ACF was not optimal.

On the contrary, the VEGA model IRFMN/JRC v1.0.0 predicted metolachlor deschloro as inactive towards the endpoint, but the prediction was considered inconsistent. Also, in this case, the prediction presented critical aspects. Indeed, most molecules in the training set were associated with an experimental value different from the provided prediction. Note that the accuracy and concordance indexes were assessed as of higher relevance for the prediction reliability rather than the ACF index. Accordingly, with the active prediction of the CAESAR v2.1.6 model, OSIRIS, the model recognized four high-risk fragments indicating irritating effects (Figure 14). The QSARToolbox profiling did not identify metolachlor as a sensitizer, but structural alerts for OECD protein binding were shown.

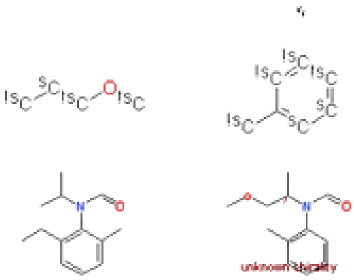


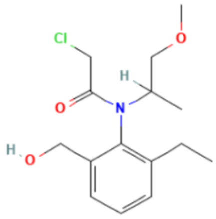
Figure 14. Risks fragments identified by OSIRIS irritating for metolachlor deschloro (OSIRIS, 2022)

Table 14. In silico hazard assessment of metolachlor deschloro for genotoxicity, carcinogenicity, developmental and reproductive toxicology, endocrine disruption, skin sensitization, and Cramer class evaluation. The prediction was 🟥 = positive, 🟨 = intermediate, 🟩 = negative, or ⬜ = inconclusive. The Applicability Domain Index (ADI) scores, thus the internal validation of the models, are reported (see Methods section paragraph 1.2.2).

endpoint		software	model	prediction & score
genotoxicity	mutagenicity	VEGA	CONSENSUS v1.0.3	⬜ 0.15
			CEASAR v2.1.13	🟥 0.768
			SarPy/IRFMN v1.0.7	⬜ 0.647
			ISS v1.0.2	⬜ 0
			KNN/*Read-Across* v1.0.0	⬜ 0
		ToxRead	*Read-across*	🟩 0.69
			QSAR consensus	🟨 0.25
		CompTox	Consensus Ames mutagenicity	🟩 -
		OSIRIS	Mutagenic	🟥 -
		QSARToolbox	Mutagenicity	🟥 -
	chromosomal aberration	VEGA	CORAL v1.0.0	⬜ 0
			IRFMN *In vitro* micronucleus v1.0.0	⬜ 0.638
			IRFMN *In vivo* micronucleus v1.0.1	🟩 0.768
		QSARToolbox	Chromosomal aberration	🟩 -
carcinogenicity		VEGA	CEASAR v2.1.9	⬜ 0
			ISS v1.0.2	⬜ 0
			IRFMN/Antares v1.0.0	⬜ 0.618
			IRFMN/ISSCAN-CGX v1.0.0	🟥 0.9
			IRFMN carcinogenicity oral classification v1.0.0	⬜ 0
			IRFMN carcinogenicity inhalation classification v1.0.0	⬜ 0
		OSIRIS	Tumorigenic	🟥 -
		QSARToolbox	Carcinogenicity	🟩 -

developmental/reproductive toxicology	VEGA	CEASAR v2.1.7	0.779
		Developmental/Reproductive Tox library v.1.1.0	0.76
		IRFMN/CORAL Zebrafish embryo AC50 v1.0.0	3000.06 µg/L
	CompTox	Developmental toxicity	🟥
	OSIRIS	Reproductive effective	🟥
	QSARToolbox	DART scheme	🟥
endocrine disruption	VEGA	NRMEA Thyroid Receptor Alpha effect v1.0.0	0.952
		NRMEA Thyroid Receptor Beta effect v1.0.0	0.952
		IRFMN Aromatase activity v1.0.0	0.672
		IRFMN Estrogen Receptor Relative Binding Affinity v1.0.1	0.946
		IRFMN/CERAPP Estrogen Receptor-mediated effect v1.0.0	0.958
		IRFMN/COMPARA Androgen Receptor-mediated effect v1.0.0	0.801
	CompTox	Estrogen Receptor Binding	🟩
	QSARToolbox	OECD Estrogen binding	🟩
skin sensitization	VEGA	CEASAR v2.1.6	0.759
		IRFMN/JRC v1.0.0	0.751
	OSIRIS	Irritant	🟥
	QSARToolbox	OECD protein binding	🟥

4.4 2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-methoxypropan-2-yl)acetamide



name	DTXSID50914542
IUPAC	2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-methoxypropan-2-yl)acetamide
route	Biotransformation
likelihood	High* predicted by three prediction TPs tool
Canonical SMILES	CCC1=CC=CC(=C1NC(=O)CCl)C
Isomeric SMILES	CCC1=CC=CC(=C1N([C@@H](C)COC)C(=O)CCl)CO
formula	$C_{15}H_{22}ClNO_3$
mass	299.13
CAS	96394-97-7

Predicted by all the considered models for biotransformation, it is relevant for RSF and WWT. It was the only chlorinated S-metolachlor TP prioritized in this research. As a product of biotransformation,

No toxicological literature information was found in PubMed, Science Direct, or Scopus; therefore, an *in silico* prediction of the likelihood of finding 2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-methoxypropan-2-yl)acetamide in the water was performed.

The water solubility predicted by CompTox for 2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-methoxypropan-2-yl)acetamide was 401.241 mg/L. The predicted solubility by CTS was 2.03 mg/L., thus lower solubility than the parent compound S-metolachlor. Moreover, Kow predicted by CTS (geometric mean based on four different models) was 1.35, and the measured data was available, equal to 2.48. A Kow higher than one shows higher lipophilicity than the hydrophilicity of the chemical. Therefore, the S-metolachlor TPs are shown to be more unlikely to be found in the water rather than in the parent compound.

In silico hazard assessment

2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-methoxypropan-2-yl)acetamide was associated with potential genotoxicity activity, both with mutagenic and chromosomal aberration modes of action. Also, *in silico*, pieces of evidence that may exert carcinogenic, developmental/ reproductive, and irritant effects were collected. On the contrary, predictions of inactivity towards the endocrine disruption endpoint were shown. The *in silico* results are available in the supplementary material (see Annex 11) and summarized in Table 15.

Genotoxicity

2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-methoxypropan-2-yl)acetamide was associated with possible activity for the genotoxicity endpoint.

The S-metolachlor TP was predicted active for genotoxicity by most models (5 out of 7) present in VEGA. On one side, as regards mutagenicity, three out of four models predicted activity for the endpoint. Among the applied models, the ISS v1.0.2 offered the prediction associated with higher reliability. However, the KNN/*Read-Across* v1.0.0 was not considered reliable as the accuracy index; thus, the agreement of the prediction with the experimental values for the molecules in the training set was not satisfying, leading to an ADI of 0. As a consequence, the CONSENSUS model for mutagenicity (v 1.0.3) predicted 2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-methoxypropan-2-yl)acetamide to be mutagenic, with a consensus score of 0.525. The structural alert SA8 (CAESAR and ISS model), SM45, SM73, SM106, and SM161 (SarPy model), already recognized in S-metolachlor, were found. For chromosomal aberration, two out of three models predicted positivity for the endpoint. On the other side, for chromosomal aberration, two out of three models assessed 2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-methoxypropan-2-yl)acetamide to be active. Only the model IRFMN *In vivo* micronucleus v1.0.1 deviated from the other predictions. However, the parameters were not ideal; therefore, the compound could be outside the model's applicability domain.

The *read-across* assessment provided by ToxRead BETA 0.23 was mutagenic with a mutagenic score of 0.82, and the QSAR consensus assessment was mutagenic with a mutagenic score of 0.525. Four SarPy alerts for mutagenicity were identified (n'45, 106, 73). Furthermore, the structural alert SA8 aliphatic halogens were reported. One alert for non-mutagenicity was found, which was maintained from S-metolachlor. The overall judgment offered by the software was mutagenic.

Aligned with ToxRead, QSARToolbox identified the structural alert for *in vitro* and *in vivo* mutagenicity of 'Aliphatic halogens'. Furthermore, a possible protein binding relevant to chromosomal aberration was found: SN2 for Alpha-Activated Haloalkanes. Also, OSIRIS detected in the chemical structure of 2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-methoxypropan-2-yl)acetamide structural alerts related to mutagenicity: one high-risk fragment and two medium-risk fragments indicating mutagenicity, already found in S-metolachlor.

On the contrary, the consensus model for Ames mutagenicity provided by CompTox was negative, as none of the models predicted positivity to the endpoint.

Carcinogenicity

2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-methoxypropan-2-yl)acetamide was predicted active towards the carcinogenicity toxicological endpoint. In VEGA, four models provided reliable predictions positive for the carcinogenicity endpoint. The statistical assessment provided by the models was optimal for two out of six applied models. The CAESAR model v.2.1.9 and the ISS model v.1.0.2 for carcinogenicity were associated with an ADI of 0.913. Indeed, since all the training set molecules agreed with the prediction and were noticeably similar to the target compound. Furthermore, the OSIRIS software recognized two high-risk fragments indicating tumorigenicity already found in S-metolachlor (see Figure 5). Moreover, the structural alert for genotoxic carcinogenicity represented by 'Aliphatic Halogens' was identified.

Reproductive and developmental toxicology

The results obtained in VEGA are inconsistent as regards reproductive and developmental toxicology. Indeed, only one model predicted the activity of 2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-methoxypropan-2-yl)acetamide towards the endpoint. On the other hand, IRFMN/CORAL Zebrafish embryo AC50 v1.0.0 predicted a higher value (5026.02 µg/L) than the parent compound, indicating a decrease in potency due to the transformation processes.

On the other side, in CompTox, the estrogen receptor binding model predicted positivity to the endpoint for 2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-methoxypropan-2-yl)acetamide, as the hierarchical clustering was positive, even though a single model revealed negativity to the endpoint. Furthermore, the profiling applied in QSARTool recognized one high-risk fragment and three medium-risk fragments into 2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-methoxypropan-2-yl)acetamide, indicating reproductive effects. The same risk fragments were already found in S-metolachlor (see Figure 6), therefore maintained over the transformation processes. Nevertheless, no alerts were found by the DART scheme for 2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-methoxypropan-2-yl)acetamide.

Even though structural alerts were present in the molecule, the collected data were insufficient to conclude the reproductive and developmental potential of the molecules. A structural alert without a *read-across* confirmation might not be sufficient to characterize the hazard related to a chemical structure, despite giving indications of the need for prioritization and further research.

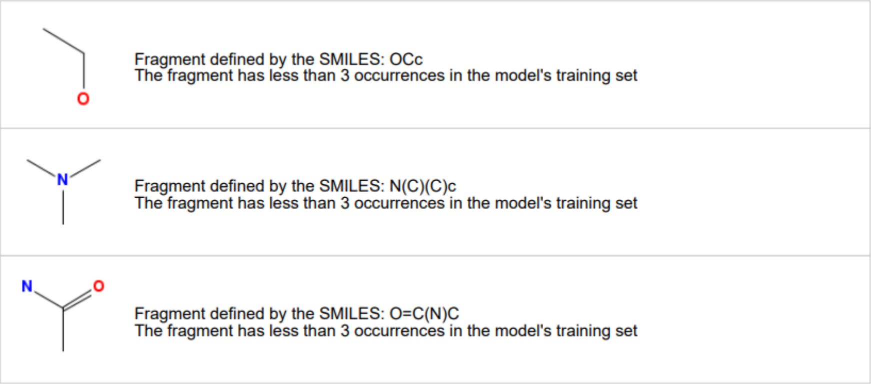
Endocrine disruption

All the models in VEGA predicted negativity to the endpoint, with noticeable high reliability assessed by the model. Also, in QSARToolbox 2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-methoxypropan-2-yl)acetamide was predicted by the tool to be non-binder to the estrogen receptor. In contrast, the developmental toxicology endpoint prediction offered by OSIRIS suggested positivity to the endpoint, and all the models were in agreement. Notably, more than the evaluated endpoints will be needed to assess the endocrine disruption activity, as the mechanisms of action involved exceed the endpoints characterized here.

Skin sensitization/irritation

VEGA suggested activity towards the endpoint, but only one model supported the prediction. The IRFMN/JRC Skin sensitization model provided an optimistic endpoint prediction with high reliability assessed by the model (3/3). However, the similarity index was not ideal, as some molecules in the training set differed from the target compound. Moreover, the CAESAR model (v2.1.6) did not provide a reliable prediction as some relevant

Atom Centered Fragments present in 2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-methoxypropan-2-yl)acetamide were not found in the training set (Figure 15).



Fragment defined by the SMILES: OCc
The fragment has less than 3 occurrences in the model's training set

Fragment defined by the SMILES: N(C)(C)c
The fragment has less than 3 occurrences in the model's training set

Fragment defined by the SMILES: O=C(N)C
The fragment has less than 3 occurrences in the model's training set

Figure 15. Atom Centered Fragments present in 2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-methoxypropan-2-yl)acetamide not found in the training set of the CAESAR skin sensitization model (VEGA, 2022).

To support the predicted activity of the model, OSIRIS recognized five high-risk fragments indicating irritating effects (Figure 16), and QSARToolbox identified a protein binding alert relevant for skin sensitization (SN2 for alpha-activated haloalkanes) as well. However, no inclusion rules for skin irritation/corrosion were found in QSARToolbox. Lastly, VEGA did not provide conclusive results for skin sensitization.

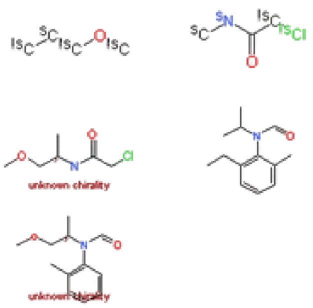


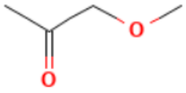
Figure 16. Risks fragments identified by OSIRIS irritating for 2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-methoxypropan-2-yl)acetamide (OSIRIS, 2022)

Table 15. In silico hazard assessment of 2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-methoxypropan-2-yl)acetamide for genotoxicity, carcinogenicity, developmental and reproductive toxicology, endocrine disruption, skin sensitization, and Cramer class evaluation. The prediction was 🟥 *= positive,* 🟨 *= intermediate,* 🟩 *= negative, or* ⬜ *= inconclusive. The Applicability Domain Index (ADI) scores, thus the internal validation of the models, are reported (see Methods section paragraph 1.2.2).*

endpoint		software	model	prediction & score
genotoxicity	mutagenicity	VEGA	CONSENSUS v1.0.3	🟥 0.525
			CEASAR v2.1.13	🟥 0.765
			SarPy/IRFMN v1.0.7	🟥 0.765
			ISS v1.0.2	🟥 0.913
			KNN/*Read-Across* v1.0.0	⬜ 0
		ToxRead	*Read-across*	🟥 0.82
			QSAR consensus	🟥 0.525
		CompTox	Consensus Ames mutagenicity	🟩
		OSIRIS	Mutagenic	🟥
		QSARToolbox	Mutagenicity	🟥
	chromosomal aberration	VEGA	CORAL v1.0.0	🟥 0.926
			IRFMN *In vitro* micronucleus v1.0.0	🟥 0.881
			IRFMN *In vivo* micronucleus v1.0.1	🟩 0.769
		QSARToolbox	Chromosomal aberration	🟥
carcinogenicity		VEGA	CEASAR v2.1.9	🟥 0.913
			ISS v1.0.2	🟥 0.913
			IRFMN/Antares v1.0.0	🟥 0.764
			IRFMN/ISSCAN-CGX v1.0.0	🟥 0.824
			IRFMN carcinogenicity oral v1.0.0	⬜ 0.691
			IRFMN carcinogenicity inhalation classification v1.0.0	⬜ 0
		OSIRIS	Tumorigenic	🟥
		QSARToolbox	Carcinogenicity	🟥

developmental/reproductive toxicology	VEGA	CEASAR v2.1.7	0.776
		Developmental/Reproductive Tox library v.1.1.0	0
		IRFMN/CORAL Zebrafish embryo AC50 v1.0.0	5026.02 µg/L
	CompTox	Developmental toxicity	🟥
	OSIRIS	Reproductive effective	🟥
	QSARToolbox	DART scheme	🟩
endocrine disruption	VEGA	NRMEA Thyroid Receptor Alpha effect v1.0.0	0.937
		NRMEA Thyroid Receptor Beta effect v1.0.0	0.37
		IRFMN Aromatase activity v1.0.0	0.665
		IRFMN Estrogen Receptor Relative Binding Affinity v1.0.1	0.944
		IRFMN/CERAPP Estrogen Receptor-mediated effect v1.0.0	0.954
		IRFMN/COMPARA Androgen Receptor-mediated effect v1.0.0	0.814
	CompTox	Estrogen Receptor Binding	🟥
	QSARToolbox	OECD Estrogen binding	🟥
skin sensitization	VEGA	CEASAR v2.1.6	0.614
		IRFMN/JRC v1.0.0	0.877
	OSIRIS	Irritant	🟥
	QSARToolbox	OECD Protein binding	🟥

4.5 1-methoxypropan-2-one



chemical name	Methoxyacetone
IUPAC	1-methoxypropan-2-one
route	biotransformation
likelihood	Medium > predicted by more tools
SMILES	COCC(C)=O
formula	$C_4H_8O_2$
mass	88.11
CAS	5878-19-3

1-methoxypropan-2-one was predicted as a product of biotransformation, relevant for RSF and WWT.

1-methoxypropan-2-one is a ketone, a class of chemicals highly soluble in water, which characteristic is the pungent smell; therefore, it could be relevant for the organoleptic characteristics of water. In line with it, CompTox predicted a noticeable high water solubility (119446.828 mg/L). Water solubility was predicted by CTS geometric mean as 250'000 mg/L. The high solubility was expected since it is a ketone.

In silico hazard assessment

1-methoxypropan-2-one was predicted as a skin sensitizer and irritant, acting towards membrane integrity. For the other endpoint considered here, it was inactive. The *in silico* results are available in the supplementary material (see Annex 11) and summarized in Table 16.

Genotoxicity

1-methoxypropan-2-one was predicted to be non-genotoxic both through mutagenicity and chromosomal aberration by the VEGA software. Indeed, the CONSENSUS model for mutagenicity (v 1.0.3) predicted 1-methoxypropan-2-one to be non-mutagenic, with a consensus score of 0.675. Also, for chromosomal aberration, the model predicted negativity for the endpoint with high reliability assessed by the model (3/3). Accordingly, in ToxRead BETA 0.23, SarPy alerts for non-mutagenicity were found. The *read-across* assessment was non-mutagenic with a non-mutagenic score of 0.9, and the QSAR consensus assessment was non-mutagenic with a score of 0.675. The most similar molecule in the training set (similarity index = 0.929) was associated with an experimental value of non-mutagenic. Only a molecule in the training set was linked to a mutagenicity value, but the similarity index was 0.894; thus, the influence on the *read-across* was less relevant. Furthermore, CompTox agreed with the previous results, predicting negativity for the Ames mutagenicity endpoint for 1-methoxypropan-2-one, as well as in OSIRIS, no alerts were found for mutagenicity. However, QSARToolbox recognized an alert for *in vivo* mutagenicity (Micronucleous) for 1-methoxypropan-2-one. These data alone were considered insufficient to assess the toxicological potential of 1-methoxypropan-2-one towards genotoxicity.

Carcinogenicity

In VEGA, only two out of six models provided a reliable prediction of the inactivity of 1-methoxypropan-2-one towards the carcinogenic endpoint. The absence of alerts found for tumorigenic effects by OSIRIS and QSARToolbox confirmed the prediction of inactivity towards the endpoint. The results are available in the supplementary documents (Annex 11).

Reproductive and developmental toxicology

Also, for reproductive and developmental toxicology, the *in silico* results were inconclusive for 1-methoxypropan-2-one. Only the CAESAR model (v2.1.7) in VEGA offered a prediction of inactivity towards the endpoint, but the prediction alone needed to be stronger to justify the inactivity of the molecules. Indeed, the prediction presented some critical aspects, with some molecules in the training set associated with experimental values in disagreement with the overall prediction (concordance index = 0.511), and the grade of similarity between the molecules included in the *read-across* was not ideal (similarity index = 0.725). Accordingly, no alerts were found for reproductive effects for 1-methoxypropan-2-one using the OSIRIS software or applying the profiling in QSARToolbox. In contrast, CompTox predicted positivity for the endpoint for 1-methoxypropan-2-one. The consensus result was positive (true), indicating activity towards the endpoint, even though the single model gave an inactivity (false) prediction. Therefore, the prediction was considered inconsistent.

Endocrine disruption

1-methoxypropan-2-one was predicted inactive towards the endpoint, with the agreement of all the models applied in VEGA. In OSIRIS, the estrogen receptor binding model predicted negativity for 1-methoxypropan-2-one, confirming the results provided by VEGA. Another confirmation was offered by QSARToolbox profiling, which defined 1-methoxypropan-2-one as non-binder of the estrogen receptor binding.

Skin sensitization/irritation

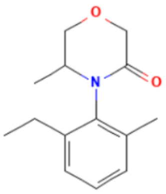
The VEGA CAESAR skin sensitization model (v2.1.6) predicted 1-methoxypropan-2-one to be active. However, the concordance index was relatively low (0.514), which indicated that several molecules in the training set had experimental values that disagreed with the prediction. The IRFMN/JRC skin sensitization model did no provide a reliable prediction; thus, the predictive data offered by VEGA are scarce to consider 1-methoxypropan-2-one a skin sensitizer. Nevertheless, in OSIRIS, one high-risk fragment was found for an irritating effect, already recognized in S-metolachlor (see Figure 7). *Moreover, QSARToolbox* identified an inclusion rule for skin irritation/corrosion, as included in the class of ketones. Therefore, there are enough structural alerts to consider 1-methoxypropan-2-one a potential hazard for skin sensitization.

Table 16. In silico hazard assessment of 1-methoxypropan-2-one for genotoxicity, carcinogenicity, developmental and reproductive toxicology, endocrine disruption, skin sensitization, and Cramer class evaluation. The prediction was 🟥 = positive, 🟨 = intermediate, 🟩 = negative, or ⬜ = inconclusive. The Applicability Domain Index (ADI) scores, thus the internal validation of the models, are reported (see Methods section paragraph 1.2.2).

endpoint		software	model	prediction & score
genotoxicity	mutagenicity	VEGA	CONSENSUS v1.0.3	🟩 0.675
			CEASAR v2.1.13	🟩 0.76
			SarPy/IRFMN v1.0.7	🟨 0.76
			ISS v1.0.2	🟩 0.911
			KNN/*Read-Across* v1.0.0	🟩 0.812
		ToxRead	*Read-across*	🟩 0.9
			QSAR consensus	🟩 0.675
		CompTox	Consensus Ames mutagenicity	🟩
		OSIRIS	Mutagenic	🟩
		QSARToolbox	Mutagenicity	🟩
	chromosomal aberration	VEGA	CORAL v1.0.0	🟩 0.91
			IRFMN *In vitro* micronucleus v1.0.0	⬜ 0
			IRFMN *In vivo* micronucleus v1.0.1	🟩 0.928
		QSARToolbox	Chromosomal aberration	🟥
carcinogenicity		VEGA	CEASAR v2.1.9	⬜ 0.485
			ISS v1.0.2	⬜ 0
			IRFMN/Antares v1.0.0	🟨 0.759
			IRFMN/ISSCAN-CGX v1.0.0	⬜ 0
			IRFMN carcinogenicity oral classification v1.0.0	🟩 0.93
			IRFMN carcinogenicity inhalation classification v1.0.0	🟩 0.93
		OSIRIS	Tumorigenic	🟩
		QSARToolbox	Carcinogenicity	🟩

developmental/reproductive toxicology	VEGA	CEASAR v2.1.7	🟩 0.72
		Developmental/Reproductive Tox library v.1.1.0	⬜ 0.652
		IRFMN/CORAL Zebrafish embryo AC50 v1.0.0	⬜ 0.621
	CompTox	Developmental toxicity	⬜ 0.54
	OSIRIS	Reproductive effective	🟩
	QSARToolbox	DART scheme	🟩
endocrine disruption	VEGA	NRMEA Thyroid Receptor Alpha effect v1.0.0	🟩 0.936
		NRMEA Thyroid Receptor Beta effect v1.0.0	🟩 0.936
		IRFMN Aromatase activity v1.0.0	🟩 0.911
		IRFMN Estrogen Receptor Relative Binding Affinity v1.0.1	🟩 0.85
		IRFMN/CERAPP Estrogen Receptor-mediated effect v1.0.0	🟨 0.92
		IRFMN/COMPARA Androgen Receptor-mediated effect v1.0.0	🟩 0.927
	CompTox	Estrogen Receptor Binding	🟩
	QSARToolbox	OECD Estrogen binding	🟩
skin sensitization	VEGA	CEASAR v2.1.6	🟥 0.754
		IRFMN/JRC v1.0.0	⬜ 0
	OSIRIS	Irritant	🟥
	QSARToolbox	Skin irritation/corrosion	🟥

4.6 Metolachlor morpholinone



name	Metolachlor morpholinone
IUPAC	4-(2-ethyl-6-methylphenyl)-5-methylmorpholin-3-one
route	Acetanilide O-dialkyl Dehalogenative Photocyclization
likelihood	Likely 77.78% (direct photolysis library)
SMILES	CCC1=CC=CC(C)=C1N1C(C)COCC1=O
formula	$C_{14}H_{19}NO_2$
mass	233.311
CAS	120375-14-6

CTS predicted metolachlor morpholinone with high reliability. It may be relevant for UV treatments, AOPs, ozonation, and chlorination. Metolachlor morpholinone was predicted as the main S-metolachlor TP by CTS direct photolysis library, thus, could be formed during UV treatments also involved in (advanced) oxidation products.

As already pointed out, metolachlor morpholinone was found in water sources, such as groundwater (Soulier et al., 2016). Moreover, it was detected in drinking water (Hladik, 2008) at mean concentrations of 8.8ng/L in the fall (2003) and 37 ng/L in the spring (2004). Conventional drinking water treatments appeared incapable of removing the S-metolachlor TP, while PAC achieved up to 100% of the removal. Ozonation appeared to be effective for removing chloroacetamide herbicide TPs, but the production of other derivated TPs was pointed out.

Metolachlor morpholinone was detected in untreated influent wastewater (IWW) at the maximum concentration level of 3304 ng/L in 2016 (Rousis, 2021). The researchers pointed out that metolachlor morpholinone in IWW could be due to environmental degradation and human metabolism. The same study also revealed the presence of S-metolachlor, showing that the parent compound could remain unaltered after drinking water treatments. The mean concentration levels of metolachlor were 4.8 µg/L, while metolachlor morpholinone was detected at mean levels of 2.0 µg/L.

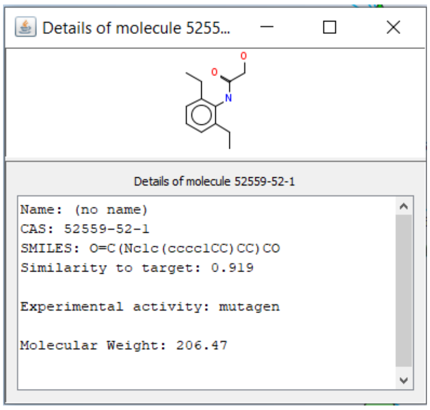
In silico hazard assessment

Some *in silico* models suggested a reliable activity for genotoxicity, mutagenicity, and chromosomal aberration. Regarding carcinogenicity and developmental and reproductive toxicology, the *in silico* results were insufficient to draw conclusions. Various models predicted the negativity to the endocrine disruption endpoint, while positivity to skin sensitization/irritation endpoints was shown. The *in silico* results are available in the supplementary material (see Annex 11) and summarized in Table 17.

Genotoxicity

The results offered by VEGA for metolachlor morpholinone were scarce as regards genotoxicity. One model only predicted the activity of metolachlor morpholinone towards the genotoxicity endpoint with a chromosomal mechanism of action. The CONSENSUS model for mutagenicity (v 1.0.3) predicted metolachlor morpholinone to be non-mutagenic, even though none of the single models on which the consensus prediction was based have provided a reliable prediction. However, none of the models provided a reliable prediction, so the CONSENSUS prediction was considered inconclusive. On the other hand, as regards chromosomal aberration, the CORAL model (v1.0.0) predicted metolachlor morpholinone to be active for the endpoint. However, the other models did not provide reliable predictions or contradicted the activity prediction.

The ToxRead BETA 0.23 *read-across* assessment was non-mutagenic (Figure 17) with a non-mutagenic score of 0.84, and the QSAR consensus assessment was non-mutagenic with a non-mutagenic score of 0.2. Two SarPy alerts for non-mutagenicity were found (n'63 and 161). However, the most similar molecule in the training set (similarity index = 0.919) was associated with an experimental value of mutagenicity; thus, the prediction presents possible inconstancy.



Details of molecule 52559-52-1

```
Name: (no name)
CAS: 52559-52-1
SMILES: O=C(Nc1c(cccc1CC)CC)CO
Similarity to target: 0.919

Experimental activity: mutagen

Molecular Weight: 206.47
```

Figure 17. Most similar molecules were extracted in the training set of ToxRead for metolachlor morpholinone and its experimental value (ToxRead, 2022).

In CompTox, the consensus model for Ames mutagenicity was false, as none of the models predicted positivity to the endpoint. However, OSIRIS recognized one medium-risk fragment for mutagenicity, already found in S-metolachlor. Aligned with that, QSARToolbox recognized an alert for *in vivo* mutagenicity (Micronucleous) for metolachlor morpholinone.

Carcinogenicity

In VEGA, only two models provided reliable predictions for the carcinogenicity endpoint for metolachlor morpholinone, but the results are contradicting. The IRFMN/ISSCAN-CGX was the only model to provide a reliable positive prediction, assessing metolachlor morpholinone carcinogenic for the recognition of the carcinogenicity alert number 42 (SMARTS Nc1ccccc1). On the contrary, the IRFMN carcinogenicity oral classification was the only model to provide a reliable negative prediction. Therefore, the overall VEGA results did not allow for the assessment of the carcinogenetic endpoint for metolachlor morpholinone with good reliability.

To support the positive prediction towards the endpoint, OSIRIS recognized one medium-risk fragment indicating tumorigenicity for metolachlor morpholinone, the same structural alert identified for mutagenicity and already found in S-metolachlor. On the other side, QSARToolbox did not recognize an alert for carcinogenicity for 1-methoxypropan-2-one. Conclusively, the results were inconclusive for the carcinogenicity endpoint for metolachlor morpholinone.

Reproductive and developmental toxicology

Also, the results obtained in VEGA included metolachlor morpholinone for reproductive and developmental toxicology. CompTox's overall prediction was of positivity to the endpoint for metolachlor morpholinone. However, the hierarchical clustering model predicted positivity, even though a single model revealed negativity to the endpoint. Moreover, OSIRIS recognized one medium-risk fragment indicating reproductive effects for metolachlor morpholinone, already found in S-metolachlor (see Figure 6). Accordingly, QSARToolbox recognized the alkyl toluene derivates' structural alert for reproductive and developmental toxicology.

Endocrine disruption

The evaluation of metolachlor morpholinone in VEGA was of negativity towards the endpoint, supported by four predictions of inactivity towards the endpoint associated with high reliability. On the contrary, the IRFMN/COMPARA Androgen Receptor-mediated effect v1.0.0 was the only model to predict positivity for metolachlor morpholinone. However, the prediction presented some critical aspects, as the concordance index was shallow (0.489), indicating that molecules in the training set presented experimental data that disagreed with the prediction. Aligned with the negative prediction, CompTox predicted negativity towards the estrogen receptor binding for metolachlor morpholinone. Moreover, QSARToolbox recognized 1-methoxypropan-2-one as a non-binder. Therefore, the overall judgment was accepted as negative for metolachlor morpholinone.

Skin sensitization/irritation

The two models applied in the VEGA software for skin sensitization/irritation presented contradictory results for metolachlor deschloro. On one side, the CAESAR Skin sensitization model (v2.1.6) predicted positivity to the endpoint, but not all the atom-centered fragments present in metolachlor morpholinone have been found in the molecules of the training set; thus, the target could be outside the applicability domain of the model. In agreement with the CAESAR model in VEGA, OSIRIS recognized metolachlor morpholinone as three high-risk fragments indicating irritating effects, already detected in S-metolachlor (see Figure 7). *Moreover,* QSARToolbox recognized a possible protein binding (OECD rules) through acylation. However, no inclusion rules for skin irritation/corrosion were found.

On the other side, the Skin sensitization model IRFMN/JRC v1.0.0 predicted inactivity towards the endpoint for metolachlor morpholinone, but the concordance index (0.492) revealed criticisms in the prediction. However, the potential sensitization activity of metolachlor morpholinone cannot be excluded, and further research was required.

Table 17. In silico hazard assessment of metolachlor morpholinone for genotoxicity, carcinogenicity, developmental and reproductive toxicology, endocrine disruption, skin sensitization, and Cramer class evaluation. The prediction was 🟥 = positive, 🟨 = intermediate, 🟩 = negative, or ⬜ = inconclusive. The Applicability Domain Index (ADI) scores , thus the internal validation of the models, are reported (see Methods section paragraph 1.2.2).

endpoint		software	model	prediction & score
genotoxicity	mutagenicity	VEGA	CONSENSUS v1.0.3	⬜ 0.2
			CEASAR v2.1.13	⬜ 0.643
			SarPy/IRFMN v1.0.7	⬜ 0.643
			ISS v1.0.2	⬜ 0
			KNN/*Read-Across* v1.0.0	⬜ 0
		ToxRead	*Read-across*	🟥 0.915
			QSAR consensus	⬜ 0.638
		CompTox	Consensus Ames mutagenicity	🟩 0.765
		OSIRIS	Mutagenic	🟨
		QSARToolbox	Mutagenicity	🟩
	chromosomal aberration	VEGA	CORAL v1.0.0	🟥 0.915
			IRFMN *In vitro* micronucleus v1.0.0	⬜ 0.638
			IRFMN *In vivo* micronucleus v1.0.1	🟩 0.765
		QSARToolbox	Chromosomal aberration	🟥
carcinogenicity		VEGA	CEASAR v2.1.9	⬜
			ISS v1.0.2	⬜
			IRFMN/Antares v1.0.0	⬜
			IRFMN/ISSCAN-CGX v1.0.0	🟥
			IRFMN carcinogenicity oral classification v1.0.0	🟩
			IRFMN carcinogenicity inhalation classification v1.0.0	⬜
		OSIRIS	Tumorigenic	🟨
		QSARToolbox	Carcinogenicity	🟩

developmental/reproductive toxicology	VEGA	CEASAR v2.1.7	0
		Developmental/Reproductive Tox library v.1.1.0	0.759
		IRFMN/CORAL Zebrafish embryo AC50 v1.0.0	4499.53 µg/L
	CompTox	Developmental toxicity	🟥
	OSIRIS	Reproductive effective	🟨
	QSARToolbox	DART scheme	🟩
endocrine disruption	VEGA	NRMEA Thyroid Receptor Alpha effect v1.0.0	0.943
		NRMEA Thyroid Receptor Beta effect v1.0.0	0.943
		IRFMN Aromatase activity v1.0.0	0.665
		IRFMN Estrogen Receptor Relative Binding Affinity v1.0.1	0.939
		IRFMN/CERAPP Estrogen Receptor-mediated effect v1.0.0	0.948
		IRFMN/COMPARA Androgen Receptor-mediated effect v1.0.0	0.795
	CompTox	Estrogen Receptor Binding	🟩
	QSARToolbox	OECD Estrogen binding	🟩
skin sensitization	VEGA	CEASAR v2.1.6	0.756
		IRFMN/JRC v1.0.0	0.747
	OSIRIS	Irritant	🟥
	QSARToolbox	OECD Protein binding	🟥

4.7 Conclusions characterization prioritized S-metolachlor transformation products

For most predicted S-metolachlor TPs, a decrease in toxicity potential was predicted compared with the parent compound regarding genotoxicity. However, 2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-methoxypropan-2-yl)acetamide was associated with more robust predictions of activity towards genotoxicity. The reason could be the presence of the halogen group, which was maintained only in this chemical structure among the prioritized S-metolachlor TPs. On the contrary, metolachlor-2-hydroxy, metolachlor deschloroacetyl, and metolachlor deschloro appeared to be less critical regarding genotoxicity and carcinogenicity endpoints in comparison with the parent compound. That could be related to the loss of the halogen functional group. However, a higher potency was predicted for metolachlor deschloroacetyl as regards the Zebrafish embryo AC50 endpoint relevant for reproductive and developmental toxicity than the parent compound. Also, 1-methoxypropan-2-one appeared less critical than the parent compound. Lastly, *in silico* predictions were insufficient for metolachlor morpholinone to conclude the related toxicological concern, as only a few models provided a reliable prediction. That could be because the models could not find structurally similar molecules in the software database to train the *read-across* assessment. However, it could interact with the androgen receptor exerting an endocrine disruption activity. However, skin sensitization alerts were found in all the prioritized S-metolachlor TPs, which could be relevant for dermal exposure to water. The results are summarized in Table 18.

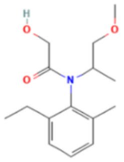
The predictions suggested inactivity towards this endpoint for all the prioritized TPs regarding endocrine disruption. Still, the only considered model for the aromatase activity did not provide a reliable prediction for the S-metolachlor TPs, which appeared to be the mechanism of action of the parent compound (Laville, 2006). Even though the decrease in the predicted toxicity of TPs was observed, all the prioritized predicted S-metolachlor TPs were associated with a priority code of toxicological concern of 3/3 (see methods chapter…), as all the prioritized S-metolachlor TPs were predicted to be active for at least one endpoint and by at least two strongly reliable models.

Therefore, the prioritized S-metolachlor TPs were attempted to be detected in treated water samples to verify whether the prediction and prioritization scheme applied could be reliable.

Table 18. In silico hazard assessment of S-metolachlor (0), metolachlor-2-hydroxy (1), metolachlor deschloroacetyl (2), metolachlor deschloro (3), 2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-methoxypropan-2-yl)acetamide (4), 1-methoxypropan-2-one (5), and metolachlor morpholinone (6).

exp = the experimental value was reported by the model


(0)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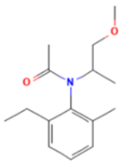
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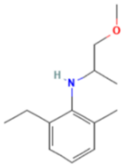
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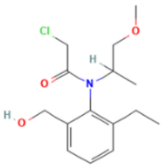
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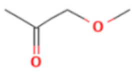
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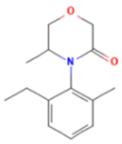
(6)

endpoint		software	model	0	1	2	3	4	5	6
TTC		ToxTree	Cramer class	III	I	I	I	III	III	III
genotoxicity	mutagenicity	VEGA[6]	CONSENSUS v1.0.3	🟥	⬜	⬜	⬜	🟥	🟩	⬜
			CEASAR v2.1.13	🟥	⬜	🟥	🟥	🟥	🟩	⬜
			Sarpy/IRFMN v1.0.7	🟥	⬜	⬜	⬜	🟥	🟨	⬜
			ISS v1.0.2	🟥	⬜	🟩	⬜	🟥	🟩	⬜
			KNN/*Read-Across* v1.0.0	⬜	⬜	⬜	⬜	⬜	🟩	⬜
		ToxRead[7]	*Read-across*	🟥	🟩	🟩	🟩	🟥	🟩	🟥
			QSAR consensus	🟥	⬜	🟩	🟨	🟥	🟩	⬜
		CompTox[8]	Consensus mutagenicity	⬜	🟩	🟩	🟩	🟩	🟩	🟩
		OSIRIS[9]	Mutagenic	🟥	🟨	🟩	🟥	🟥	🟩	🟨
		QSARToolbox[10]	Mutagenicity	🟥	🟩	🟩	🟥	🟥	🟩	🟩
	chromosomal aberration	VEGA	CORAL v1.0.0	🟥	⬜	🟩	⬜	🟥	🟩	🟥
			IRFMN *In vitro* micronucleus	⬜	🟥	🟥	⬜	🟥	⬜	⬜
			IRFMN *In vivo* micronucleus	⬜	🟩	🟩	🟩	🟩	🟩	🟩
		QSARToolbox	Chromosomal aberration	🟥	🟥	🟥	🟩	🟥	🟥	🟥
carcinogenicity		VEGA	CEASAR v2.1.9	⬜	⬜	⬜	⬜	🟥	⬜	⬜
			ISS v1.0.2	🟥	⬜	⬜	⬜	🟥	⬜	⬜
			IRFMN/Antares v1.0.0	⬜	⬜	⬜	⬜	🟥	🟨	⬜
			IRFMN/ISSCAN-CGX v1.0.0	⬜	🟥	⬜	🟥	🟥	⬜	🟥
			IRFMN carcinogenicity oral	🟩 exp	🟩	⬜	⬜	⬜	🟩	🟩
			IRFMN carcinogenicity inhalation	🟩 exp	⬜	⬜	⬜	⬜	🟩	⬜
		OSIRIS	Tumorigenic	🟥	🟨	⬜	🟥	🟥	🟩	🟨
		QSARToolbox	Carcinogenicity	🟥	🟩	🟩	🟩	🟥	🟩	🟩
developmental/reproductive toxicity		VEGA	CEASAR v2.1.7	⬜	⬜	⬜	⬜	🟥	🟩	⬜
			Dev/ReproTox library v.1.1.0	⬜	⬜	⬜	⬜	⬜	⬜	🟩
			IRFMN/CORAL Zebrafish AC50	🟨 exp	🟨	🟥	🟨	🟨	⬜	🟨
		CompTox	Developmental toxicity	🟥	🟥	⬜	🟥	🟥	⬜	🟥
		OSIRIS	Reproductive effective	🟥	🟨	🟩	🟥	🟥	🟩	🟨
		QSARToolbox	DART scheme	🟩 exp	🟥	🟥	🟥	🟩	🟩	🟩
endocrine disruption		VEGA	NRMEA Thyroid Receptor α	🟩 exp	🟩	🟩	🟩	🟩	🟩	🟩
			NRMEA Thyroid Receptor β	🟩 exp	🟩	🟩	🟩	🟩	🟩	🟩
			IRFMN Aromatase activity	🟥 exp	⬜	⬜	⬜	⬜	🟩	⬜
			IRFMN Estrogen Receptor	🟩	🟩	🟩	🟩	🟩	🟩	🟩
			CERAPP Estrogen Receptor	🟩 exp	🟩	🟩	🟩	🟩	🟨	🟩
			COMPARA Androgen Receptor	🟩 exp	🟥	🟩	⬜	🟩	🟩	🟥
		CompTox	Estrogen Receptor Binding	🟩 exp	🟥	⬜	🟩	🟥	🟩	🟩
		QSARToolbox	OECD Estrogen binding	🟥	🟩	🟩	🟩	🟥	🟩	🟩
skin sensitization		VEGA	CEASAR v2.1.6	🟥	🟥	⬜	🟥	⬜	🟥	🟥
			IRFMN/JRC v1.0.0	⬜	⬜	⬜	⬜	🟥	⬜	🟩
		OSIRIS	Irritant	🟥	🟥	🟥	🟥	🟥	🟥	🟥
		QSARToolbox	OECD protein binding	🟥	🟥	🟩	🟥	🟥	🟥	🟥

5. Detection of S-metolachlor prioritized transformation products in treated water

The prioritized predicted S-metolachlor were tentatively identified using a dataset available from Brunner et al. (2019) who analyzed water samples experimentally treated with rapid sand filtration and containg the racemic mixture metolachlor. Here are reported the main results and in the supplementary documents are available further information (Annex 12).

In 2019, the pioneering research by Brunner et al. analyzed experimentally treated water samples with ozonation and RSF – respectively used as a model for abiotic and biotic drinking water treatments – and spiked with three known contaminants of drinking water sources. Among these contaminants, they selected metolachlor; therefore, the results data were used in this research, considering the approximation of having metolachlor and not S-metolachlor. The researchers created an in-house list of suspect metolachlor TPs, which was then used to interpret the results of the HPLC-HRMS analysis applied.

Suspect list by Brunner et al. (2019) vs. the prioritized list of S-metolachlor TPs

An initial comparison was made with the suspect list presented by Brunner et al. (2019) available in the supplementary material (Annex 12, Table 15). The list presented 26 suspect metolachlor TPs identified by literature data mining or found in the databases NORMAN SusDat and STOFF-IDENT or predicted using enviPath. Among these, 14 metolachlor TPs were also predicted as S-metolachlor TPs in the current research.

Four out of six prioritized transformation products prioritized in this research were also present in the suspect list by Brunner et al. (2019). These were: metolachlor-2-hydroxy, metolachlor deschloro, 1-methoxypropan-2-one, and metolachlor morpholinone. Three metolachlor TPs reported only with their SMILES in the research conducted by Brunner et al. (2019) were identified here, moving a step further in the research on metolachlor TPs in drinking water.

The molecule associated with the SMILES CCC1=C(C(=CC=C1)C)NC(=O)CCl was identified as 2-chloro-N-(2-ethyl-6-methylphenyl)acetamide, the one with the smiles CCC1=CC=CC(=C1N(C(C)COC)C(=O)CCl)CO was identified as 2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-methoxypropan-2-yl)acetamide, and the one with the SMILES CCC1=C(C(=CC=C1)C)N(C(C)CO)C(=O)CCl as N-(2-Methyl-6-ethylphenyl)-N-((1S)-1-methyl-2-hydroxyethyl)-2-chloroacetamide. These three molecules were not prioritized based on the structural prioritization applied in this research. 2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-methoxypropan-2-yl)acetamide, predicted by UM-PPS and BioTransformer, was not present in the list by Brunne et al. (2018) Nevertheless, the structurally similar compound 2-chloro-N-(2-ethyl-6-methylphenyl)acetamide, which was prioritized over it, was present in both lists. At the same time, the other two S-metolachlor TPs present in the suspect list by Brunner et al. (2019) were predicted by enviPath and Biotransformer but were not found in the literature and therefore, not prioritized in the current research.

Six other TPs were consistent in both suspect lists. The metabolite CGA 37735 was predicted by the UM-PPS tool and found in NORMAN SusDat databases but not prioritized in this research (predicted by only one tool and not identified in the literature research - see chapter prioritization). Similarly, metolachlor OXA was predicted by the enviPath tool only, as also reported by Brunner et al. (2019), but not prioritized. On the contrary, although it was predicted by only one tool, metolachlor morpholinone was also prioritized because it was found systematically in the literature review. The S-metolachlor TPs with SMILES CCC1=C(C(=CC=C1)C)N(C(C)C=O)C(=O)CCl was predicted by both experiments by enviPath - and Biotransformer confirmed the prediction - but not included in the suspect list by Brunner et al. (2019). Lastly, three smaller molecules were pointed out by both suspect TPs lists.

Data analysis and interpretation

The samples pre and after-treatment were compared to look into the changes in the chemical composition caused by the treatments.

The HPLC/MS data by Brunner et al. (2019) was analyzed using the software Compound Discoverer 3.1 (ThermoFisher Scientific), while the new version Compound Discoverer 3.3 (ThermoFisher Scientific) was adopted to examine the provided data, namely the table containing features from the dataset and molecular information and the chromatograms and mass spectra obtained from the analysis. A filter exclusion of peaks with RT inferior to 2.4 minutes was selected to exclude the solvent peak. Furthermore, background features were removed. Two suspects TPs were tentatively identified in the positive ionization data: metolachlor morpholinone and metolachlor deschloro. The supplementary material (Annex III) shows the data underlying these results. A confirmation with a reference standard is needed to confirm the attempted identification of the compounds, as already pointed out by Kiefer et al. (2021).

The predicted fragmentation spectra of the prioritized S-metolachlor TPs obtained by CFM-ID software (Wang et al., 2021) were compared with those explored in Compound Discoverer 3.3. The link to the CFM-ID predictions is available in the supplementary material (Annex IV) to validate the tentative identification of metolachlor morpholinone and metolachlor deschloro.

The features expected to be metolachlor deschloro had a molecular weight of 249.17229 g/mol and RT 17.188 minutes, as visible in Figure 18. The compound was already present in the Brunner et al. (2019) suspect list, found in NORMAN SusDat, StoffIDENT, and predicted by enviPath. As Brunner et al. (2019) noted, dehalogenation was a diffused reaction involved with TPs formation during biodegradation experiments (represented by RSF since it involves biodegradation). The peak intensity was seven times higher after RSF treatment than before treatments, as illustrated in Figure 1, therefore compatible with the hypothesis of being formed after treatment. It should be noted that the peak intensity was very low (i.e., approximately 60000 counts, which can almost be considered a noise), and the shape of the peak was not optimal.

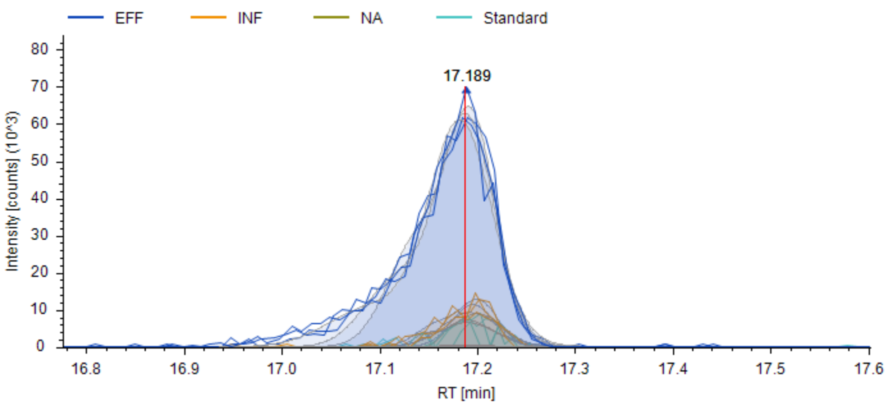

Figure 18. Chromatogram of the features, which was tentatively identified as metolachlor deschloro. The orange line indicates the presence of the compound in the water before treatment. In contrast, the blue line is associated with the elution of metolachlor deschloro after rapid sand filtration (RSF) was applied (Compound Discoverer 3.3, 2022)

In the MS spectra, the peaks are visible as the mass-to-charge ratio (m/z). During the analysis, charged ions are created when an electron is taken from the molecule (negative ionization) or given to the molecule (positive ionization). For this reason, the m/z is reported, which often corresponds to the mass (because the charge is 1). The fragments with m/z 218.15256 and m/z 176.142 were visible in the CFM-ID fragmentation spectra and were the fragments at a higher intensity. A comparison of the two spectra is reported in Figure 19.

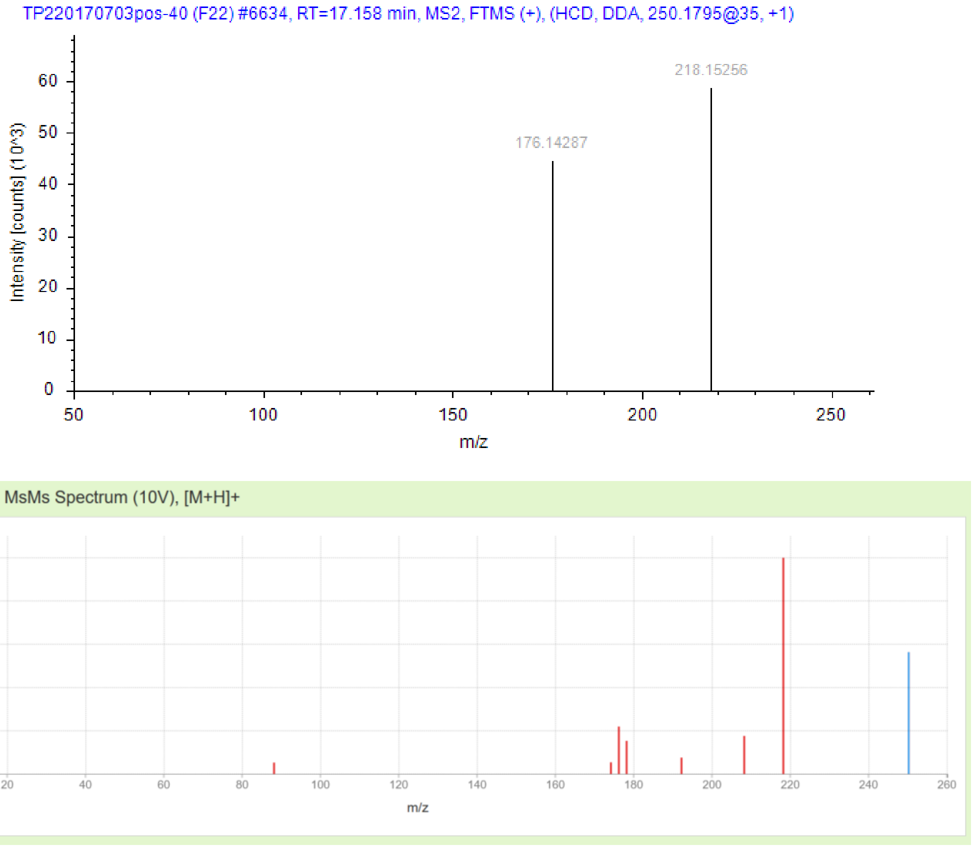

Figure 19. Experimental mass fragmentation spectrum of the features expected to be metolachlor deschloro (up) (Compound Discoverer 3.3, 2022) compared with the predicted mass fragmentation spectrum of metolachlor deschloro by CFM-ID (down) (Wang et al., 2021).

Metolachlor morpholinone was expected to be found in the RSF samples as a product of biotransformation. Also, in this case, only in the positive ionization analysis. It was found in the sample with a molecular weight of 233.1412 g/mol and RT 14.317 minutes. In this case, as illustrated in Figure 20, the peaks before and after RSF treatment application were comparable, suggesting its presence in the water independently from RSF processes. Moreover, the intensity of the peak was low. A Log 2-fold change filter was applied to exclude peaks whose intensity was not notably increased after the applied RSF. This fact explains why Brunner et al. (2019) did not point out metolachlor morpholinone, even though present in the suspect screening list.

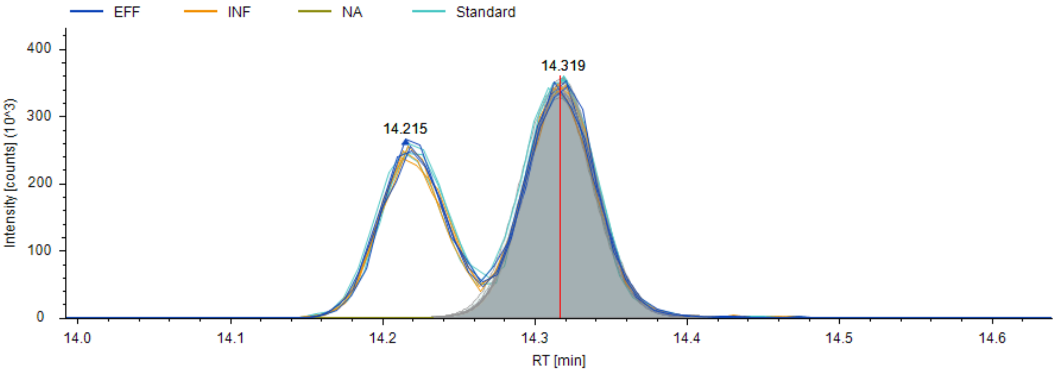

Figure 20. Chromatogram of the features, which was tentatively identified as metolachlor morpholinone. The orange line indicates the presence of the compound in the water before treatment. In contrast, the blue line is associated with the elution of metolachlor morpholinone after rapid sand filtration (RSF) was applied (Compound Discoverer 3.3, 2022)

For metolachlor morpholinone, as regards the fragmentation spectra, the fragmentation spectrum was not wholly overlapping, as shown in Figure 21. Therefore, the formation of metolachlor morpholinone was considered irrelevant to the experimental conditions applied.

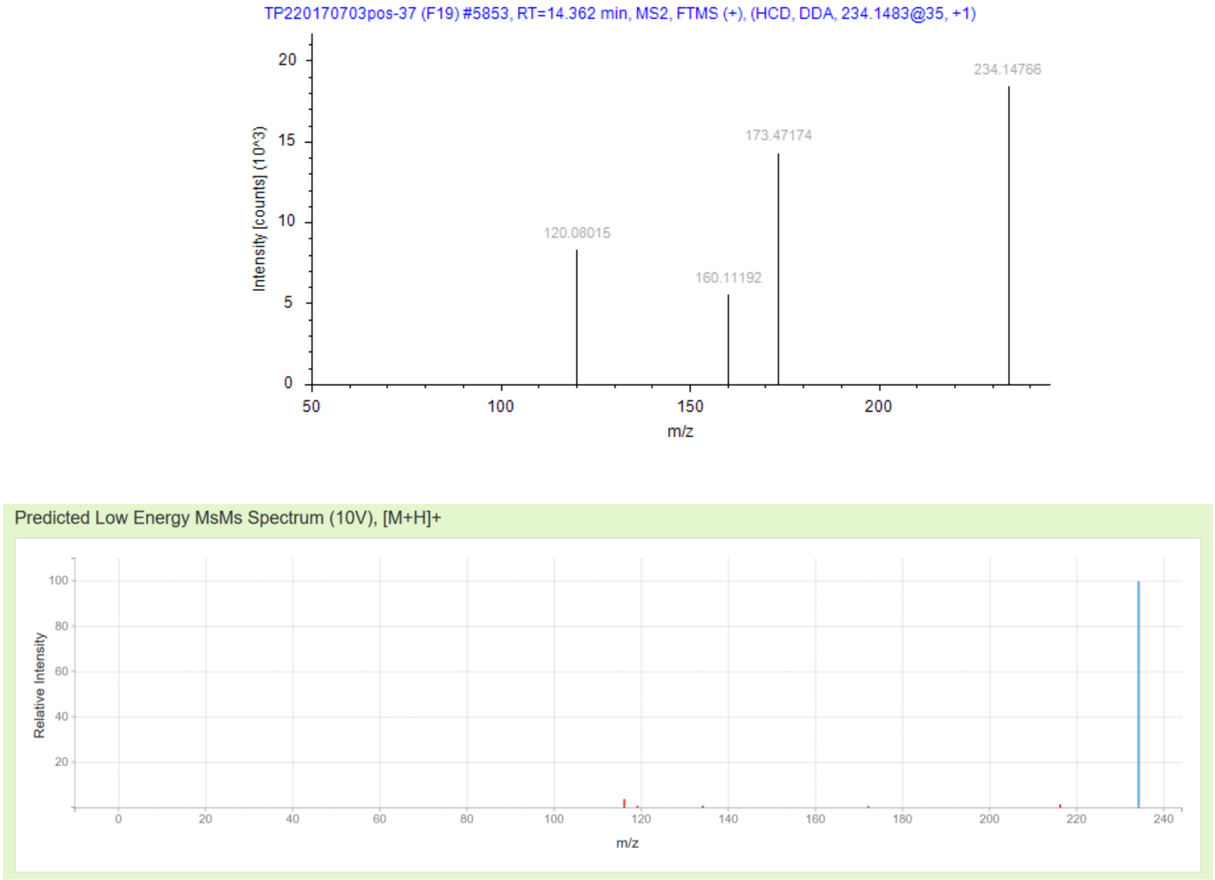

Figure 21. Experimental mass fragmentation spectrum of the features expected to be metolachlor morpholinone (up) (Compound Discoverer 3.3, 2022) compared with the predicted mass fragmentation spectrum of metolachlor deschloro by CFM-ID (down) (Wang et al., 2021).

Surprisingly, no significant decrease in metolachlor concentration was observed after RSF filtration - while it was observed by Brunner et al. (2019) after the ozonation treatment. The reduction of metolachlor after RSF was expected as it is known to be degraded in soil (DT50 of months). However, it was not observed. Brunner et al. (2019) suggested that the continuous spike-in of the compound could mask the expected reduction. Another explanation could be that the range of microorganisms present in RSF sand differed from the soil, resulting in lower transformation activity.

DISCUSSION

A considerable number (83) of unique S-metolachlor TPs were predicted, among which only thirty-five were identified in chemical databases. Six were prioritized through literature data mining and structural alert identification. The hazard assessment suggests that S-metolachlor TPs might be less active regarding genotoxicity, expect for 2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-methoxypropan-2-yl)acetamide. At the same time, other S-metolachlor TPs have been predicted as having comparable or higher toxicity than the parent compound as regards reproductive/developmental toxicity and skin irritation/corrosion. Two selected S-metolachlor TPs were tentatively identified using the non-target HPLC-HRMS screening, but further confirmation is needed. The necessary implementation and refinement of the proposed methodology are discussed here.

1. Prediction of transformation products

The present research used four different predictive *in silico* tools for abiotic (Chemical Transformation Simulator CTS) and biotic reactions (UM-PPS, enviPath, BioTransformer) to predict the formation of TPs due to reactions occurring during specific drinking water treatments. The methods chapter has already discussed the relevance of these reactions in predicting the transformation of S-metolachlor during specific drinking water treatments (see paragraph 2.2).

The selected *in silico* tools predicted 83 unique S-metolachlor TPs due to specific reactions relevant to drinking water treatments. Among these, 72 TPs were predicted by at least one model of biotransformation, while TPs formed by abiotic reaction were less often predicted. Multiple tools pointed out the same S-metolachlor TPs, predictive for different reaction pathways. The overlapping results were scrutinized to eliminate duplicates. Duplicates suggest a higher probability of their formation in the complexity of drinking water treatments.

Biotransformation was used here to represent reactions during RSF and WWT. Among the freely available biotransformation tools, UM-PPS, enviPath, and BioTransformer all contained EEA EAWAG-BBD data; therefore, some predicted TPs overlapped between the results from different methods. However, enviPath was considered more comprehensive since it provided the highest number of predicted TPs among the selected biotransformation prediction tools. Therefore, it is suggested to always include enviPath in predicting biodegradation TPs. Moreover, the UM-PPS SMILES proposed was not recognized in PubChem; consequently, it required further identification of the compound names, drawing the molecules in RMG. Here is pointed out, but not further discussed, that a tool for the translation of isomeric smiles into chemical names is necessary to speed up the identification of TPs.

Interestingly, UM-PPS indicated more S-metolachlor TPs when performing only aerobic transformations than all the transformation reactions. This observation means that the aerobic database is more extensive than the anaerobic database. Consequently, to assess biotransformation, it is recommended to choose the aerobic option, but this also depends on the specific drinking water treatment analysed.

Regarding abiotic reactions, CTS was the only freely available tool able to predict abiotic processes. Beyond assuring the evaluation of the abiotic transformation of contaminants, the CTS tool presented other advantages. Indeed, it provided the IUPAC name of the predicted compounds, already identifying the names of the predicted TPs. In contrast, an additional step of identification of the compound names was necessary with the other predictive *in silico* tools because they only provided the SMILES and chemical formula. Moreover, the CTS tool also allows inputting a batch of files to predict more chemicals' TPs simultaneously, assuring automatization. It is preferable to select the hydrolysis and photolysis independently if the aim is to assess TPs possibly originating from specific drinking water treatments alone or the combined library if the objective is a water treatment process involving both reactions. That is because the *in silico* predictions may differ due to the consideration of different reaction libraries (see 2-{2-[(2-ethyl-6-methylphenyl)amino]propoxy}acetic acid, in Results chapter paragraph 2.1). For instance, for evaluating the AOPs processes and UV treatments, it is recommended to use the combined libraries as both reactions may co-occur depending on the treatment applied.

Conclusively, it is recommended to use the combination of CTS and enviPath to predict TPs, as the former is the only freely available tool able to predict abiotic reactions, and the latter was demonstrated to be the most complete for predicting biotic reactions.

The overall *in silico* prediction succeeded in selecting TPs related to drinking water treatment processes and suggested transformation products confirmed by the literature. Nonetheless, the presented methodology presents some limitations. Hydrolysis and photolysis reactions were here used as a model for ozonation, chlorination, and AOPs. However, they may need to be more comprehensive to evaluate the impact of these drinking water treatments. For example, the selected *in silico* tools did not predict oxidation reactions.

The correspondence of *in silico* results with literature information on monitoring data was used to confirm the likelihood of production of a determinate TP, even though uncertainties about the origin of the TPs in the environment are present, because most of the literature was found about water sources. Moreover, the prioritization steps applied in this research project are limited because they entail approximations. Indeed, TPs might be relevant even though predicted by only one tool, and little structural changes might alter the toxicity of a compound. It may be interesting to investigate the other predicted S-metolachlor TPs (see Results section chapter 2) to avoid overlooking any possible consequence of transformation reactions. However, the purpose was here to apply the selected freely available *in silico* tools to a limited group of high-concern TPs. Therefore, it was chosen to select the most often predicted and strongly associated with a Cramer class III.

2. Characterization of transformation products

The current research used literature data mining combined with *in silico* methodologies to collect relevant physicochemical and toxicological information about S-metolachlor and its predicted TPs. Combining different methodologies is an efficient approach to characterizing data-poor chemicals, contributing to the weight-of-evidence approach for the risk assessment (Hardy et al., 2017). On the one hand, experimental data can validate *in silico* predictions, indicating the model's reliability, while *in silico* prediction can fill data gaps found in the literature. For the same reasons, other NAMs, such as bioassays, may compensate assessment deficiencies in available prediction tools. Moreover, experimental data guarantee the implementation of the models' algorithms. On the other hand, *in silico* predictions can steer experimental analysis, helping to reduce and refine animal testing. A schematic representation of the mutual contribution between *in silico*, *in vitro*, and *in vivo* methods is illustrated in Figure 22.

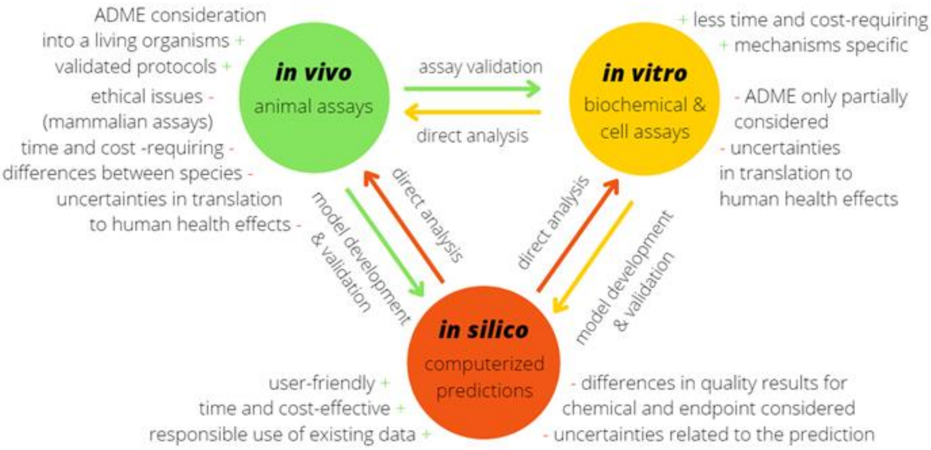

Figure 22. Mutual implementation between in silico, in vitro, and in vivo methodologies.

Integrating the *in silico* results with bioassays can strengthen the presented results, adopting a weight of evidence (WoE) approach based on considering multiple sources of evidence to support the hazard assessment (Hardy et al., 2017). In REACH regulation, the WoE approach is defined as the correlation of several independent sources of information to assume that a compound has (or does not) a toxic property (Hardy et al., 2017). The current research project provided evidence of toxicological concern but was insufficient to assess the hazard of predicted TPs, other methodologies must be considered in the future, such as bioassays and, when strictly necessary, *in vivo* experiments to clarify specific endpoints.

2.1 *In silico* prediction of physicochemical characterization

Water solubility was considered the most relevant PCC for the presence of chemicals in the water and the only parameter ubiquitously considered for S-metolachlor TPs.

Among the applied *in silico* tools for predicting water solubility, higher performance (confirmed with the comparison with literature data) was noted for the WATERNT^{TM} model (v1.1) present in EPISuite^{TM}. Indeed, the predicted value by EPISuite^{TM} was half the experimental reported value for S-metolachlor. Using the other selected software (CTS and CompTox), the difference was almost ten-fold different than the experimental reported water solubility. Moreover, EPISuite^{TM} allows for predicting other parameters relevant to the characterization of the environmental fate of chemicals. In addition, CTS was here conveniently applied as already being used for predicting TPs. The tool allowed for the direct characterization of TPs after their prediction. Similarly, CompTox was also used to predict water solubility while performing the *in silico* hazard assessment.

2.2 *In silico* hazard assessment.

The prediction results were compared with literature information and structural alerts, and possibly hazardous S-metolachlor TPs were prioritized. Structural alert relationships (SARs) and *read-across* were used to characterize the hazard for relevant endpoints for the prioritized S-metolachlor TPs. The qualitative *in silico* hazard assessment provided relevant insight into the predicted TPs' potential toxicity and validated the prioritization scheme applied because it selected S-metolachlor TPs of possible high toxicological concern.

For five out of six prioritized S-metolachlor TPs, all considered *in silico* tools agreed upon a decreased genotoxicity. However, the predicted toxicity was equal to or more probable for developmental and reproductive toxicity and endocrine disruption endpoints (see Results chapter paragraph 4.7).

Biometabolism usually creates more hydrophilic compounds that are less critical from a toxicological point of view (Garefalaki et al., 2021). Therefore, metabolism should lead to the formation of less toxic compounds. However, this was only sometimes true. The presence of the halogen group appeared to be critical for genotoxicity, as the only prioritized predicted S-metolachlor TP that maintained the functional group (2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-methoxypropan-2-yl)acetamide) was predicted to preserve the genotoxic activity of the parent compound.

The reliability of *in silico* tools for the identification and hazard assessment of TPs increases when the mechanism of action (MoA) is well understood. The models' reliability strongly depends on the experimental data available for the molecules selected in the training set: if poor or no data are available, the algorithm was seen to be poorly predictive. Indeed, the selected models offered more reliable predictions for genotoxicity, skin sensitization, or receptor binding rather than carcinogenicity and reproductive/developmental toxicology. For the former endpoints, the understanding of the MoA is more well understood (EPA, 2005) and, thus, predictable *in silico*. On the contrary, complex endpoints such as developmental toxicity and carcinogenicity were less reliably predicted by the applied models. Therefore, further software implementation for these endpoints is necessary.

Some critical MoA, like ligands of hormone receptors, were investigated for the endocrine disruption endpoint. The only model available was VEGA, but it could not reliably predict the activity for the S-metolachlor prioritized TPs. The interaction with the aromatase, the experimentally proven MoA of S-metolachlor (Laville et al., 2006), was only evaluated by one VEGA model, and the predictions were considered inconclusive. The only compound for which it provided a reliable prediction was 1-methoxypropan-2-one.

Within the VEGA software, different models were available to assess the mutagenicity endpoint, and overall reliable predictions were obtained. Moreover, VEGA offers multiple models for all the considered endpoints; therefore, it was considered the most helpful software to assess TPs structural alerts and to direct further analysis quickly. Also, the software is easy-to-use and provides an internal statistical assessment of the prediction reliability; therefore, it helps the expert to judge the prediction's validity.

Expert judgment must always be applied, as a critical evaluation of the *in silico* results may reveal incorrect values assigned by the model. The comparison between different software and models for the same endpoint is always recommended (ECHA, 2016). For instance, when using the software VEGA to assess the mutagenicity endpoint, it is always recommended to consider the reliability of the single models rather than blindly accept the overall CONSENSUS model. In other words, the single model predictions need to be considered to justify the

CONSENSUS prediction. Indeed, the CONSENSUS prediction was active for metolachlor deschloroacetyl, while none of the model predicted positivity for mutagenicity with good reliability. Similarly, for metolachlor morpholinone, the CONSENSUS mutagenicity model gave an inactive prediction for the endpoint; however, none of the models provided a reliable prediction; therefore, the CONSENSUS result was considered inconsistent.

Understanding the scores assigned by the model and critically assessing them is needed case by case. Still, applying thresholds to interpret *in silico* results is also convenient. The *a priori* selected threshold value of 0.75 preselected for the Applicability Domain Index (ADI) provided by the models was a valuable tool to exclude unreliable predictions. Indeed, none of the predictions with an ADI < 0.75 were considered reliable, evaluating the molecules of the training set. Nonetheless, expert judgment is still needed to assess predictions associated with an ADI slightly above the threshold value of 0.75, as some parameters were assessed to have a higher impact on the prediction reliability than others. Therefore, it was noticed that even though the ADI is above 0.75, if other parameters did not confirm the prediction, this was considered inconsistent. Therefore, a corrected threshold value for acceptance of the prediction is proposed at 0.8.

Moreover, the similarity and concordance indexes were assessed as of higher relevance for the prediction's reliability than the ACF index because they of higher impact on the reliability of the prediction (see Results chapter paragraph 4.3 about skin sensitization/irritation). The similarity between molecules of the training set and the target molecule and the concordance between the prediction and the experimental data reported for the molecule of the training set was therefore considered more important than the correspondence of atom-cantered fragments between the target and the training set. It would be interesting to investigate whether the defined reasoning may apply to other predictions. However, the reliability of parameters is expected to depend on the specific molecular structure considered; therefore, it is always recommendable to evaluate them specifically (ECHA, 2016).

3. Detection of transformation products

The detection of the prioritized S-metolachlor TPs was attempted as a proof of principle for the proposed framework for prioritizing TPs assessment in drinking water. The prioritized TPs were tentatively identified using the data of the HPLC-HRMS non-target screening by Brunner et al. (2019). The researchers applied a novel methodology for evaluating metolachlor TPs formation during drinking water treatment processes, using respectively ozonation and rapid sand filtration (RSF) as representative of biotic and abiotic treatment processes of drinking water. However, only the data regarding RSF were here analysed.

Two predicted TPs were tentatively identified in the dataset made available by Brunner et al. (2019).

Metolachlor deschloro was expected to be found in RSF as predicted by *in silico* tools of biodegradation. Indeed, it was predicted to be formed by hydrogenolysis and biotransformation. It was also present in the NORMAN SusDat and Stoffident databases and has already been detected in drinking water by previous research (Hladik et al., 2008; Guelfi et al., 2018).

Conversely, metolachlor morpholinone was not expected to be found in the analysed water samples by Brunner et al. (2019) since it was not predicted to be formed by biodegradation. Indeed, it was a predicted photolysis product relevant for UV treatments, AOPs, ozonation, and chlorination. Therefore, targeted screening of metolachlor morpholinone in drinking water samples which implied the use of other treatment processes rather than RSF could be a possible direction for research. However, Brunner et al. (2019) did not detect metolachlor morpholinone in the ozonation experiments.

Even though here tentatively identified, the detected signal for S-metolachlor TPs was low (nearby the detection limit). Moreover, it is crucial to note that the concentration of the parent compound used in the experiment was relatively high (10 µg/L) – one to two orders of magnitude higher compared to the levels at which it was found in surface water and groundwater (see Results chapter, Table 6). Also, the signal was considered insignificant for metolachlor morpholinone; therefore, its formation was considered absent. Consequently, the formation of the prioritized S-metolachlor TPs during the RSF experiments performed by Brunner et al. (2019) seemed not critical at the applied experimental conditions. Nevertheless, identifying traces of predicted S-metolachlor TPs supports the predictive workflow applied here.

The results were a tentative identification of TPs, which should be validated with reference standards. The HPLC-HRMS requires an internal standard to confirm the suspect compound (Keifer et al., 2021). Also, the ionization pattern can vary depending on the instrument used and the type of ionization selected - electrospray ionization (ESI) or higher energy collisional dissociation (HCD) available in the instrument used for the analysis by Brunner et al. (2019) – thus, the energy involved (Ho et al., 2003). Therefore, the formation of the predicted S-metolachlor TPs cannot be excluded.

The specific applied experimental conditions could not have induced the formation of the predicted TPs, but they may have been formed under other conditions. Also, the applied experimental conditions could differ from those in drinking water treatment plans. As Brunner et al. (2019) noticed, the composition of the microorganisms involved could influence the types of emerging reactions. In conclusion, the formation of the predicted TPs that were not tentatively identified cannot be excluded from being formed during RSF. Therefore, more targeted research needs to clarify their presence in drinking water.

The limited screening done here was applied as proof of the principle of the methodology, thus, might be extended. The detection of the predicted TPs in drinking water could further confirm the applicability of the proposed methodology for predicting high-concern TPs originating from drinking water treatments.

4. Further research

Prediction and prioritization of TPs

Further research is needed to consider other reactions that were not included in the *in silico* tools applied here. Future research may include the QSARToolbox direct oxidation model to amplify its predictivity for AOPs.

The prioritization steps were done manually, increasing the chances of biases. It would be interesting to check the reliability of the expert judgment here applied, comparing the presented results with experimental data. ToxTree comparison was used to justify the prioritization of only one of the TPs gathered for structure similarities. This prioritization method was considered sufficient for the scope of the present research. However, this methodology would not be applicable for the risk assessment in a regulatory context because the grouping of molecules needs to be done following the EFSA and OECD guidelines (OECD, 2017; EFSA, 2021).

Physicochemical characterization

Considering different PCC besides water solubility might be necessary to understand both the environmental fate of the parent compound – therefore, its availability in drinking water sources – and the persistence of TPs after water treatments. Partitioning coefficients were investigated only for the parent compound to understand the likelihood of finding it in water sources. However, further research should be aimed at understanding the environmental fate of the predicted S-metolachlor TPs. In the present research, only water solubility was considered, as the TPs were assumed not to enter into contact with other environmental matrixes. In reality, the tap water enters the sewer, comes into contact with metal tubes, and is ultimately released into the environment.

In silico hazard assessment

The *in silico* hazard assessment methodology proposed here requires implementation, as some crucial endpoints (e.g., developmental/reproductive toxicology and carcinogenicity) still need to be reliably predictable *in silico*. The sharing of existing data is pivotal to allow the development of more inclusive algorithms. Data openness is primarily considered the basis of the scientific method (Elbe, 2018), and the reuse of information is necessary to optimize the resources (Jacobsen et al., 2020). In 2016, the FAIR Guiding Principles were defined to improve the Findability, Accessibility, Interoperability, and Reuse of digital assets (Wilkinson et al., 2016).

The OECD QSARToolbox application was limited to the profiling of molecules in this research, while the *read-across* assessment can enhance the reliability of the predictions. A structural alert without a *read-across* confirmation might not be sufficient to characterize the hazard related to a chemical structure but can give indications of the need for prioritization and further research. Therefore, a more extensive application of the software is suggested.

Moreover, the evaluation of other endpoints not here evaluated should be investigated, such as the repeat dose toxicity, since the possible exposure to TPs could be repeated over time, or respiratory toxicity. Although ingestion is considered the main route of exposure, research has shown that the highest increase in the internal dose of DBPs was found after the shower, rendering inhalation and dermal exposure relevant route (Gordon et al., 2006). The findings might also be relevant for S-metolachlor TPs, but further research is required to consider respiratory toxicology. Also, other NAMs, such as bioassays, are suggested to investigate the activity of S-metolachlor TPs against the aromatase. An example could be the granulosa cell aromatase bioassay (Liu et al., 2021).

Health risks considerations

TPs in drinking water does not necessarily determine a direct risk to human health. Besides the related capacity to cause harmful effects (hazard), the exposure must be considered to evaluate potential risks (Costa & Teixeira, 2014). In other words, a hazardous chemical in drinking water alone is not a health risk, as exposure to humans must be assessed.

Intermittent exposure to TPs can be expected since pesticide exposure is inclined to temporal variation (Boonstra et al., 2022). The temporal variation should, thus, be considered for the risk assessment of TPs. Also, bioaccumulation is to be considered because, for repeated doses, TPs can be bioaccumulated in living organisms, as reviewed by Maculewicz et al. (2022) for pharmaceuticals.

Moreover, TPs can be formed by different reactions (here predicted by different tools) and, therefore, even though formed at low concentrations, may be found at higher aggregates in the environment. In fact, in the groundwater, higher concentrations of TPs than the corresponding pesticides were found (Kiefer et al., 2019). However, an explanation of the increase in concentrations of TPs compared to the parent compounds was not discussed by the researchers, leaving space to further research.

Detoxification

Moreover, conjugation reactions typical of the biotransformation of pesticides in living organisms (Konuk et al., 2022) might detoxify the compounds in humans after their absorption. The conjugated TPs are the more favourable from a thermodynamic point of view as conjugation decrease the energy of the system and increases stability (Garefalaki et al., 2021). Therefore, conjugation reactions and detoxification still need to be evaluated to assess the health risk relevance of transformation processes.

Detection of S-metolachlor TPs

The applied methodology tentatively identified two predicted S-metolachlor TPs at low-intensity signals in drinking water treated with RSF; however, extensive research is needed to confirm their presence in drinking water. Firstly, confirmation of their presence with internal standards is needed to confirm the HPLC-HRMS analysis. Moreover, analysis of water treated with processes other than RSF or other microorganism compositions for RSF is still missing. The data for identifying S-metolachlor TPs resulted from an analysis of only RSF, and other evaluated reaction processes could occur during water treatments not represented by RSF. Hence further analysis needs to investigate whether the predicted S-metolachlor TPs may be formed during other treatment processes (such as chlorination or UV treatments). The same authors of the non-target screening suggested that the method could be applied to further research on TPs derived from other drinking water treatments (Brunner et al., 2019). Full-scale research is suggested to consider the overall effect of multiple drinking water treatment processes, including biotic or abiotic processes.

The same available non-target screening data collected by Brunner et al. (2019) could also be analysed to detect the other predicted S-metolachlor that were not prioritized in this research. Unidentified TPs' features exceeded the number of annotated compounds (Brunner et al., 2019), leaving space for future retrospective research, which can fasten the risk assessment of emerging contaminants (Creusot et al., 2020).

S-metolachlor TPs elimination

Moreover, further research is needed to understand if the TPs can persist in drinking water after their formation. The *in silico* tools for predicting PCC could also be adopted to evaluate the possible elimination of TPs due to the adsorption processes involved in drinking water treatment (i.e., sludge treatment or activated carbon purification). The evaluation of the elimination of TPs done by treatment processes was outside the scope of this study, but further research is advised to better understand the possible exposure to humans via drinking water, thus the relevance for human health. Indeed, TPs could be removed after being formed by specific drinking water treatments; however, the efficiency and efficacy must be evaluated. Guide et al. (2021) identified over 200 newly formed TPs derived from known micropollutants when ozonation was applied as water treatment. Of these, only 13% were removed by rapid sand filtration (RSF). Also, Kiefer et al. (2020) reported partial effectiveness of drinking water treatments in removing TPs. Matsushita et al. (2018) suggested that the effort required to remove pesticide TPs from the water via PAC and ozonation was higher than for the precursor pesticides. TPs' removal depends on the PCC of the TPs and the drinking water treatment processes involved.

Environmental processes

The focus of the presented research was evaluating the formation of TPs as a direct consequence of drinking water treatments, and the consideration of the environmental processes was outside the scope of this research. However, active substances in water sources can encounter transformation processes even before the treatment processes are applied, increasing the number of unpredicted parent compounds to assess. Therefore, understanding the environmental processes of transformation is critical for understanding TPs formation during drinking water treatments and direct mitigation planning. The biotic reactions included in the prediction tools used as models for drinking water treatments may also represent environmental processes, but further research is needed.

Evaluation of mixtures

Furthermore, although investigated separately in this research, TPs are found in water sources in mixtures. Exposure to mixtures tends to be more realistic and critical than pesticides alone (Hayes et al., 2006); therefore, the risk assessment of pesticides – and their TPs – should be cumulative (EFSA, 2020). Therefore, the toxicological effect of low-concentration mixtures of S-metolachlor TPs, such as other active substances, should be investigated.

Evaluation of small structural changes

Finally, the difference between S-metolachlor and the racemic mixture metolachlor was not considered relevant based on the collected literature data since S-metolachlor is the active portion of the racemic product (Shaner et al., 2006). Therefore, R-metolachlor was not expected to exert a toxicological effect. Further research still needs to confirm whether this assumption is acceptable or not. It would also be interesting to investigate how structural changes influence the toxicological properties of TPs, for example, by assessing the hazard of structurally similar compounds. This investigation can be used to validate whether the prioritization of 2-chloro-N-[2-ethyl-6-(hydroxymethyl)phenyl]-N-(1-hydroxypropan-2-yl)acetamide over two structurally similar compounds was a good approach.

5. Conclusions

Drinking water treatment processes must consider TPs formation. The final aim of this research was to evaluate how *in silico* tools can contribute to assess the possible impact of drinking water treatment processes on S-metolachlor and, therefore, the health risks related to contamination of water. The rational scheme applied successfully predicted possible S-metolachlor TPs, confirmed by the literature, and predicted possible hazards to direct further analysis for the risk assessments of these newly formed chemicals.

The applied predictive tools can steer the identification of TPs, helping to prioritize follow-up research and targeted analysis to directly assess contaminants' impact on water quality and the implementation of water treatment processes. The effectiveness of freely available *in silico* tools in predicting, prioritizing, and evaluating TPs was discussed in the previous chapter. S-metolachlor TPs were used as proof of the applicability of the methodology, which was effective in prioritizing S-metolachlor TPs of high toxicological concern and likely to be produced during water treatments. The reliability of the methods varies depending on the specific reaction pathway, PCC, or endpoint considered. The combination of the Chemical Transformation Simulator (CTS) and enviPath were suggested for predicting TPs originating from drinking water treatments. EpiSuite^{TM} was recommended for the PCC evaluation, and VEGA QSAR for the hazard prioritization.

Two prioritized S-metolachlor TPs were tentatively identified in the water samples after RSF treatment. However, the detected signal for the selected features was near the detection limit. Therefore, the formation of S-metolachlor TPs during RSF treatment applied by Brunner et al. (2019) was not critical at the observed transformation rates. Further research is needed to confirm the attempted identification of RSF-treated water samples with standards to assess the collected data precisely.

Conclusively, the proposed methodology is a valuable starting point in evaluating the formation of TPs in drinking water, also considering the reduced costs and time required in comparison to experimental research. The proposed rational scheme could be applied to the assessment of active substances worldwide and represent a step forward to understanding the relevance of transformation reactions generated during drinking water treatments.

6. Highlights

1. The reliability of the methods varies depending on the specific reaction pathway or endpoint considered.
2. It is suggested to always include CTS and enviPath in predicting TPs for considering abiotic and biotic reactions.
3. For the prediction of physicochemical characteristics, EPISuite^{TM} offered the higher reliability.
4. VEGA QSAR offers user-friendly and reliable models to prioritize TPs of high toxicological concern. Thresholds rules to accept predictions were proposed.
5. There is a need for an automation workflow tool to collect the tools here applied.

Supplementary documents

The supplementary documents are available here Supplementary documents.pdf

During the research project at KWR Water Research Institute, the results were shared with the scientific community through three deliverables.

1. Article for Water Matters, published in June 2022 in the English version available at the link Article H2O Water Matters.eng.pdf and the Dutch version available at the link Article Water Matters.dutch.pdf

2. Poster for NVT Meetings 2022 available here: nvt

3. Poster for International Congress of Toxicology ICT2022 available here: ICT2022__e-poster_SOC-VI-10.pdf

Literature

Anagnostopoulou, K., Nannou, C., Evgenidou, E., & Lambropoulou, D. (2022, April 1). *Overarching issues on relevant pesticide transformation products in the aquatic environment: A review*. Science of the Total Environment. Elsevier B.V. https://doi.org/10.1016/j.scitotenv.2021.152863

Antanasijević, D., Antanasijević, J., Trišović, N., Ušćumlić, G., & Pocajt, V. (2017). *From Classification to Regression Multitasking QSAR Modeling Using a Novel Modular Neural Network: Simultaneous Prediction of Anticonvulsant Activity and Neurotoxicity of Succinimides*. Molecular Pharmaceutics, 14(12), 4476–4484. https://doi.org/10.1021/acs.molpharmaceut.7b00582

Basant, N., Gupta, S., & Singh, K. P. (2016). *QSAR modeling for predicting reproductive toxicity of chemicals in rats for regulatory purposes.* Toxicology Research, 5(4), 1029–1038. https://doi.org/10.1039/c6tx00083e

Benfenati, E., Manganaro, A., & Gini, G. (2013). *VEGA-QSAR: AI Inside a Platform for Predictive Toxicology*. 2nd Workshop on Popularize Artificial Intelligence, PAI 2013, Held in Conjunction With AI*IA 2013, 21–28. https://ceur-ws.org/Vol-1107/paper8.pdf

Benigni, R., & Bossa, C. (2008). *Predictivity of QSAR*. Journal of Chemical Information and Modeling, 48(5), 971–980. https://doi.org/10.1021/ci8000088

Benigni, R., & Bossa, C. (2011, April 13). *Mechanisms of chemical carcinogenicity and mutagenicity: A review with implications for predictive toxicology*. Chemical Reviews. https://doi.org/10.1021/cr100222q

Benigni, R., & Bossa, C. (2019). *Data-based review of QSARs for predicting genotoxicity: The state of the art*. Mutagenesis, 34(1), 25–32. https://doi.org/10.1093/mutage/gey028

Beretsou, V. G., Psoma, A. K., Gago-Ferrero, P., Aalizadeh, R., Fenner, K., & Thomaidis, N. S. (2016). *Identification of biotransformation products of citalopram formed in activated sludge*. Water Research, 103. pp. 205–214. https://doi.org/10.1016/j.watres.2016.07.029

Berggren, E., White, A., Ouedraogo, G., Paini, A., Richarz, A. N., Bois, F. Y., … Mahony, C. (2017). *Ab initio chemical safety assessment: A workflow based on exposure considerations and non-animal methods.* Computational Toxicology, 4, 31–44. https://doi.org/10.1016/j.comtox.2017.10.001

Boonstra, H., de Baat, M. L., van der Meer, F., Roessink, I., & Kraak, M. H. (2022). *Temporal Variation of Ecotoxicological Risks in Agricultural Waterbodies.* SSRN Electronic Journal. https://doi.org/10.2139/ssrn.4204476

Brumovský, M., Bečanová, J., Kohoutek, J., Borghini, M., & Nizzetto, L. (2017). *Contaminants of emerging concern in the open sea waters of the Western Mediterranean.* Environmental Pollution, 229, 976–983. https://doi.org/10.1016/j.envpol.2017.07.082

Brunner, A. M., Bertelkamp, C., Dingemans, M. M. L., Kolkman, A., Wols, B., Harmsen, D., … ter Laak, T. L. (2020). *Integration of target analyses, non-target screening and effect-based monitoring to assess OMP related water quality changes in drinking water treatment*. Science of the Total Environment, 705. https://doi.org/10.1016/j.scitotenv.2019.135779

Brunner, A. M., Vughs, D., Siegers, W., Bertelkamp, C., Hofman-Caris, R., Kolkman, A., & ter Laak, T. (2019). *Monitoring transformation product formation in the drinking water treatments rapid sand filtration and ozonation*. Chemosphere, 214, 801–811. https://doi.org/10.1016/j.chemosphere.2018.09.140

Bura, L., Friel, A., Magrans, J. O., Parra-Morte, J. M., & Szentes, C. (2019). *Guidance of EFSA on risk assessments for active substances of plant protection products that have stereoisomers as components or impurities and for transformation products of active substances that may have stereoisomers*. EFSA Journal, 17(8). https://doi.org/10.2903/j.efsa.2019.5804

Cao, P., Wang, X., Liu, F., Zhao, E., & Han, L. (2008). *Dissipation and residue of S-metolachlor in maize and soil.* Bulletin of Environmental Contamination and Toxicology, 80(5), 391–394. https://doi.org/10.1007/s00128-008-9359-z

Cassano, A., Manganaro, A., Martin, T., Young, D., Piclin, N., Pintore, M., … Benfenati, E. (2010). *CAESAR models for developmental toxicity.* Chemistry Central Journal, 4(SUPPL. 1). https://doi.org/10.1186/1752-153X-4-S1-S4

Cormier, G., Barbeau, B., Arp, H. P. H., & Sauvé, S. (2015). *The degradation behaviour of nine diverse contaminants in urban surface water and wastewater prior to water treatment*. Environmental Sciences: Processes and Impacts, 17(12), 2051–2065. https://doi.org/10.1039/c5em00338e

Costa, L. G., Giordano, G., Guizzetti, M., & Vitalone, A. (2008). *Neurotoxicity of pesticides: A brief review. Frontiers in Bioscience*. Bioscience Research Institute. https://doi.org/10.2741/2758

Costa, S., & Teixeira, J. (2014). *Toxicology*. Encyclopedia of Toxicology, 718–720. https://doi.org/10.1016/b978-0-12-386454-3.00440-1

Covaci, A. (2014). *Environmental Fate and Behavior*. In Encyclopedia of Toxicology: Third Edition (pp. 372–374). Elsevier. https://doi.org/10.1016/B978-0-12-386454-3.01041-1

Creusot, N., Casado-Martinez, C., Chiaia-Hernandez, A., Kiefer, K., Ferrari, B. J., Fu, Q., Munz, N., Stamm, C., Tlili, A., & Hollender, J. (2020). *Retrospective screening of high-resolution mass spectrometry archived digital samples can improve environmental risk assessment of emerging contaminants: A case study on* *antifungal azoles*. Environment International, 139, 105708. https://doi.org/10.1016/j.envint.2020.105708

de Souza, R. M., Seibert, D., Quesada, H. B., de Jesus Bassetti, F., Fagundes-Klen, M. R., & Bergamasco, R. (2020). *Occurrence, impacts and general aspects of pesticides in surface water: A review*. Process Safety and Environmental Protection, pp. 135, 22–37. https://doi.org/10.1016/j.psep.2019.12.035

Di Marcantonio, C., Bertelkamp, C., van Bel, N., Pronk, T. E., Timmers, P. H., van der Wielen, P., & Brunner, A. M. (2020). *Organic micropollutant removal in full-scale rapid sand filters used for drinking water treatment in The Netherlands and Belgium*. Chemosphere, p. 260, 127630. https://doi.org/10.1016/j.chemosphere.2020.127630

Dix, D. J., Houck, K. A., Martin, M. T., Richard, A. M., Setzer, R. W., & Kavlock, R. J. (2007). *The Toxcast program for prioritizing toxicity testing of environmental chemicals.* Toxicological Sciences, 95(1), 5–12. https://doi.org/10.1093/toxsci/kfl103

Dos Santos, J. R. N., Alves, I. C. B., Marques, A. L. B., & Marques, E. P. (2022). *Bibliometric analysis of global research progress on electrochemical degradation of organic pollutants*. Environmental Science and Pollution Research, 29(36), 54769–54781. https://doi.org/10.1007/s11356-022-19534-y

EC Regulation *n° 1107/2009 of the European Parliament and of the Council of October 21, 2009 concerning the placing of plant protection products on the market* https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:309:0001:0050:en:PDF

ECHA. (2008). *Guidance on information requirements and chemical safety assessment. Chapter R.6: QSARs and grouping of chemicals*. https://echa.europa.eu/documents/10162/13632/information_requirements_r6_en.pdf/77f49f81-b76d-40ab-8513-4f3a533b6ac9

ECHA. (2016). *Practical guide: How to use alternatives to animal testing to fulfil your information requirements for REACH registration*. European Chemical Agency. ECHA-16-B-25-EN. https://echa.europa.eu/documents/10162/13655/practical_guide_how_to_use_alternatives_en.pdf/148b30c7-c186-463c-a898-522a888a4404

ECHA. (2022a). *C&L Inventory Metolachlor-2-hydroxy*. https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/281026

ECHA. (2022b). *C&L Inventory Deschloroacetylmetolachlor*. https://echa.europa.eu/information-on-chemicals/cl-inventory-database/-/discli/details/276686

ECHA. (2022c). *Chemicals Strategy for Sustainability*. European Chemical Agency. https://echa.europa.eu/hot-topics/chemicals-strategy-for-sustainability

EFSA. (2012). *Review of the existing maximum residue levels (MRLs) for S-metolachlor according to Article 12 of Regulation (EC) n° 396/2005*. EFSA Journal, 10(2). https://doi.org/10.2903/j.efsa.2012.2586

EFSA. (2018, November 29). *Public consultation on the active substance S-metolachlor. European Food Safety Authority*. https://www.efsa.europa.eu/en/consultations/call/181129-1

EFSA. (2019). *Guidance on the use of the Threshold of Toxicological Concern approach in food safety assessment.* https://doi.org/10.2903/j.efsa.2019.5708

EFSA. (2020, April 29). *Cumulative risk assessment of pesticides: FAQ*. European Food Safety Authority. https://www.efsa.europa.eu/en/news/cumulative-risk-assessment-pesticides-faq

EFSA. (2021, December 17). *Guidance Document on Scientific criteria for grouping chemicals into assessment groups for human risk assessment of combined exposure to multiple chemicals*. European Food Safety Authority. https://www.efsa.europa.eu/en/efsajournal/pub/7033

Egger, M., Higgins, J. P. T., & Smith, D. G. (2022). *Systematic Reviews in Health Research: Meta-Analysis in Context* (3rd ed.). BMJ Books. ISBN: 978-1-405-16050-6. O-Book. pp. 17-33

Elbe, S. (2018, January 13). *A Global Health Epidemic Is A Ticking Time Bomb - But Virus Databases Can And Are Helping To Save Lives*. HuffPost UK. https://www.huffingtonpost.co.uk/professor-stefan-elbe/a-global-health-epidemic-_b_14129608.html

EPA. (1995). *R.E.D. FACTS Metolachlor*. In Environmental Protection Agency (EPA-738-F-95-007). https://www3.epa.gov/pesticides/chem_search/reg_actions/reregistration/fs_PC-108801_1-Apr-95.pdf

EPA. (2005). *Guidelines for carcinogen risk assessment* (EPA/630/P-03/001F). Risk Assessment Forum U.S. Environmental Protection Agency Washington, DC. https://www.epa.gov/sites/default/files/201309/documents/cancer_guidelines_final_3-25-05.pdf

EU Directive n° 2020/2184 *of the European Parliament and of the Council of December 16 2020 on the quality of water intended for human consumption*. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32020L2184&from=EN

EU Regulation n° 528/2012 *of the European Parliament and of the Council of May 22 2012 concerning the making available on the market and use of biocidal products.* https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2012:167:0001:0123:en:PDF

European Commission. (2004). *Review report for the active substance S-Metolachlor Finalised in the Standing Committee on the Food Chain and Animal Health at its meeting on October 8 2004 in view of the inclusion of S-Metolachlor in Annex I of Directive 91/414/EEC*. In European Commission (SANCO/1426/2001-rev. 3). European Commission. https://efsa.onlinelibrary.wiley.com/doi/pdf/10.2903/j.efsa.2012.2586

European Commission. (2022a). *Approval of active substances*. EU Commission. https://food.ec.europa.eu/plants/pesticides/approval-active-substances_en

European Commission. (2022b). *Skin Sensitisation*. EU Science Hub. https://joint-research-centre.ec.europa.eu/eu-reference-laboratory-alternatives-animal-testing-eurl-ecvam/alternative-methods-toxicity-testing/validated-test-methods-health-effects/skin-sensitisation_en

European Commission. (2022c). *Skin Irritation*. EU Science Hub. https://joint-research-centre.ec.europa.eu/eu-reference-laboratory-alternatives-animal-testing-eurl-ecvam/alternative-methods-toxicity-testing/validated-test-methods-health-effects/skin-irritation_en

European Commission. (2022d). *Water Treatment*. CDC Center for Disease Control and Prevention. https://www.cdc.gov/healthywater/drinking/public/water_treatment.html

European Commission. (2022e) *Commission Implementing Regulation (EU) 2022/708 of May 5 2022 amending Implementing Regulation (EU) n° 540/2011 as regards the extension of the approval periods of the active substances 2,5-dichlorobenzoic acid methylester, acetic acid, aclonifen, aluminium ammonium sulphate, aluminium phosphide, aluminium silicate, beflubutamid, benthiavalicarb, boscalid, calcium carbide, captan, cymoxanil, dimethomorph, dodemorph, ethephon, ethylene, extract from tea tree, fat distilation residues, fatty acids C7 to C20, fluoxastrobin, flurochloridone, folpet, formetanate, gibberellic acid, gibberellins, hydrolysed proteins, iron sulphate, magnesium phosphide, metam, metamitron, metazachlor, metribuzin, milbemectin, phenmedipham, pirimiphos-methyl, plant oils/clove oil, plant oils/rape seed oil, plant oils/spear mint oil, propamocarb, proquinazid, prothioconazole, pyrethrins, quartz sand, fish oil, repellents by smell of animal or plant origin/sheep fat, S-metolachlor, Straight Chain Lepidopteran Pheromones, sulcotrione, tebuconazole and urea*. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32022R0708&qid=1653634154057

Eysseric, E., Gagnon, C., & Segura, P. A. (2022). *Identifying congeners and transformation products of organic contaminants within complex chemical mixtures in impacted surface waters with a top-down non-targeted screening workflow.* Science of the Total Environment, 822, 153540. https://doi.org/10.1016/j.scitotenv.2022.153540

Farlin, J., Gallé, T., Bayerle, M., Pittois, D., Köppchen, S., Krause, M., & Hofmann, D. (2018). *Breakthrough dynamics of s-metolachlor metabolites in drinking water wells: Transport pathways and time to trend reversal*. Journal of Contaminant Hydrology, 213, 62–72. https://doi.org/10.1016/j.jconhyd.2018.05.002

Ferrari, T., Cattaneo, D., Gini, G., Golbamaki Bakhtyari, N., Manganaro, A., & Benfenati, E. (2013). *Automatic knowledge extraction from chemical structures: The case of mutagenicity prediction.* SAR and QSAR in Environmental Research, 24(5), 365–383. https://doi.org/10.1080/1062936X.2013.773376

Finckh, S., Beckers, L. M., Busch, W., Carmona, E., Dulio, V., Kramer, L., Krauss, M., Posthuma, L., Schulze, T., Slootweg, J., Von der Ohe, P. C., & Brack, W. (2022). *A risk based assessment approach for chemical mixtures from wastewater treatment plant effluents.* Environment International, 164, 107234. https://doi.org/10.1016/j.envint.2022.107234

Fishbein, J. C., Heilman, J. M., & Kostal, J. (2016). Advances in Molecular Toxicology [ISBN 9780128047002]. In *Chapter Four - Computational Chemistry in Predictive Toxicology: status quo et quo vadis?* (1st ed., Vol. 10). Academic Press. pp. 145-162 https://doi.org/10.1016/B978-0-12-804700-2.00004-0

Fjodorova, N., Vračko, M., Novič, M., Roncaglioni, A., & Benfenati, E. (2010). *New public QSAR model for carcinogenicity*. Chemistry Central Journal, 4(SUPPL. 1). https://doi.org/10.1186/1752-153X-4-S1-S3

Föllmann, W., Degen, G., Oesch, F., & Hengstler, J. G. (2013). *Ames Test.* In Brenner's Encyclopedia of Genetics: Second Edition (pp. 104–107). Elsevier Inc. https://doi.org/10.1016/B978-0-12-374984-0.00048-6\

Gago-Ferrero, P., Bletsou, A. A., Damalas, D. E., Aalizadeh, R., Alygizakis, N. A., Singer, H. P., Hollender, J., & Thomaidis, N. S. (2020). *Wide-scope target screening of >2000 emerging contaminants in wastewater samples with UPLC-Q-ToF-HRMS/MS and smart evaluation of its performance through the validation of 195 selected representative analytes.* Journal of Hazardous Materials, p. 387, 121712. https://doi.org/10.1016/j.jhazmat.2019.121712

Garefalaki, V., Papavergi, M. G., Savvidou, O., Papanikolaou, G., Felföldi, T., Márialigeti, K., Fakis, G., & Boukouvala, S. (2021). *Comparative Investigation of 15 Xenobiotic-Metabolizing N -Acetyltransferase (NAT) Homologs from Bacteria.* Applied and Environmental Microbiology, 87(19). https://doi.org/10.1128/aem.00819-21

Gellatly, N., & Sewell, F. (2019, August 1). *Regulatory acceptance of in silico approaches for the safety assessment of cosmetic-related substances*. Computational Toxicology. Elsevier B.V. https://doi.org/10.1016/j.comtox.2019.03.003

Giaginis, C., Zira, A., Theocharis, S., & Tsantili-Kakoulidou, A. (2009). *Application of quantitative structure-activity relationships for modeling drug and chemical transport across the human placenta barrier: A multivariate data analysis approach.* Journal of Applied Toxicology, 29(8), 724–733. https://doi.org/10.1002/jat.1466

Gini, G., & Zanoli, F. (2020). *Machine learning and deep learning methods in ecotoxicological QSAR modeling.* In Methods in Pharmacology and Toxicology (pp. 111–149). Humana Press Inc. https://doi.org/10.1007/978-1-0716-0150-1_6

Glassmeyer, S. T., Furlong, E. T., Kolpin, D. W., Batt, A. L., Benson, R., Boone, J. S., … Wilson, V. S. (2017). *Nationwide reconnaissance of contaminants of emerging concern in source and treated drinking waters of the United*

States. Science of the Total Environment, 581–582, 909–922. https://doi.org/10.1016/j.scitotenv.2016.12.004

Global Data. (2019). *Global Company Intelligence*. Retrieved from http://www.globalcompanyintelligence.com

Gogoi, A., Mazumder, P., Tyagi, V. K., Tushara Chaminda, G. G., An, A. K., & Kumar, M. (2018, March 1). *Occurrence and fate of emerging contaminants in water environment: A review*. Groundwater for Sustainable Development. Elsevier B.V. https://doi.org/10.1016/j.gsd.2017.12.009

Gordon, S. M., Brinkman, M. C., Ashley, D. L., Blount, B. C., Lyu, C., Masters, J., & Singer, P. C. (2006). *Changes in Breath Trihalomethane Levels Resulting from Household Water-Use Activities*. Environmental Health Perspectives, 114(4), 514–521. https://doi.org/10.1289/ehp.8171

Guelfi, D. R. V., Gozzi, F., Machulek Jr., A., Sirés, I., Brillas, E., & de Oliveira, S. C. (2018). *Degradation of herbicide S-metolachlor by electrochemical AOPs using a boron-doped diamond anode*. Catalysis Today, 313, 182–188. https://doi.org/10.1016/j.cattod.2017.10.026

Halbach, K., Möder, M., Schrader, S., Liebmann, L., Schäfer, R. B., Schneeweiss, A., … Reemtsma, T. (2021). *Small streams–large concentrations? Pesticide monitoring in small agricultural streams in Germany during dry weather and rainfall.* Water Research, 203. https://doi.org/10.1016/j.watres.2021.117535

Hansen, K., Mika, S., Schroeter, T., Sutter, A., Laak, A. T., Thomas, S. H., … Müller, K. R. (2009). *Benchmark data set for in silico prediction of Ames mutagenicity*. Journal of Chemical Information and Modeling, 49(9), 2077–2081. https://doi.org/10.1021/ci900161g

Hardy, A., Benford, D., Halldorsson, T., Jeger, M. J., Knutsen, H. K., … Younes, M. (2017). *Guidance on the use of the weight of evidence approach in scientific assessments.* EFSA Journal, 15(8). https://doi.org/10.2903/j.efsa.2017.4971

Hartnett, S., Musah, S., & Dhanwada, K. R. (2013). *Cellular effects of metolachlor exposure on human liver (HepG2) cells*. Chemosphere, 90(3), 1258–1266. https://doi.org/10.1016/j.chemosphere.2012.09.077

Hartung, T., & Rovida, C. (2009, August 27). *Chemical regulators have overreached*. Nature. https://doi.org/10.1038/4601080a

Hayes, T. B., Case, P., Chui, S., Chung, D., Haeffele, C., Haston, K., … Tsui, M. (2006). *Pesticide mixtures, endocrine disruption, and amphibian declines: Are we underestimating the impact?* Environmental Health Perspectives, 114(SUPPL.1), 40–50. https://doi.org/10.1289/ehp.805

Hinnenkamp, V., Balsaa, P., & Schmidt, T. C. (2021). Target, suspect and non-target screening analysis from wastewater treatment plant effluents to drinking water using collision cross section values as additional identification criterion. *Analytical and Bioanalytical Chemistry*, *414*(1), 425–438. https://doi.org/10.1007/s00216-021-03263-1

Hladik, M. L., Roberts, A. L., & Bouwer, E. J. (2005). *Removal of neutral chloroacetamide herbicide degradates during simulated unit processes for drinking water treatment*. Water Research, 39(20), 5033–5044. https://doi.org/10.1016/j.watres.2005.10.008

Hladik, M., Bouwer, E., & Roberts, A. (2008). *Neutral degradates of chloroacetamide herbicides: Occurrence in drinking water and removal during conventional water treatment*. Water Research, 42(20), 4905–4914. https://doi.org/10.1016/j.watres.2008.09.008

Ho, C. S., Lam, C. W. K., Chan, M. H. M., Cheung, R. C. K., Law, L. K., Lit, L. C. W., & Tai, H. L. (2003). *Electrospray ionisation mass spectrometry: principles and clinical applications.* The Clinical Biochemist. Reviews / Australian Association of Clinical Biochemists, PMID: 18568044. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1853331/

Hollender, J., Rothardt, J., Radny, D., Loos, M., Epting, J., Huggenberger, P., … Singer, H. (2018). *Comprehensive micropollutant screening using LC-HRMS/MS at three riverbank filtration sites to assess natural attenuation and potential implications for human health*. Water Research X, 1. https://doi.org/10.1016/j.wroa.2018.100007

Hollender, J., Rothardt, J., Radny, D., Loos, M., Epting, J., Huggenberger, P., Borer, P., & Singer, H. (2018). *Comprehensive micropollutant screening using LC-HRMS/MS at three riverbank filtration sites to assess natural attenuation and potential implications for human health*. Water Research X, 1, 100007. https://doi.org/10.1016/j.wroa.2018.100007

Jacobsen, A., de Miranda Azevedo, R., Juty, N., Batista, D., Coles, S., Cornet, R., Courtot, M., Crosas, M., Dumontier, M., Evelo, C. T., Goble, C., Guizzardi, G., Hansen, K. K., Hasnain, A., Hettne, K., Heringa, J., Hooft, R. W., Imming, M., Jeffery, K. G., . . . & Schultes, E. (2020). *FAIR Principles: Interpretations and Implementation Considerations*. Data Intelligence, 2(1–2), 10–29. https://doi.org/10.1162/dint_r_00024

Jensen, G. E., Niemelä, J. R., Wedebye, E. B., & Nikolov, N. G. (2008). *QSAR models for reproductive toxicity and endocrine disruption in regulatory use - A preliminary investigation*. In SAR and QSAR in Environmental Research (Vol. 19, pp. 631–641). https://doi.org/10.1080/10629360802550473

Ji, C., Song, Q., Chen, Y., Zhou, Z., Wang, P., Liu, J., Sun, Z., & Zhao, M. (2020). *The potential endocrine disruption of pesticide transformation products (TPs): The blind spot of pesticide risk assessment*. Environment International, 137, 105490. https://doi.org/10.1016/j.envint.2020.105490

Jursík, M., & Holec, J. (2019). *Future of weed management in sugar beet in central europe*. Listy Cukrovarnicke a Reparske, 135(5–6), 180–186. http://www.cukr-listy.cz/

Kavlock, R. J., Bahadori, T., Barton-Maclaren, T. S., Gwinn, M. R., Rasenberg, M., & Thomas, R. S. (2018, May 21). *Accelerating the Pace of Chemical Risk Assessment*. Chemical Research in Toxicology. American Chemical Society. https://doi.org/10.1021/acs.chemrestox.7b00339

Khan, K., Sanderson, H., & Roy, K. (2020). *Ecotoxicological QSARs of personal care products and biocides*. In Methods in Pharmacology and Toxicology (pp. 357–386). Humana Press Inc. https://doi.org/10.1007/978-1-0716-0150-1_16

Kiefer, K., Du, L., Singer, H., & Hollender, J. (2021). *Identification of LC-HRMS nontarget signals in groundwater after source related prioritization*. Water Research, 196, 116994. https://doi.org/10.1016/j.watres.2021.116994

Kiefer, K., Müller, A., Singer, H., & Hollender, J. (2019). *New relevant pesticide transformation products in groundwater detected using target and suspect screening for agricultural and urban micropollutants with LC-HRMS*. Water Research, 165, 114972. https://doi.org/10.1016/j.watres.2019.114972

Kirkland, D. J., Aardema, M., Banduhn, N., Carmichael, P., Fautz, R., Meunier, J. R., & Pfuhler, S. (2007). *In vitro approaches to develop weight of evidence (WoE) and mode of action (MoA) discussions with positive in vitro genotoxicity results*. Mutagenesis. Oxford University Press. https://doi.org/10.1093/mutage/gem006

Kleinstreuer, N. C., Hoffmann, S., Alépée, N., Allen, D., Ashikaga, T., Casey, W., Clouet, E., Cluzel, M., Desprez, B., Gellatly, N., Göbel, C., Kern, P. S., Klaric, M., Kühnl, J., Martinozzi-Teissier, S., Mewes, K., Miyazawa, M., Strickland, J., van Vliet, E., . . . Petersohn, D. (2018). *Non-animal methods to predict skin sensitization (II): an assessment of defined approaches*. Critical Reviews in Toxicology, *48*(5), 359–374. https://doi.org/10.1080/10408444.2018.1429386

Klutzny, S., Kornhuber, M., Morger, A., Schönfelder, G., Volkamer, A., Oelgeschläger, M., & Dunst, S. (2022). *Quantitative high-throughput phenotypic screening for environmental estrogens using the E-Morph Screening Assay in combination with in silico predictions*. Environment International, 158. https://doi.org/10.1016/j.envint.2021.106947

Knoell. (2022). *In silico Models*. KNOELL. https://www.knoell.com/en/solutions/in-silico-models

Knudsen, T. B., Martin, M. T., Kavlock, R. J., Judson, R. S., Dix, D. J., & Singh, A. V. (2009). *Profiling the activity of environmental chemicals in prenatal developmental toxicity studies using the U.S. EPA's ToxRefDB*. Reproductive Toxicology, 28(2), 209–219. https://doi.org/10.1016/j.reprotox.2009.03.016

Konuk, M., Abed, A. B., Liman, R., Aydın, B., Korcan, S. E., & Ciğerci, I. (2022). *Genotoxic effects and bacteria-related bioremediation of pesticides.* Pesticides Remediation Technologies From Water and Wastewater, 385–410. https://doi.org/10.1016/b978-0-323-90893-1.00018-0

Koroša A., Mali N. (2022). *Control of organic contaminants in groundwater by passive sampling and multivariate statistical analysis.* Journal of Environmental Management, Volume 318, 115440, ISSN 0301-4797, https://doi.org/10.1016/j.jenvman.2022.115440

Kotthoff, L., Keller, J., Lörchner, D., Mekonnen, T. F., & Koch, M. (2019b). *Transformation Products of Organic Contaminants and Residues—Overview of Current Simulation* Methods. Molecules, 24(4), 753. https://doi.org/10.3390/molecules24040753

la Cecilia, D., Dax, A., Ehmann, H., Koster, M., Singer, H., & Stamm, C. (2021). *Continuous high-frequency pesticide monitoring to observe the unexpected and the overlooked*. Water Research X, 13. https://doi.org/10.1016/j.wroa.2021.100125

Lai, A., Singh, R. R., Kovalova, L., Jaeggi, O., Kondić, T., & Schymanski, E. L. (2021). *Retrospective non-target analysis to support regulatory water monitoring: from masses of interest to recommendations via in silico workflows.* Environmental Sciences Europe, 33(1). https://doi.org/10.1186/s12302-021-00475-1

Laville, N., Balaguer, P., Brion, F., Hinfray, N., Casellas, C., Porcher, J. M., & Aït-Aïssa, S. (2006). *Modulation of aromatase activity and mRNA by various selected pesticides in the human choriocarcinoma JEG-3 cell line.* Toxicology, 228(1), 98–108. https://doi.org/10.1016/j.tox.2006.08.021

Lewis, K. A., Tzilivakis, J., Warner, D. J., & Green, A. (2016). *An international database for pesticide risk assessments and management*. Human and Ecological Risk Assessment: An International Journal, 22(4), 1050–1064. https://doi.org/10.1080/10807039.2015.1133242

Li, N., Qi, J., Wang, P., Zhang, X., Zhang, T., & Li, H. (2019). *Quantitative structure-activity relationship (QSAR) study of carcinogenicity of polycyclic aromatic hydrocarbons (PAHs) in atmospheric particulate matter by random forest (RF).* Analytical Methods, 11(13), 1816–1821. https://doi.org/10.1039/c8ay02720j

Liu, H., Ye, W., Zhan, X., & Liu, W. (2006). *A comparative study of rac- and S-metolachlor toxicity to Daphnia magna*. Ecotoxicology and Environmental Safety, 63(3), 451–455. https://doi.org/10.1016/j.ecoenv.2005.02.002

Liu, S., Wang, L., Chen, K., Yang, H., Ling, M., Wu, L., Zhou, X., Ma, G., & Bai, L. (2022). *Combined effects of S-metolachlor and benoxacor on embryo development in zebrafish (Danio rerio).* Ecotoxicology and Environmental Safety, *238*, 113565. https://doi.org/10.1016/j.ecoenv.2022.113565

Liu, T., Huang, Y., & Lin, H. (2021). *Estrogen disorders: Interpreting the abnormal regulation of aromatase in granulosa cells (Review).* International Journal of Molecular Medicine, 47(5). https://doi.org/10.3892/ijmm.2021.4906

Maculewicz, J., Kowalska, D., Świacka, K., Toński, M., Stepnowski, P., Białk-Bielińska, A., & Dołżonek, J. (2022). *Transformation products of pharmaceuticals in the environment: Their fate, (eco)toxicity and bioaccumulation potential*. Science of the Total Environment, 802, 149916. https://doi.org/10.1016/j.scitotenv.2021.149916

Marín-Benito, J. M., Herrero-Hernández, E., Ordax, J. M., Sánchez-Martín, M. J., & Rodríguez-Cruz, M. S. (2021). *The role of two organic amendments to modify the environmental fate of S-metolachlor in agricultural soils*. Environmental Research, 195, 110871. https://doi.org/10.1016/j.envres.2021.110871

Martin, M. T., & Judson, R. (2010). *ToxRefDB - Release user-friendly web-based tool for mining ToxRefDB*. U.S. Environmental Protection Agency, Washington, DC. https://cfpub.epa.gov/si/si_public_record_report.cfm?Lab=NCCT&dirEntryId=227139

Martin, M. T., Judson, R. S., Reif, D. M., Kavlock, R. J., & Dix, D. J. (2009). *Profiling chemicals based on chronic toxicity results from the US EPA ToxRef database.* Environmental Health Perspectives, 117(3), 392–399. https://doi.org/10.1289/ehp.0800074

Martin, M. T., Knudsen, T. B., Reif, D. M., Houck, K. A., Judson, R. S., Kavlock, R. J., & Dix, D. J. (2011). *Predictive model of rat reproductive toxicity from ToxCast high throughput screening.* Biology of Reproduction, 85(2), 327–339. https://doi.org/10.1095/biolreprod.111.090977

Martin, Y. C. (1996). *Exploring QSAR: Hydrophobic, Electronic, and Steric Constants* C. Hansch, A. Leo, and D. Hoekman. American Chemical Society, Washington, DC. 1995. pp. 22. ISBN 0-8412-2993-7

Marzo, M., Kulkarni, S., Manganaro, A., Roncaglioni, A., Wu, S., Barton-Maclaren, T. S., … Benfenati, E. (2016). *Integrating in silico models to enhance predictivity for developmental toxicity*. Toxicology, 370, 127–137. https://doi.org/10.1016/j.tox.2016.09.015

Mathias, F. T., Romano, R. M., Sleiman, H. K., de Oliveira, C. A., & Romano, M. A. (2012*). Herbicide Metolachlor Causes Changes in Reproductive Endocrinology of Male Wistar Rats*. ISRN Toxicology, 2012, 1–7. https://doi.org/10.5402/2012/130846

Matsushita, T., Morimoto, A., Kuriyama, T., Matsumoto, E., Matsui, Y., Shirasaki, N., … Kameya, T. (2018). *Removals of pesticides and pesticide transformation products during drinking water treatment processes and their impact on mutagen formation potential after chlorination*. Water Research, 138, 67–76. https://doi.org/10.1016/j.watres.2018.01.028

Matthews, E. J., Kruhlak, N. L., Daniel Benz, R., Ivanov, J., Klopman, G., & Contrera, J. F. (2007). *A comprehensive model for reproductive and developmental toxicity hazard identification: II. Construction of QSAR models to predict activities of untested chemicals.* Regulatory Toxicology and Pharmacology, 47(2), 136–155. https://doi.org/10.1016/j.yrtph.2006.10.001

Menger, F., Boström, G., Jonsson, O., Ahrens, L., Wiberg, K., Kreuger, J., & Gago-Ferrero, P. (2021). *Identification of Pesticide Transformation Products in Surface Water Using Suspect Screening Combined with National Monitoring Data*. Environmental Science and Technology, 55(15), 10343–10353. https://doi.org/10.1021/acs.est.1c00466

Mesnage, R., & Antoniou, M. (2021). *Mammalian toxicity of herbicides used in intensive GM crop farming.* In Herbicides: Chemistry, Efficacy, Toxicology, and Environmental Impacts (pp. 143–180). Elsevier. https://doi.org/10.1016/B978-0-12-823674-1.00007-9

Michel, C., Baran, N., André, L., Charron, M., & Joulian, C. (2021). *Side Effects of Pesticides and Metabolites in Groundwater: Impact on Denitrification.* Frontiers in Microbiology, 12. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8155494/

More, S., Bampidis, V., Benford, D., Boesten, J., Bragard, C., Halldorsson, T., … Schlatter, J. (2019). *Genotoxicity assessment of chemical mixtures*. EFSA Journal, 17(1), 1–11. https://doi.org/10.2903/j.efsa.2019.5519

Muendo, B. M., Shikuku, V. O., Getenga, Z. M., Lalah, J. O., Wandiga, S. O., & Rothballer, M. (2021). *Adsorption-desorption and leaching behavior of diuron on selected Kenyan agricultural soils*. Heliyon, 7(2). https://doi.org/10.1016/j.heliyon.2021.e06073

Murrell, K. A., Teehan, P. D., & Dorman, F. L. (2021). *Determination of contaminants of emerging concern and their transformation products in treated-wastewater irrigated soil and corn*. Chemosphere, p. 281. https://doi.org/10.1016/j.chemosphere.2021.130735

Muter, O., & Bartkevics, V. (2020, December 1). *Advanced analytical techniques based on high-resolution mass spectrometry for the detection of micropollutants and their toxicity in aquatic environments*. Current Opinion in Environmental Science and Health. Elsevier B.V. https://doi.org/10.1016/j.coesh.2020.05.002

Nagarathna, P. K. M., Johnson Wesley, M., Sriram Reddy, P., & Reena, K. (2013, October). *Review on genotoxicity, its molecular mechanisms and prevention*. International Journal of Pharmaceutical Sciences Review and Research. https://globalresearchonline.net/journalcontents/v22-1/43.pdf

National Library of Medicine. (2022). *Download Carcinogenic Potency Database (CPDB) Data*. NIH. https://www.nlm.nih.gov/databases/download/cpdb.html

NIH. (2022). *S-metolachlor*. PubChem. https://pubchem.ncbi.nlm.nih.gov/compound/S-Metolachlor

Nolte, T. M., Chen, G., van Schayk, C. S., Pinto-Gil, K., Hendriks, A. J., Peijnenburg, W. J., & Ragas, A. M. (2020). *Disentanglement of the chemical, physical, and biological processes aids the development of quantitative structure-biodegradation relationships for aerobic wastewater treatment.* Science of the Total Environment, 708, 133863. https://doi.org/10.1016/j.scitotenv.2019.133863

NORMAN. (2022). *NORMAN Substance Database*. https://www.norman-network.com/nds/susdat

Novič, M., & Vračko, M. (2010, March). *QSAR models for reproductive toxicity and endocrine disruption activity*. Molecules. https://doi.org/10.3390/molecules15031987

NPIC. (2022). *Water Solubility*. National Pesticides Information Center. NPIC. http://npic.orst.edu/envir/watersol.html

O'Connell, P. J., Harms, C. T., & Allen, J. R. F. (1998). *Metolachlor, S-metolachlor and their role within sustainable weed-management.* Crop Protection, 17(3), 207–212. https://doi.org/10.1016/S0261-2194(98)80011-2

OECD. (2004). *OECD principles for the validation, for regulatory purposes, of (quantitative) structure-activity relationships models*. Biotechnology, (November), pp. 1–2. Retrieved from www.oecd.org/dataoecd/33/37/37849783.pdf

OECD. (2016). *OECD Guideline for the testing of chemicals: n° 421: Reproduction/Developmental Toxicity Screening Test*. In OECD (ISSN: 20745788). https://www.oecd-ilibrary.org/environment/test-no-421-reproduction-developmental-toxicity-screening-test_9789264264380-en

OECD. (2017). *Guidance on Grouping of Chemicals. Series on Testing and Assessment Number 80*. ENV/JM/MONO(2007)28. Organisation for Economic Cooperation and Development, Paris, France. 2nd edition. https://www.oecd.org/publications/guidance-on-grouping-of-chemicals-second-edition-9789264274679-en.htm

Otto, S., Pappalardo, S. E., Cardinali, A., Masin, R., Zanin, G., & Borin, M. (2016). *Vegetated Ditches for the Mitigation of Pesticides Runoff in the Po Valley*. PLOS ONE, 11(4), e0153287. https://doi.org/10.1371/journal.pone.0153287

Pallocca, G., Moné, M. J., Kamp, H., Luijten, M., Van de Water, B., & Leist, M. (2022). *Next-generation risk assessment of chemicals - Rolling out a human-centric testing strategy to drive 3R implementation: The RISK-HUNT3R project perspective*. ALTEX, 39(3), 419–426. https://doi.org/10.14573/altex.2204051

Papamokos, G., & Silins, I. (2016). *Combining QSAR modeling and text-mining techniques to link chemical structures and carcinogenic modes of action*. Frontiers in Pharmacology, 7(AUG). https://doi.org/10.3389/fphar.2016.00284

Paparella, M., Bennekou, S. H., & Bal-Price, A. (2020). *An analysis of the limitations and uncertainties of in vivo developmental neurotoxicity testing and assessment to identify the potential for alternative approaches*. Reproductive Toxicology, 96, 327–336. https://doi.org/10.1016/j.reprotox.2020.08.002

Pearl, G., Livingston-Carr, S., & Durham, S. (2005). *Integration of Computational Analysis as a Sentinel Tool in Toxicological Assessments*. Current Topics in Medicinal Chemistry, 1(4), 247–255. https://doi.org/10.2174/1568026013395074

Pelkonen, O. (2010). *Predictive toxicity: Grand challenges*. Frontiers in Pharmacology, APR. https://doi.org/10.3389/fphar.2010.00003

Petrie, B., Barden, R., & Kasprzyk-Hordern, B. (2015). *A review on emerging contaminants in wastewaters and the environment: Current knowledge, understudied areas and recommendations for future monitoring.* Water Research, 72, 3–27. https://doi.org/10.1016/j.watres.2014.08.053

Pfeiffer, M. (2010). *Groundwater Ubiquity Score (GUS)*. Pesticide Training Resources. Ptrpest.Com, pp. 40–41.

Pico’ Y., and Barcelo’ D., *Transformation products of emerging contaminants in the environment and high-resolution mass spectrometry: a new horizon*. Anal Bioanal Chem 407, 6257–6273 (2015). https://doi.org/10.1007/s00216-015-8739-6

Piersma, A. H., Rorije, E., Beekhuijzen, M. E. W., Cooper, R., Dix, D. J., Heinrich-Hirsch, B., … Hakkert, B. C. (2011). *Combined retrospective analysis of 498 rat multi-generation reproductive toxicity studies: On the impact of parameters related to F1 mating and F2 offspring*. Reproductive Toxicology, 31(4), 392–401. https://doi.org/10.1016/j.reprotox.2010.11.013

Rallo, R., Espinosa, G., & Giralt, F. (2005). *Using an ensemble of neural based QSARs for the prediction of toxicological properties of chemical contaminants*. Process Safety and Environmental Protection, 83(4 B), pp. 387–392. https://doi.org/10.1205/psep.04389

RIVM. (2022). *Replacement, reduction, refinement of animal use*. National Institute for Public Health and the Environment. https://www.rivm.nl/en/replacement-reduction-refinement-of-animal-use

Rorije, E., Muller, A., Beekhuijzen, M. E. W., Hass, U., Heinrich-Hirsch, B., Paparella, M., … Piersma, A. H. (2011). *On the impact of second generation mating and offspring in multi-generation reproductive toxicity studies on classification and labelling of substances in Europe*. Regulatory Toxicology and Pharmacology, 61(2), 251–260. https://doi.org/10.1016/j.yrtph.2011.08.005

Rousis, N. I., Denardou, M., Alygizakis, N., Galani, A., Bletsou, A. A., Damalas, D. E., … Thomaidis, N. S. (2021). *Assessment of environmental pollution and human exposure to pesticides by wastewater analysis in a seven-year study in athens, greece*. Toxics, 9(10). https://doi.org/10.3390/toxics9100260

Schollée, J. E., Schymanski, E. L., Stravs, M. A., Gulde, R., Thomaidis, N. S., & Hollender, J. (2017). *Similarity of High-Resolution Tandem Mass Spectrometry Spectra of Structurally Related Micropollutants and Transformation Products*. Journal of the American Society for Mass Spectrometry, 28(12), 2692–2704. https://doi.org/10.1007/s13361-017-1797-6

Shaner, D. L., Brunk, G., Belles, D., Westra, P., & Nissen, S. (2006). *Soil dissipation and biological activity of metolachlor and S-metolachlor in five soils*. Pest Management Science, 62(7), 617–623. https://doi.org/10.1002/ps.1215

Sharma, A., Kumar, V., Shahzad, B., Tanveer, M., Sidhu, G. P. S., Handa, N., … Thukral, A. K. (2019, November 1). *Worldwide pesticide usage and its impacts on ecosystem*. SN Applied Sciences. Springer Nature. https://doi.org/10.1007/s42452-019-1485-1

Skanes, B., Warriner, K., & Prosser, R. S. (2021). *Hazard assessment using an in-silico toxicity assessment of the transformation products of boscalid, pyraclostrobin, fenbuconazole and glyphosate generated by exposure to an advanced oxidative process*. Toxicology *in* Vitro, 70. https://doi.org/10.1016/j.tiv.2020.105049

Soulier, C., Coureau, C., & Togola, A. (2016). *Environmental forensics in groundwater coupling passive sampling and high resolution mass spectrometry for screening*. Science of the Total Environment, 563–564, 845–854. https://doi.org/10.1016/j.scitotenv.2016.01.056

Speight, J. G. (2016). *Chapter 6 - Introduction Into the Environment* [ScienceDirect]. In Environmental Organic Chemistry for Engineers: Vol. ISBN 9780128044926 (1st ed.). Butterworth-Heinemann. pp. 263-303 https://doi.org/10.1016/B978-0-12-804492-6.00006-X

Suciu, N., Farolfi, C., Zambito Marsala, R., Russo, E., De Crema, M., Peroncini, E., Tomei, F., Antolini, G., Marcaccio, M., Marletto, V., Colla, R., Gallo, A., & Capri, E. (2020). *Evaluation of groundwater contamination sources by plant protection products in hilly vineyards of Northern Italy*. Science of the Total Environment, 749, 141495. https://doi.org/10.1016/j.scitotenv.2020.141495

Suman, T. Y., Kim, S. Y., Yeom, D. H., & Jeon, J. (2022b). *Transformation Products of Emerging Pollutants Explored Using Non-Target Screening: Perspective in the Transformation Pathway and Toxicity Mechanism—A Review*. Toxics, 10(2), 54. https://doi.org/10.3390/toxics10020054

Syafrudin, M., Kristanti, R. A., Yuniarto, A., Hadibarata, T., Rhee, J., Al-Onazi, W. A., ... Al-Mohaimeed, A. M. (2021, January 2). *Pesticides in drinking water-a review. International Journal of Environmental Research and Public Health*. MDPI AG. https://doi.org/10.3390/ijerph18020468

Tang, Y., Yin, M., Yang, W., Li, H., Zhong, Y., Mo, L., & Sun, X. (2019, October 1). *Emerging pollutants in water environment: Occurrence, monitoring, fate, and risk assessment*. Water Environment Research. John Wiley and Sons Inc. https://doi.org/10.1002/wer.1163

Tröger, R., Ren, H., Yin, D., Postigo, C., Nguyen, P. D., Baduel, C., Golovko, O., Been, F., Joerss, H., Boleda, M. R., Polesello, S., Roncoroni, M., Taniyasu, S., Menger, F., … & Wiberg, K. (2021). *What's in the water? – Target and suspect screening of contaminants of emerging concern in raw water and drinking water from Europe and Asia*. Water Research, 198. https://doi.org/10.1016/j.watres.2021.117099

UNESCO. (2020). *Water resources an essential part of the solution to climate change*. *UNESCO website*. https://www.unesco.org/en/articles/water-resources-essential-part-solution-climate-change

University of Hertfordshire. (2022). *Pesticide Properties DataBase* [Dataset]. In S-metolachlor (Ref: CGA 77102). https://sitem.herts.ac.uk/aeru/ppdb/en/Reports/1027.htm

Valerio, L. G. (2009, December 15). *In silico toxicology for the pharmaceutical sciences*. Toxicology and Applied Pharmacology. https://doi.org/10.1016/j.taap.2009.08.022

Wang, F., Liigand, J., Tian, S., Arndt, D., Greiner, R., & Wishart, D. S. (2021). *CFM-ID 4.0: More Accurate ESI-MS/MS Spectral Prediction and Compound Identification*. Analytical Chemistry, 93(34), 11692–11700. https://doi.org/10.1021/acs.analchem.1c01465

Watford, S., Ly Pham, L., Wignall, J., Shin, R., Martin, M. T., & Friedman, K. P. (2019). *ToxRefDB version 2.0: Improved utility for predictive and retrospective toxicology analyses*. Reproductive Toxicology, pp. 89, 145–158. https://doi.org/10.1016/j.reprotox.2019.07.012

WHO. (2022). *Guidelines for drinking-water quality: Fourth edition incorporating the first and second addenda*. ISBN: 978-92-4-004506-4. https://www.who.int/publications/i/item/9789240045064

Wilkinson, M. D., Dumontier, M., Aalbersberg, I. J., Appleton, G., Axton, M., Baak, A., Blomberg, N., Boiten, J. W., da Silva Santos, L. B., Bourne, P. E., Bouwman, J., Brookes, A. J., Clark, T., Crosas, M., Dillo, I., Dumon, O., Edmunds, S., Evelo, C. T., Finkers, R., . . . Mons, B. (2016). *The FAIR Guiding Principles for scientific data management and stewardship.* Scientific Data, 3(1). https://doi.org/10.1038/sdata.2016.18

WISE. (2021). *Water Framework Directive*. WISE - Freshwater information system for Europe. https://water.europa.eu/freshwater/europe-freshwater/water-framework-directive

Wołejko, E., Kaczyński, P., Łozowicka, B., Wydro, U., Borusiewicz, A., Hrynko, I., … Malinowski, P. (2017). *Dissipation of S-metolachlor in plant and soil and effect on enzymatic activities.* Environmental Monitoring and Assessment, 189(7). https://doi.org/10.1007/s10661-017-6071-7

Worth, A., Fuart-Gatnik, M., Lapenna, S., & Serafimova, R. (2011). *Applicability of QSAR analysis in the evaluation of developmental and neurotoxicity effects for the assessment of the toxicological relevance of metabolites and degradates of pesticide active substances for dietary risk assessment*. EFSA Supporting Publications, 8(6). https://doi.org/10.2903/sp.efsa.2011.en-169

Yang, L., Ivantsova, E., Souders, C. L., & Martyniuk, C. J. (2021). *The agrochemical S-metolachlor disrupts molecular mediators and morphology of the swim bladder: Implications for locomotor activity in zebrafish (Danio rerio)*. Ecotoxicology and Environmental Safety, 208. https://doi.org/10.1016/j.ecoenv.2020.111641

Yang, Y., Zhang, X., Jiang, J., Han, J., Li, W., Li, X., … Alvarez, P. J. J. (2022, January 4). *Which Micropollutants in Water Environments Deserve More Attention Globally?* Environmental Science and Technology. American Chemical Society. https://doi.org/10.1021/acs.est.1c04250

Zahn, D., Mucha, P., Zilles, V., Touffet, A., Gallard, H., Knepper, T. P., & Frömel, T. (2019). *Identification of potentially mobile and persistent transformation products of REACH-registered chemicals and their occurrence in surface waters*. Water Research, 150, 86–96. https://doi.org/10.1016/j.watres.2018.11.042

Zambito Marsala, R., Capri, E., Russo, E., Bisagni, M., Colla, R., Lucini, L., … Suciu, N. A. (2020). *First evaluation of pesticides occurrence in groundwater of Tidone Valley, an area with intensive viticulture*. Science of the Total Environment, 736. https://doi.org/10.1016/j.scitotenv.2020.139730

Zemolin, C. R., Avila, L. A., Cassol, G. V., Massey, J. H., & Camargo, E. R. (2014, July 1). *Environmental fate of S-metolachlor - A review*. Planta Daninha. Sociedade Brasileira da Ciencia das Plantas Daninha. https://doi.org/10.1590/S0100-83582014000300022

Zemolin, C., Avila, L., Cassol, G., Massey, J., & Camargo, E. (2014). *Environmental fate of S-Metolachlor: a review*. Planta Daninha, 32(3), 655–664. https://doi.org/10.1590/s0100-83582014000300022

Zorn, K. M., Foil, D. H., Lane, T. R., Hillwalker, W., Feifarek, D. J., Jones, F., … Ekins, S. (2020). *Comparing Machine Learning Models for Aromatase (P450 19A1)*. Environmental Science and Technology, 54(23), 15546–15555. https://doi.org/10.1021/acs.est.0c05771

Zou, E. (2020, July 1). *Invisible endocrine disruption and its mechanisms: A current review*. General and Comparative Endocrinology. Academic Press Inc. https://doi.org/10.1016/j.ygcen.2020.113470