TOXICITY ASSESSMENT OF PESTICIDES WITH NO ECOTOXICOLOGICAL DATA TO FRESHWATER ALGAE

by

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ABSTRACT

TOXICITY ASSESSMENT OF PESTICIDES WITH NO ECOTOXICOLOGICAL DATA TO FRESHWATER ALGAE

Widespread contamination of water bodies by pesticides is indeed a matter of great concern. Therefore, their adverse effects on non-target organisms including algae, invertebrates, and fish should be investigated. In this study, freshwater algal toxicity assay was carried out for 8 pesticides (acetamiprid, acetochlor, boscalid, diclofop, diphenamid, gibberellic acid, ioxynil, and 2,4,5-T) selected from the list of chemicals with no ecotoxicological data recently announced by TUBITAK. The effect of selected pesticides on the growth rate of *Chlorella vulgaris* was measured using the Organisation for Economic Cooperation and Development (OECD) test number 201. The order of 96-h algal toxicity is acetochlor>ioxynil>diclofop>2,4,5-T. The toxicity value for boscalid could not be determined in its solubility limit. Acetamiprid, diphenamid and gibberellic acid revealed IC_{50} values > 100 mg/L.The experimental results are compared to the predicted toxicity values of tested chemicals from the literature QSAR models. Also, the toxicity values were predicted from an algae-algae (*Chlorella vulgaris -Pseudokirchneriella subcapitata*) toxicity relationship. Low correlation was found between the toxicity and hydrophobicity implying that these chemicals exert their toxicity in different mode of action other than baseline toxicity. Global half-life, persistence bioaccumulation and toxicity (PBT) indices were calculated for all chemicals. Boscalid and diclofop were found to be PBT chemicals. The calculated low-toxic-effect concentrations (LOEC, NOEC and IC_{20}) were used in risk assessment and Acute to Chronic Ratio (ACR) were evaluated to control if the ACR values are within the safe level of chemicals set for algae.

ÖZET

EKOTOKSİKOLOJİK VERİ EKSİKLİĞİ OLAN PESTİSİTLERİN TATLI SU ALG TOKSİSİTELERİNİN BELİRLENMESİ

Su kaynaklarının pestisitler tarafından yaygın olarak kontaminasyonu, oldukça büyük bir endişe teşkil etmektedir. Bu nedenle, pestisitlerin algler, omurgasızlar ve balıklar dahil olmak üzere hedefte olmayan organizmalar üzerindeki olumsuz etkileri araştırılmalıdır. Bu çalışmada, son zamanlarda TÜBİTAK tarafından açıklanan ekotoksikolojik veri eksikliği bulunan kimyasallar listesinden seçilen 8 pestisit (asetamiprid, asetoklor, boscalid, diklofop, difenamid, gibberellik asit, iyoksinil ve 2,4,5-T) için tatlı su alg toksisitesi deneyi gerçekleştirilmiştir. Seçilen pestisitlerin *Chlorella vulgaris*'in büyüme hızı üzerindeki etkisi, OECD'nin 201 numaralı testine göre ölçülmüştür. 96 saatlik alg toksisitesi sırasıyla asetoklor>iyoksinil>diklofop>2,4,5-T olarak bulunmuştur. Boscalid'in toksisite değeri, çözünürlük sınırları içinde belirlenememiştir. Asetamiprid, difenamid ve gibberellik asit, IC_{50} değerleri > 100 mg /L olarak bulunmuştur. Deney sonuçları, literatür QSAR modellerinden test edilen kimyasalların tahmini toksisite değerleri ile karşılaştırılmıştır. Ayrıca, toksisite değerleri bir alg-alg (*Chlorella vulgaris* - *Pseudokirchneriella subcapitata*) toksisite ilişkisinden tahmin edilmiştir. Düşük toksisite ve hidrofobisite ilişkisi, bu kimyasalların toksisitesini temel toksisite dışındaki farklı etki modlarında uyguladıklarını gösteren düşük bir ilişki bulunmuştur. Tüm kimyasallar için global yarı ömür ve kalıcılık biyobirikim ve toksisite endeksleri hesaplanmıştır. Boscalid ve asetoklor PBT özelliği olan kimyasallar olarak bulunmuştur. Risk değerlendirmesinde hesaplanan düşük toksik etki konsantrasyonları (LOEC, NOEC ve IC_{20}) kullanılmıştır ve ACR verileri kimyasal konsantrasyonlarının alg için belirlenmiş olan güvenli seviyesinde olup olmadığının kontrolü bakımından değerlendirilmiştir.

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LIST OF SYMBOLS/ABBREVIATIONS

Symbol	Explanation
CO_2	Carbondioxide
R^2	Coefficient of Determination
µg/L	Micrograms per Liter

Abbreviation	Explanation	Unit
2,4,5-T	2,4,5-trichlorophenoxyacetic acid	
2D	2-dimensional	
3,5-DCP	3,5-dichlorophenol	
ACR	Acute to Chronic Toxicity Ratio	
ACR_{EC20}	Acute to Chronic Toxicity Ratio Based on 20% Inhibition	
ACR_{NOEC}	Acute to Chronic Toxicity Ratio Based on No Observed Effect Concentration	
AD	Applicability Domain	
AF	Assessment Factor	
APHA	American Public Health Association	
ASD	Autism Spectrum Disorder	
B	Bioaccumulative	
B05[C-S]	Presence/Absence of C–S at Topological Distance 5	
3 N BBM+V	Bold Basal Medium with 3-fold Nitrogen and Vitamins	
BCF	Bioconcentration Factor	
C. riparius	*Chironomus riparius*	
C. vulgaris	*Chlorella vulgaris*	
CAS	Chemical Abstracts Service	
CATS2D_02_AP	CATS2D Acceptor-Positive at Lag 02	
CHD	Congenital Heart Defects	
ChV	Chronic Value	
CLP	Classification, Labelling and Packaging	

CMR	Carcinogenic, Mutagenic and Reproductive Toxicity	
CoA	Coenzyme A	
D. magna	*Daphnia magna*	
DM	Diclofop-methyl	
DMSO	Dimethylsulfoxide	
EC_{10}	10% Effective Concentration	mg/L
EC_{20}	20% Effective Concentration	mg/L
EC_{50}	50% Effective Concentration	mg/L
ECHA	European Chemical Agency	
ECOSAR	Ecological Structure Activity Relationships	
EC_x	x% Effective Concentration	mg/L
EFSA	European Food Safety Authority	
E_{LUMO}	Lowest Unoccupied Molecular Orbital Energy	
E_{HOMO}	Highest Occupied Molecular Orbital Energy	
EU	Europen Union	
EU TGD	European Union Technical Guidance Document	
F03[C-N]	Frequency of C–N at Topological Distance 3	
GA3	Gibberellic acid	
GGI8	Topological Charge Index of Order 8	
GHLI	Global Half-Life Index	
GHS	Globally Harmonised System	
h	Hat Value	
h^*	Critical Hat Value	
HPV	High Production Volume	
IC_{10}	10% Inhibitory Concentration	mg/L
IC_{20}	20% Inhibitory Concentration	mg/L
IC_{50}	50% Inhibitory Concentration	mg/L
LC_{50}	50% Lethal Concentration	mg/L

ICp	Linear Interpolation Combined with Bootstrapping	
Ig(%)	Percent Growth Inhibition	
IOMC	The Inter-Organization Programme for the Sound Management of Chemicals	
IUPAC	International Union of Pure and Applied Chemistry	
K_a	Acid Dissociation Constant	
K_{ow}	*n*-octanol-water Coefficient	
L. variegatus	*Lytechinus variegatus*	
LOEC	Lowest Observed Effective Concentration	
Log D	Logarithm of pH Dependent Hydrophobicity	
Log P	Logarithm of *n*-octanol-water Partition Coefficient	
M. aeruginosa	*Microcystis aeruginosa*	
MATC	Maximum Acceptable Toxicant Concentration	
MAXDP2	Maximal Electrotopological Positive Variation	
maxHBa	Maximum E-States for (Strong) Hydrogen Bond Acceptors	
MDA	Malondialdehyde	
mg	Miligram	
minHother	Minimum Atom-type H E-State: H on aaCH, dCH2 or dsCH	
minsCl	Minimum Atom-type E-State: -Cl	
mM	Milimolar	
mmHg	Milimeter of Mercury	
MoA	Mode of Action	
Mor31p	Signal 31/weighted by polarizability	
MLOGP2	Squared Moriguchi *n*-octanol-water Partition Coefficient	
Mp	Mean Atomic Polarizabilities (scaled on carbon atom)	
MW	Molecular Weight	mol/L

nAChR	Nicotinic Acetylcholine Receptor	
nBondsM	Number of Multiple Bonds	
nBondsS2	Total Number of Single Bonds (including bonds to hydrogens, excluding aromatic bonds)	
NdsCH	Number of Atoms of Type dsCH	
ng/g	Nanograms per Gram	
nHBDon_Lipinski	Number of Hydrogen Bond Donors, Using Lipinski's Definition	
NOEC	No Observed Effective Concentration	mg/L
nm	Nanometers	
non-POPs	Non-Persistent Organic Pollutants	
NTD	Neural Tube Defects	
nX	Number of Halogen Atoms	
OECD	Organisation for Economic Co-operation and Development	
p	Probability Value	
P	Persistence	
P. subcapitata	*Pseudokirchneriella subcapitata*	
PBT	Persistence, Bioaccumulation and Toxicity	
PCA	Principal Component Analysis	
PCP	Personal Care Products	
PEC	Predicted Environmental Concentration	µg/L
pEC_{50}	Negative Logarithm of 50% Effective Concentration	mM or M
pH	Potential Hydrogen	
piPC6	Conventional Bond Order ID Number of Order 6 (ln(1+x)	
pK_a	The Negative Logarithm of the Acid Dissociation Constant	
PNEC	Predicted No Effect Concentration	
POPs	Persistent Organic Pollutants	
pT	Negative Logarithm of Toxicity Value	mM or M
QSAR	Quantitative Structure-Activity Relationship	

QSPR	Quantitative Structure -Property Relationship
QSTR	Quantitative Structure-Toxicity Relationship
QTTR	Quantitative Toxicity-Toxicity Relationship
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
RF	Risk Factor
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
SPAM	Average Span R
T	Toxicity
TRI	Toxic Release Inventory
TUBITAK	Scientific and Technological Research Council of Turkey
TÜBİTAK-ARDEB	Türkiye Bilimsel ve Teknolojik Araştırma Kurumu – Araştırma Destek Programları Başkanlığı
UN GHS	The United Nations Globally Harmonised System of Classification and Labelling of Chemicals
USEPA	United States Environmental Protection Agency
VE3_Dt	Logarithmic Coefficient Sum of The Last Eigenvector from Detour Matrix
VCH-6	Valence Chain, Order 6
VLCFA	Very Long Chain Fatty Acids
vPvB	Very Persistent and Very Bioaccumulative

1. INTRODUCTION

Anthropocene is defined as a period when human activities trigger global environmental changes. During this period, which is thought to have begun with the Industrial Revolution of the 19th century, there has been a pressure on some important planetary boundaries as a result of human activities. One of these boundaries is chemical pollution. With the rapid growth of chemical industry, nearly 100,000 chemicals were manufactured and utilized all over the world (Egeghy et al., 2012). Most of these chemicals can contaminate aquatic environment after direct or indirect releases from agriculture, industries and households (Afkar et al., 2010). It is certain that besides their benefits, these chemicals have some adverse effects on environment and especially on aquatic ecosystems (Fochtman et al., 2000). Agriculture is one of the few activities where chemicals, namely pesticides, are integrated into the environment on purpose. There are various types of pesticides applied for specific purposes. The term "pesticide" is a composite term which contains all chemicals used to kill or control pests. In agriculture, this covers herbicides (to control weeds), insecticides (to control insects), fungicides (to control fungi), nematocides (to control nematodes), and rodenticides (vertebrate poisons). Figure 1.1. shows the percentage of pesticide types in world market.

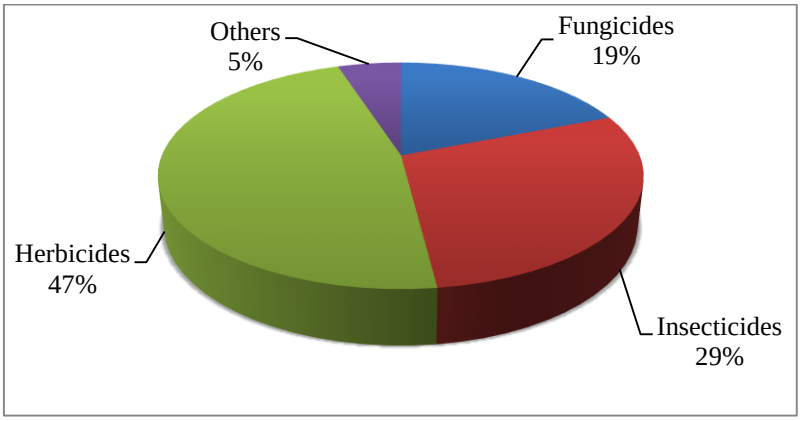


Figure 1.1. World pesticide expenditures at user level by pesticide type (Grube et al., 2011).

A new EU chemicals regulation, Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) aims to increase the protection of human health and environment, provides the most extensive dataset on individual chemicals (ecotoxicity, uses and exposure), that should be provided in material safety data sheet of each chemical. Authorisation is required for use of chemicals that cause Cancer, Mutations or problems with Reproduction (CMR), that are Persistent,

Bioaccumulative and Toxic (PBT) or very Persistent and very Bioaccumulative (vPvB), or that are identified from scientific evidence as causing probably serious effects on humans or the environment. On 1 June 2007, it entered into force as a law.

REACH–like legislation in Turkey as National (Turkish) REACH regulation has been prepared by Environment and City Planning Ministry and published in the official newspaper in June 23rd, 2017 with the number of 30105 and the regulation title of "KİMYASALLARIN KAYDI, DEĞERLENDİRİLMESİ, İZNİ VE KISITLANMASI HAKKINDA YÖNETMELİK". This kind of legislations suggest an increase in the global need of aquatic toxicity results in the near future.

Of the minimum required data set for the assessment of environmental and human hazards PBT (Persistence (P), Bioaccumulation (B) and ecoToxicology (T)) properties are of major concern as well as CMR (Carcinogenic, Mutagenic and Reproductive toxicity) properties. However this information is not available for the majority of the existing chemicals.

Risk assessment for individual chemicals uses normally a basic dataset on (eco)toxicity studies. More often acute than chronic ecotoxicological data are provided. The retrieved endpoints are used to derive limit values, like the Predicted No Effect Concentration (PNEC). An environmental risk assessment is performed by comparing the Predicted No-Effect Concentration (PNEC) with the exposure values, the Predicted Environmental Concentrations (PECs). In other words, along with exposure data, risk management measures can be developed. These data are also used for the environmental hazard identification of the substances, that is, the toxicity threshold estimation in the Persistent, Bioaccumulative and Toxicity (PBT) assessment. As a chemical is desired to be registered, it must be indicated whether it belongs to a PBT substance or vPvB substance or not.

In the EU, aquatic toxicity data are generated for chemicals, plant protection products, pharmaceuticals, biocides and feed additives. The assessment of short-term algal toxicity data provide data on plants, one out of three trophic levels. Therefore, algae have a significant role in aquatic ecosystems. They are the primary producers generating oxygen and located in the beginning of food chain in most of aquatic environments (Shigeoka et al., 1988). Also, algae have a very big role in biochemical cycles, like nitrogen and carbon cycles (Boyce et al., 2010). Besides their vital roles in the aquatic ecosystems, wide distribution throughout the globe, easy collection and culturing, fast growth etc. make them a suitable test organism to be used in algal toxicity bioassays (DeLorenzo, 2009).

1.1. Aim of the Study

The purpose of this study is to use freshwater algae, namely *Chlorella vulgaris* as a test organism and determine 96-h growth inhibition values (96-h IC_{50}, 50% effect concentration in terms of inhibition of growth rate) of 8 chemicals using the standardized test protocols (APHA et al., 2012; OECD, 2011), to obtain both the inhibitory concentrations of the chemicals that result in a 50% reduction (IC_{50}) and low-toxic-effect concentrations, LOEC (Lowest Observed Effect Concentration) and NOEC (No Observed Effect Concentration), statistically determined to be used in environmental hazard assessments. The chemicals were selected from the list of chemicals with no ecotoxicological data which were recently announced by TUBITAK-ARDEB (SU0303, 2015). The second objective of the present study is to compare the experimental algal toxicity values with the predicted toxicity values from various models reported in the literature. Within this scope, we aimed to use previously published four quantitative structure-toxicity relationship (QSTR) models and one quantitative toxicity-toxicity relationship (QTTR) model. The QTTR model is based on algae–algae (*Chlorella vulgaris* - *Pseudokirchneriella subcapitata*) relationship and the independent variable was the 72-h toxicity values of *Pseudokirchneriella subcapitata*. The third objective of this study is the assessment of Global Half-Life Index (GHLI) and PBT Index as implemented in QSARINS 2.2.1 software for the selected pesticides. A final objective of this study was to comment on risk assessment perspective by calculating various terms (e.g., low-toxic-effect concentrations, Predicted No Effect Concentration (PNEC), and Acute to Chronic Ratio (ACR), etc.) used in risk assessment.

2. LITERATURE REVIEW

2.1. REACH Regulation

The diffusion of anthropogenic chemicals into different divisions of environment is the main object for water quality and subject for increasing both public and scientific concern (Brinkmann et al., 2017). According to CAS Registry (https://www.cas.org/about/cas-content), 142 million organic and inorganic substances were adverted in literature from the early 1800s, and it is also known that each year, thousands of new chemicals are produced. Although, the information on the physico-chemical properties, environmental reactivity and biological activity for newly produced chemicals is available, the same can not be said for the great number of "existing" chemicals (including High Production Volume (HPV) compounds) (Gramatica, 2013). The European Council and the European Parliament established the REACH reagulation to protect the environment and as a result human health from the harmful effects of exposure to environmental chemicals (Schwarzman and Wilson, 2009). It provides a single body of knowledge with related data on the properties and activities of all “existing” and “new” commercialized chemicals for a safer chemical management. Also, under this legislation, manufacturers and suppliers are required to register chemical information on their physicochemical properties and possible risks on human, animals, organisms or environment in a central database of the European Chemicals Agency (ECHA) (Zarfl and Matthies, 2013). Toxicity should be provided for representative species of all trophic levels, such as destruents (bacteria), producers (algae) and invertebrates and vertebrate consumers (daphnids and fish, respectively) according to the aquatic risk assessment (Brinkmann et al., 2017). Depending on the increased production quantities, different test requirements must be established for these trophic levels (ECHA, 2016; Schulte et al., 2012). Additionally, PBT/vPvB assessment of a substance according to REACH regulation should be made according to the criteria specified in Annex XIII (REACH, 2006). However, according to the criteria indicated, the PBT screening is still a problematic process due to both low quality and quantity of available Persistence, Bioaccumulation and Toxicity data (Arnot and Mackay, 2008).

2.2. Growth Inhibition Test with Algae

Aquatic toxicity data are needed to assess the effects of chemical substances on living organisms. Algae play a major role in the collection of ecotoxicity data, as they constitute about half of the primary producers and the first ring of the food chain. Algae are also preferred in toxicology tests because they form the majority of the aquatic ecosystem, are easy to sample, take up less space in testing, grow fast and respond quickly to tests (DeLorenzo, 2009). Algae are more responsive to chemicals than fish, suggesting that the number of fish required for regulatory toxicity testing can be reduced in the presence of reliable algal endpoints (Hoekzema et al., 2006; Önlü and Saçan, 2017). On the other hand, reported algal toxicity data are less than for fish and crustaceans (Cronin et al., 2004).

The short-term (acute) toxicity is generally defined as the concentration that causes the elimination of 50% of the organisms (Effective Concentration, EC_{50}; Lethal Concentration, LC_{50}; Inhibitory Concentration, IC_{50}) (Netzeva et al., 2008). The IC_{50} value is generally expressed in mg/L, mol/L, and mmol/L. The IC_{50} value and toxicity are inversely proportional terms, i.e. a compound with a lower IC_{50} value is more toxic than the one with a higher IC_{50}. In the present study, the aquatic toxicity experiments were carried out using the standardized test protocols (APHA et al., 2012; OECD, 2011), to obtain IC_{50}, LOEC (Lowest Observed Effect Concentration) and NOEC (No Observed Effect Concentration), statistically determined) values to be used in environmental risk assessments.

The main purpose of the algal growth inhibition test is to observe any side effects of the tested chemicals on the growth of algae. The response of the organisms is not affected by the individual tolerance of the organisms, since a great amount of cells from a single algal species are exposed to algal bioassays (Christensen et al., 2009). The main point of these tests is to apply the test substance in increasing concentrations to an exponentially growing test organisms in batch cultures over a specific test period (generally from 48 to 96 hours). The response is measured as a reduction in the algal growth subjected to the increasing concentration of a test substance in a specific time.

The algal growth is quantified including cell counts, optical density, fluorescence, etc. The logarithmic increase in the number of algal cells gives out the endpoint. From the laboratory experiments, average specific growth rate data are recorded and a specific inhibition % is determined. Additionally, OECD (2011) states another response variable, which is the definition of

biomass at the end of the exposure period minus the biomass at the start of the exposure period, as it is called as the yield. The x% inhibition yield is calculated by the collected data from the experiments. These algal tests give out useful data for the usage in environmental hazard evaluations.

According to OECD (2011), algal growth inhibition test can be applied on different microalgae and cyanobacteria species. The recommended suitable strains by OECD were listed in Appendix A (Table A1). On the other hand, many toxicity studies have shown that *C. vulgaris* is a usually preferred test organism, since it has a widespread distribution and it can be found in freshwater ecosystems naturally (Borecka et al., 2016; Camuel et al., 2017; Dauda et al., 2017; Geiger et al., 2016; Yuan et al., 2016).

2.2.1. *Chlorella vulgaris*

Chlorella vulgaris is a spherical green microscopic cell with a mean diameter of 5-10 μm (Illman et al., 2000; Yamamoto et al., 2005) (Figure 2.1). Because *C. vulgaris* is a non-motile and very proliferating cell, a *C. vulgaris* cell that is amplified in optimal conditions within 24 hours reproduces with the most common asexual reproduction form of autosporulation in algae (Safi et al., 2014). Scientific classification of *C. vulgaris* is shown in Table 2.1.

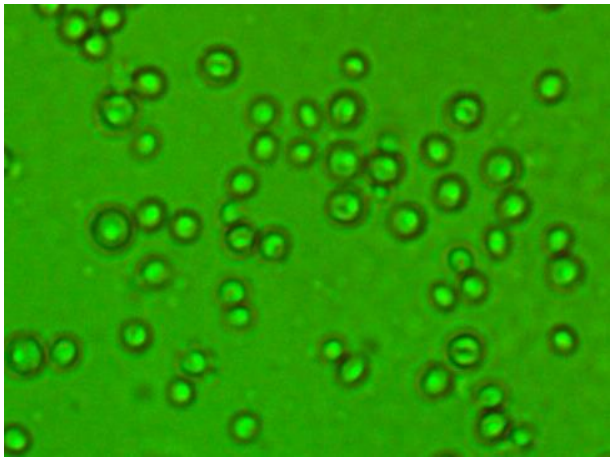


Figure 2.1. *Chlorella vulgaris* viewed in binocular microscope (40x, BM-250/S/P).

Table 2.1. Scientific classification of *Chlorella vulgaris*

Domain	Eukaryota
Kingdom	Plantae
Division	Chlorophyta
Class	Trebouxiophyceae
Order	Chlorellales
Family	Chlorellaceae
Genus	Chlorella
Species	*Chlorella vulgaris*

In addition to being reported as one of the eight most tolerant genera, many studies have shown that rapid growth, efficient organic and nutrient uptake from waste water, reduced toxicity from waste water (waste water treatment), and the dominance of wastewater worldwide, including temperate climates, made *Chlorella sp.* as the model species (Abdelaziz et al., 2014; Dahmani et al., 2016; Franchino et al., 2016; Ge et al., 2017; Hoh et al., 2016). Furthermore, according to previous studies (Singh and Singh, 2014; Yang et al., 2015), *C. vulgaris* can be considered as a preferred species for CO_2 fixation since it can tolerate CO_2 at high concentrations and has a high capacity of photosynthesis.

2.3. Pesticides

Pesticides are agricultural chemicals which are applied to prevent plants from the harmful effects of pests. Among pesticide types; herbicides are used to inhibit weeds, fungicides inhibit diseases due to fungi infections, and insecticides inhibit bugs. It is also known that these pesticides do not only affect the target organisms, but also they affect the non-target organisms, i.e., aquatic organisms. In the following section information about only the selected pesticides was given.

2.3.1. Pesticides Used in This Study

2.3.1.1. Acetamiprid. Acetamiprid ((E)-N1-[(6-chloro-3-pyridyl)methyl]-N2-cyano-N1-methyl acetamidine) is a neonicotinoid insecticide which is used to manage some insects on some vegetables, fruits and tea (Mateu-Sánchez et al., 2003). The chemical structure of acetamiprid is shown in Figure 2.2. The neonicotinoid group insecticides are the largest insecticide and seed treatment sales category in the global market (Jeschke et al., 2011). Because of the relatively low

toxicity of acetamiprid, it is used instead of some organophosphate insecticides which cause a severe environmental pollution (Yao et al., 2006). Acetamiprid has a half-life of 2.8-15 days as reported by Singh and Kumar (2008). Environmental fate of acetamiprid was evaluated and it is found that acetamiprid poses a low risk on ecosystem because of its rapid dissipation (Pitam et al., 2013). However, it is proven that acetamiprid showed adverse effects on greenhouse workers, soil microorganisms, and non-target insects (Fitzgerald, 2004; Marín et al., 2004; Yao et al., 2006). It is also reported that the group of neonicotinoids found in nature showed harmful effects to a wide scale of invertebrate and vertebrate (Gibbons et al., 2015; Pisa et al., 2014). Additionally, developmental disorders like congenital heart defects (CHD), neural tube defects (NTD) and autism spectrum disorder (ASD) were mentioned among its harmful effects regarding the risks for human health (Carmichael et al., 2014; Keil et al., 2014; Yang et al., 2014).

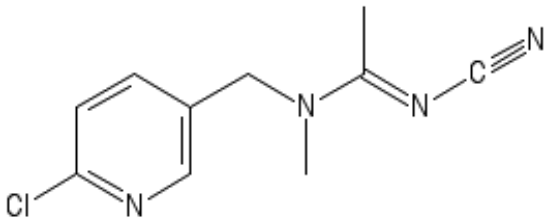


Figure 2.2. The chemical structure of acetamiprid (Structure was drawn using PubChem Sketcher v.2.4).

2.3.1.2. Acetochlor. Acetochlor is a U.S. Environmental Protection Agency approved selective systematic (Wang et al., 2018), chloroacetacetanilide herbicide used on soil as a pre-emerge or post-emerge treatment (Nemeth-Konda et al., 2002). The chemical structure of acetochlor is shown in Figure 2.3. It has been widely applied all around the world, especially in China, with more than 104 tonnes/yr usage (Xiao et al., 2006). Also, acetochlor is one of the 4638 chemicals in "The 2007, OECD List of High Production Volume Chemicals" (IOMC, 2009) which lists chemicals produced or imported at levels greater than 1,000 tonnes per year in at least one member country/region. A study shows that acetochlor has a half-life of 6.3 days in warm and moist conditions which are favorable for rapid degradation of herbicides (Mueller et al., 1999). Also, it is found that the half-life of acetochlor was between 10 and 16 days under anaerobic conditions (Loor-Vela et al., 2003). Due to their comprehensive application, there are some concerns about possible toxic effects of acetochlor on non-target organisms (Wang et al., 2018). For example; Tousova et al., (2018) reported that acetochlor is one of the top-ranking pesticides which is not allowed to be used in plant

protection products or biocides in the Czech Republic, indicating that their non-agricultural entrance into aquatic environment via wastewater treatment plants should be carefully noted.

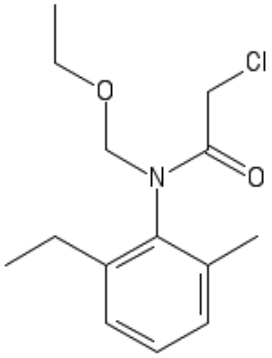


Figure 2.3. The chemical structure of acetochlor (Structure was drawn using PubChem Sketcher v.2.4).

Wang et al. (2018) investigated aquatic toxicity of acetochlor on a nonaxenic unialgal culture of *Chlorella* and zebrafish (*Danio rerio*) embryos, and reported that the solution containing 20 mg/L acetochlor showed an inhibiting effect on the *Chlorella* growth, and zebrafish hatching. Also, some studies on acetochlor toxicity reported its thyroid hormone disrupting effects (Jin et al., 2008; Turque et al., 2005). Additionally, Nowell et al. (2018) reported that acetochlor is one of the herbicides with the highest frequencies and concentrations, and one of the most dominant contributors of potential fish toxicity in Midwestern U.S. streams.

2.3.1.3. Boscalid. Boscalid (2-chloro-N-[2-(4-chlorophenyl)phenyl]pyridine-3-carboxamide) is a pesticide which is reported as an effective systemic, fat-soluble and persistent fungicide for canola and soybean (Matheron and Porchas, 2004; Simon-Delso et al., 2017). The chemical structure of boscalid is shown in Figure 2.4. Generally, boscalid has a slow biodegradation in most soils and it increases the potential for both groundwater and surface water contamination. It is also indicated that boscalid more likely accumulates in surface water bodies than groundwater. Boscalid has a half-life of 108 days (USEPA, 2003). Whether boscalid shows a low acute toxicity to mammals, it poses an important risk to human health due to the threshold for concern in mammals' short-term diet with respect to the ecotoxicology database of the International Union of Pure and Applied Chemistry (IUPAC) (Qian et al., 2018). A recent study shows that boscalid is a very common pesticide found in many bee matrices, also in aquatic environment, and at high concentrations around 36 μg/L in main coastal estuaries in California (Qian et al., 2018). Gaillard et al. (2016) reported that boscalid is found in more than 50% of the samples collected from the fishponds in the Lorraine Region, north-eastern France. Boscalid was also detected in bumblebees, pollens, and

overwintered honey sample up to 9.8 ng/g, 38 ng/g and 2.3 ng/g respectively (David et al., 2016; Jabot et al., 2016; Ostiguy and Eitzer, 2014).

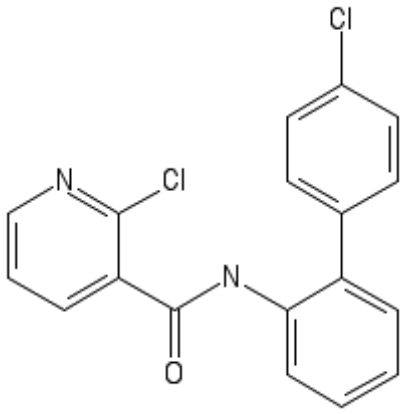


Figure 2.4. The chemical structure of boscalid (Structure was drawn using PubChem Sketcher v.2.4).

Qian et al., (2018) reported 96-h LC_{50} of boscalid on zebrafish embryos is 2.65 (2.506–2.848) mg/L and confirmed that boscalid has a considerable effect on development mechanisms of zebrafish embryos.

2.3.1.4. Diclofop. Methyl 2-[4-(2,4-dichlorophenoxy) phenoxy] propionate also called diclofop-methyl (or DM) is a post-emergence herbicide registered by Farbwerke Hoechst AG for annual grass control in several oil seed, cereal and legume types. The chemical structure of diclofop is shown in Figure 2.5. Diclofop acid which is the active structure of diclofop methyl herbicide is a chiral molecule with one stereogenic center.

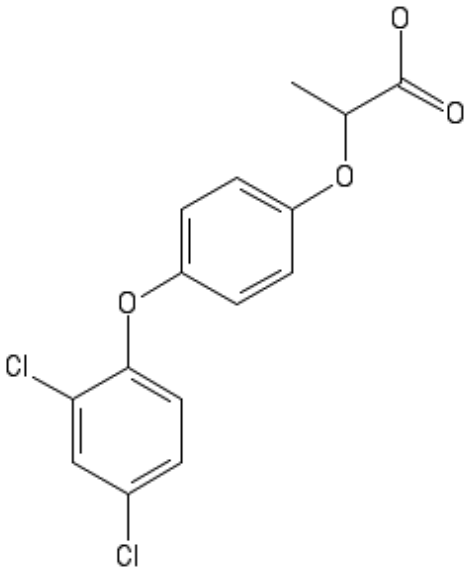


Figure 2.5. The chemical structure of diclofop (Structure was drawn using PubChem Sketcher v.2.4).

Studies have shown that, if the water conditions are alkaline, DM forms its acid form (diclofop) rapidly in water with hydrolysis reaction shown in Figure 2.6 (Cai et al., 2005). Diclofop acid has a higher solubility in water than diclofop methyl, so it tends to enter water systems after rainfall more than diclofop methyl making it to be found in surface waters substantially (Liu et al., 1991).

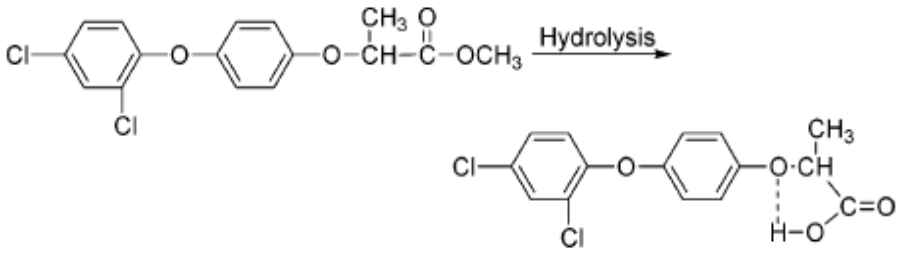


Figure 2.6. Hydrolysis of diclofop methyl to diclofop.

Between 1987 and 1996, the total use of diclofop methyl in the USA was approximately 340,200 kg of active substance per year, and the use in 2006 was 1 to 5 kilograms in China, while in Canada, the total use in 1986 was over one million kilograms (Ye et al., 2013).

Smith et al. (1986) reported that during the treatment process with diclofop methyl, as much as 73% of the active ingredient spreads onto soil surface instead of target weeds.

Ye et al. (2014) studied the effects of diclofop acid on *Microcystis aeruginosa*, and reported that diclofop acid exposure showed an increase in malondialdehyde (MDA) concentration and superoxide dismutase (SOD) activity in *M. aeruginosa* sooner than the exposure of R- and the S-enantiomer. Also, R-enantiomer resulted with more reactive oxygen species (ROS) generation, superoxide dismutase (SOD) activity, and toxin synthesis and release than the S-enantiomer. S-diclofop-methyl was found to be more toxic to the leaves of rice seedlings, while R-diclofop acid showed higher toxicity to the roots (Xie et al., 2018).

Cai et al., (2008) stated that the diclofop acid enantiomers showed different ecotoxicities to three freshwater algae; *Chlorella pyrenoidosa, Chlorella vulgaris,* and *Scenedesmus obliquus*. Also, it is reported that different enantiomers showed different effects on non-target plants than target ones, because the inactive enantiomer showed a similar or higher toxicity to non-target plants than the actively used enantiomer. As a result, herbicidal activity is not always directly related to environmental safety for chiral pesticides.

2.3.1.5. Diphenamid. Diphenamid (N,N-dimethyl-2,2-diphenylacetamide) is a herbicide which is being sold under the trade name Dymid, Enid (Islam and Tanaka, 2004), and applied as pre-emergence control on annual grasses and some broad-leaved weeds (Liang et al., 2010). The chemical structure of diphenamid is shown in Figure 2.7. A thorough search of the relevant literature yielded no related article about the toxicity tests for diphenamid.

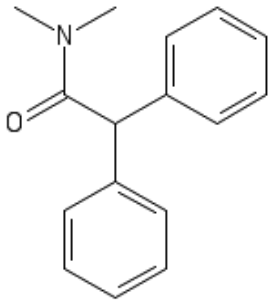


Figure 2.7. The chemical structure of diphenamid (Structure was drawn using PubChem Sketcher v.2.4).

2.3.1.6. Gibberellic acid. Gibberellic acid (GA3) is a plant growth hormone which provides seed germination, stem elongation, leaf expansion, flowering and fruit development as well as secondary metabolite production in plants (Iqbal and Ashraf, 2013). The chemical structure of gibberellic acid is shown in Figure 2.8. As a plant growth regulator, gibberellic acid production and use will result

in its direct release to the environment, but no detailed information was given about the route of degradation of gibberellic acid (EFSA, 2012).

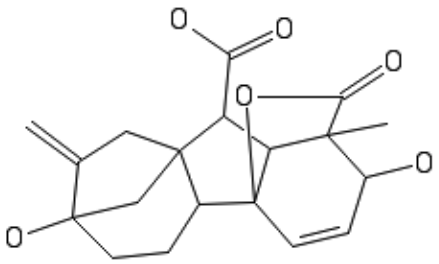


Figure 2.8. The chemical structure of gibberellic acid (Structure was drawn using PubChem Sketcher v.2.4).

Wang et al. (2011) investigated the effect of gibberellic acid on *Daphnia neonate* and the embryo stage. The 48-h EC_{50} values of neonatal daphnids and *D. magna* embryos for gibberellic acid was found to be 22.5 mg/L and 19.4 mg/L, respectively.

2.3.1.7. Ioxynil. Ioxynil (3,5-diiodo-4-hydroxybenzonitrile) which is mainly applied for the control of broad-leaved weeds in cereal crops belongs to a group of benzonitrile herbicides (Holtze et al., 2008). The chemical structure of ioxynil is shown in Figure 2.9. Ioxynil is a herbicide which is commercially marketed and widely used for agricultural purposes (Mäenpää et al., 2003). Ioxynil is used worldwide as a postemergent herbicide, especially for crops, vegetable and cotton, and ioxynil inhibits photosynthesis and oxidative phosphorylation (Tomlin, 1994). According to soil studies, ioxynil is expected to biodegrade in both soil and water. It is expected that ioxynil biodegrade rapidly under aerobic conditions with half-life of 9-10 days in soil (Nielsen et al., 2007).

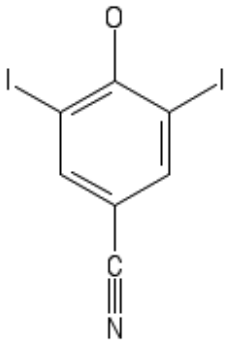


Figure 2.9. The chemical structure of ioxynil (Structure was drawn using PubChem Sketcher v.2.4).

Mäenpää et al. (2003) studied its toxicities on *L. variegatus* and *C. riparius* larvae by estimating its water concentration and the critical tissue concentration. They reported that LC_{50} values for ioxynil are 1.79 and 2.79 mg/L for *L. variegatus* and *C. riparius*, respectively.

2.3.1.8. 2,4,5-Trichlorophenoxyacetic acid. 2,4,5-Trichlorophenoxyacetic acid (2,4,5-T), one of the most common phenoxy herbicides in agriculture, can cause a damage in brain and central nervous system in humans, besides its low biodegradability due to its chlorine atom on the aromatic ring (An et al., 2014). The chemical structure of 2,4,5,-T is shown in Figure 2.10. Because 2,4,5-T is likely to biodegrade in soil, and it is mobile in most of the soil types, ground water contamination can only occur by rapid flow through large channels. Studies show that 2,4,5-T has an average half-life of 14 days (Howard, 1989).

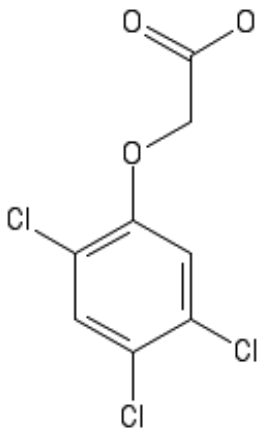


Figure 2.10. The chemical structure of 2,4,5-T (Structure was drawn using PubChem Sketcher v.2.4).

Phenoxy herbicides are known to show a wide range of toxic effects, such as myotonia, myopathy, and embryotoxicity (i.e. developmental defects, skeletal muscle toxicity during embryonic development) (Graillet and Girard, 1994).

2.4. Quantitative Structure - Activity Relationships (QSARs)

When considering the number of chemicals produced and the number of new chemicals to be produced so far, it is not difficult to imagine how much toxicological and ecotoxicological data is needed under REACH and how much animal experimentation is required to cover this lack of data. For this reason REACH promotes the substitution of laboratory testing (i.e., *in vitro* and *in vivo* methods) with alternative methods (i.e. non-experimental *in silico* methods) under 3R principle

(i.e., reduction, replacement, refinement) (Brinkmann et al., 2014). Therefore, scientists have developed quantitative structure-activity/toxicity relationships (QSARs/QSTRs) to predict toxicity of a group of chemicals to fill the data gap in ecotoxicity. For a valid QSAR model, external validation is an important requirement for both scientific and regulatory purposes.

It is obvious that there is still insufficient algal toxicity data for a majority of chemicals. Therefore, there is always a need for robust and predictive QSAR models for the estimation of algal toxicity to fulfill the data gaps in risk assessment, evaluation of algal toxicity screening, prioritization of chemicals, and making decisions on future testing in a scientific and regulatory framework. There are 5 principles to take into consideration during a model development. The first principle is the use of a defined endpoint. To end up with an accurate endpoint prediction by a constructed model, given endpoints should be consistent. Different experimental methods will give different endpoint values. Therefore, it is very significant to define the experimental procedure which is modelled in QSAR studies (OECD, 2007). The second principle is the use of an unambiguous algorithm, to guarantee a transparent model algorithm which generates enpoint predictions by the use of physicochemical properties and chemical structure information. The third principle necessitates a defined domain of applicability to inform the model user about the reliability of the resulting endpoint predictions. The fourth principle indicates that a valid QSAR/QSTR should have appropriate measures of goodness of fit, robustness and predictivity. The last principle for a valid QSAR/QSTR is a mechanistic interpretation, if any physicochemical interpretation can be done between the descriptors and the known mechanism of action of the chemicals used in the model development. Though, the last principle is not mandatory where there is not enough information. Dearden et al. (2009) highlighted the steps for how not to develop a quantitative structure-activity or structure-property relationship (QSAR/QSPR).

2.5. Inter-species Toxicity Correlations

While the toxicity of a chemical to a specific species is known, its toxicity to another species can be estimated by generating inter-species models. However, to obtain such models, a common toxicity data of both species is needed. Unfortunately this is nearly impossible, expensive, and slow, also unethical to carry out individual toxicity tests for every living organism exposed to all chemicals. However, calculation tools such as the QSAR are intended to fill in data gaps where the existing experimental data is limited. One of the major challenges in ecological risk assessment has been making estimations from limited toxicity data on a wide variety of species exposed to pollutants. Quantitative toxicity-toxicity relationship (QTTR) has a potential to estimate the toxicity

of a compound to a particular biological organism by evaluating the toxicity data of another species. Kar et al. (2016) have extensively reviewed inter-species models and outlined their toxicity mechanisms, the importance of mode of action (MoA) in species-specific toxicities and the importance of both the cost of various toxicity tests, and animal use reduction. For this reason, it is important to investigate toxicity relationships between species and to make toxicity estimates for algae using other species.

Inter-species toxicity relationships and their probability of successful predictions have already been studied previously. Inter-species correlations of toxicity for 141 organic chemicals between species of *Vibrio fischeri*, river bacteria, algae, *Daphnia magna*, carp, *Tetrahymena pyriformis*, fathead minnow and guppy (Zhang et al., 2010), for pharmaceuticals between *Daphnia magna* and fish (Kar and Roy, 2010), for narcotics between ciliate, water flea and fish (Dimitrov et al., 2000), also for aldehydes between variety of aquatic organisms (Dimitrov et al., 2004). Moreover, Tugcu et al. (2017) studied specifically with *Chlorella vulgaris* on inter-species correlations of toxicity for phenol derivatives, and developed QTTR models for *Chlorella vulgaris* and other species, *Pseudokirchneriella subcapitata* and *Tetrahymena pyriformis* respectively.

Even though it is not very good in terms of modeling, the inter-species toxicity relationship may not always be proportional. Zhang et al. (2010) found poor inter-species toxicity relationships between *T. pyriformis* and *D. magna* for a wide range of chemicals, since their mechanisms of action was different between these species.

2.6. Global Half-Life and Persistence, Bioaccumulation and Toxicity Indices

When it comes to identification of persistent organic pollutants (POPs) and the precedency of the hazardous chemicals, the environmental persistence is stated as a crucial factor. Environmental persistence studies can include many results that can be obtained from the half-life experiments, although the lack of experimental data arises as a significant problem. Gramatica and Papa (2007) studied on the Global Half-Life Index (GHLI) of 250 POPs to obtain a ranking with respect to their degradation half-life using a QSAR approach with several theoretical molecular descriptors. They applied GHLI model for the POPs screening. The use of this application can lead to the determination of the precedency of unknown POP chemicals, based on their molecular structure and can be used to develop a safer non-POPs alternative.

Many chemicals in the environment are naturally broken down by sunlight, wiped off by some environmental factors (such as; change in the temperature, humidity, light, rain, etc.) or metabolized by naturally occurring bacteria (De and Roy, 2018). A group of substances that cannot be easily broken down are represented by persistent, bioaccumulative and toxic (PBT) substances (Dórea, 2006). These substances may accumulate in various organisms and cause acute or chronic toxicity. Over time, some of the chemicals in the environment accumulate in human nutrition by accumulating in living creatures, not deteriorated by physical, chemical and biological reactions. These compounds may show resistance to biotic and abiotic degradation, and accumulate in environment for long periods of time; can cause different toxic effects in humans, plants and animals such as cancer, endocrine disruption, reproductive dysfunction, behavioral disorders, birth defects, immune system disorders, and damage to the liver and nervous system (De and Roy, 2018).

A more recent study Gramatica et al. (2015) was reported Persistent Bioaccumulation and Toxicity (PBT) Index model which is derived from experimental and reliable predicted data for a set of 180 heterogeneous organic chemicals by Principal Component Analysis (PCA) from half-life, bioconcentration factor (BCF) and P.promelas toxicity. Thus, the PBT index model allows for a broader interpretation, including bioaccumulation and toxicity in addition to the persistence of chemicals covered by the GHLI model.

Persistence can be described as the duration of a chemical found in the environment without being destroyed by the natural events, that is, without being converted into another substance (MacKay and Fraser, 2000). Bioaccumulative chemicals are substances that accumulate in living organisms in different ways, such as water, sediment, and biota, and cause concentrations in body tissues to increase (De and Roy, 2018).

In the Toxic Release Inventory (TRI) of The United States Environmental Protection Agency (US-EPA), data from different industry sectors on the emission of detrimental chemicals into the environment is collected (Arora and Cason, 1995). The US-EPA labeled a group of chemicals as PBT substances which show resistance to degradation (persistence), accumulate in body tissue (bioaccumulation), and show carcinogenic or adverse effects on living organisms (toxicity) (De and Roy, 2018).

2.7. Mode of Action, Algal Toxicity and Hydrophobicity

Mode of Action (MoA) is a term which is used to describe the response of a chemical or a substance when in contact with the exposed organism. Development of such classification system is a very exhausting task. Baseline toxicity, also called as narcosis, is the minimal toxicity of any hydrophobic pollutant shows. One of the most essential targets of the toxic effects is the biological membranes. If a hydrophobic compound is in contact with the membranes, it leads to some disturbances in the structure and function of membranes, because of its baseline toxicity (Escher and Schwarzenbach, 2002). The narcosis is the most important toxic action mode due to the fact that 70% of all organic industrial chemicals act via narcosis (Kar et al., 2016). Many researchers developed a method to characterize a chemical via its MoA with the help of the placement of narcosis as a base.

Studies show that, anilines may be more toxic to *Daphnia magna* while they act as a narcotic to fish (Netzeva et al., 2008). Hence, the exposed organism plays a major role in the characterization process (Jager et al., 2007). To eliminate dull conclusions, the mechanism of action should be interpreted correctly. There may be differences for a specific response in the means of the mechanism and the mode of action. The mode of action is described by the incomplete physiological, biochemical or behavioral responses, in spite of that, the mechanism requires much specific and detailed data for the toxicity of the chemical.

Establishing models according to their MoA is very important in terms of regulatory and scientific purposes. Thus, the estimated endpoints of the models are more reliable when used in real life scenarios. Still, as mentioned above, the MoA of chemicals varies according to many different views (Papa et al., 2005). For this reason, it is difficult to state the actual MoA. As Aptula et al. (2005) pointed out, the MoA may even be species-specific and may depend on the metabolism and distribution of the chemicals *in vivo*. For this reason, predictive toxicity models are usually generated with independent of the toxic mechanism of the chemicals (Ren, 2003). Toxicity of a chemical on an organism can be considered to have side effects by reaching the active site of action. In fact, it is usually related to its hydrophobicity. Therefore, hydrophobicity expressed as log P is a widely used chemical property appearing in aquatic toxicity models. In this study, log P is used for log K_{ow}, logarithm of *n*-octanol-water coefficient (K_{ow}).

Various models with log P were recommended for the use of toxicity-hydrophobicity relationships in the European Union Technical Guidance Document (EU TGD, 2003) in previous aquatic risk assessment.

It is well known that there is a strong correlation between hydrophobicity and the toxicity of polar and non-polar narcotics (Vighi et al., 2009). However, warning is issued for chemicals with a different mode of action (MoA) than baseline toxicity. Compounds with other MoAs are often "more toxic" (potent) than these base-line toxicants, at least if the toxicity data are interpreted on a log P scale (Russom et al., 1997).

In the case of some chemicals which partly or completely ionize at the pH of the test media, the distribution coefficient (log D) which is a pH corrected hydrophobicity can be used to consider the ionization. For a nonionizable compound, its log D equals to log P. When measured or predicted log D value is not available at a specific pH, it can be calculated by the following equations (Eq. 2.1 and Eq. 2.2);

For acidic chemicals:

$$\text{Log D} = \text{Log P} - \log(1 + 10^{\text{pH} - \text{pKa}}) \quad (2.1)$$

For basic chemicals:

$$\text{Log D} = \text{Log P} - \log(1 + 10^{\text{pKa} - \text{pH}}) \quad (2.2)$$

where, pK_a is the negative logarithm of the acid dissociation constant (K_a), and pH is the negative logarithm of the hydrogen ion concentration.

Although ionized species are probably able to partition into the lipid phase, at least together with a counter ion, the partition coefficient of such an ionic species is usually much lower than the partition coefficient of the neutral species (i.e., if log $P_{neutral}$ is 4, log P_{ion} is about 1). It is, thus, easily understandable that log D is lower when the ionized species of a chemical is mostly present at the investigated pH.

The pH corrected hydrophobicity (log D) is used to account for ionization. In this context, Ertürk and Saçan (2013) reported that ionization of phenols in the *C. vulgaris* test medium had a

considerable impact on their toxicity to *C. vulgaris*. Accordingly, using log D instead of log P yielded better results in explaining the toxicity of polar narcotic phenols. On the other hand, Tugcu et al. (2017) reported that descriptors other than a hydrophobicity term are required to explain the algal toxicity of chemiclas acting through more reactive mechanisms than polar narcosis.

2.8. Risk Assessment

There is a risk for a chemical to have an impact on environment from its production, to the disposal. These impacts on every compartment of the environment should be calculated. Since the environmental concentration of a newly synthesized chemicals cannot be measured, its concentration in each environmental compartment must be modelled/calculated/estimated.

There are many reports on gathering data for the existing substances in numerous environmental compartments. The regulatory assessment of chemicals includes the assessment of Predicted No Effect Concentrations (PNEC) and Predicted Environmental Concentrations (PECs). The PNEC is a level for which lower concentrations are considered to cause no adverse effects to the aquatic organisms. The PNEC is calculated by dividing the lowest long term NOEC value or short-term (acute) LC_{50} value by a relevant assessment factor. The assessment factors depend on the available toxicity data and they reflect the degree of uncertainty when extrapolating the laboratory data to the practical environment. The assessment factor ranges from 10 to 10,000 and the highest factor is applied if there are limited number of acute toxicity data. On the other hand, the lowest factor is applied when chronic NOECs are applicable for three trophic levels and two taxonomic groups. The assessment factor decreases when applied for long-term tests, since the uncertainty of the extrapolation from experimental data to the natural environment is reduced (EU TGD, 2003). The PEC to PNEC ratio can be applied to show the possibility of the adverse effect. This ratio is also noted as the Risk Factor (RF) (Figure 2.11). When the ratio is above 1, this means that there is a potential risk from the substance towards the environment. By interpreting this ratio, the risk scale can be understood roughly. The concern levels can be classified as follows; 1-10 low concern, 10-100 additional data needed and over 100 is major concern. If the ratio is over 100, there must be an action to reduce the risk to the environment.

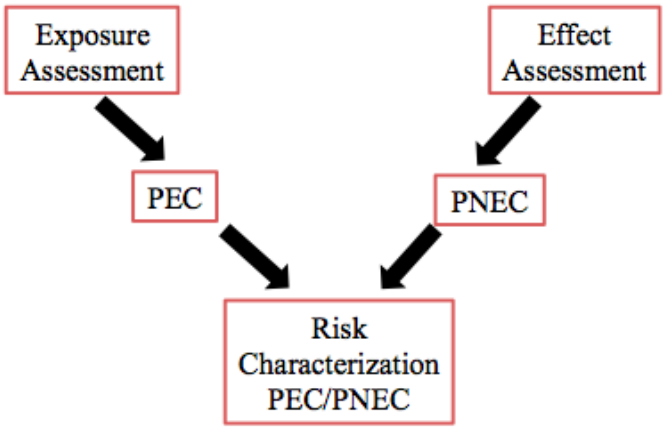


Figure 2.11. Simplified scheme of the process for risk characterization (Vighi and Villa, 2013).

Low-toxic-effect parameters such as NOEC, LOEC, EC_x (x% effective concentration) are used for risk assessment purposes. The LOEC value is described as the lowest tested concentration which is significantly different from the control. The NOEC is described as the tested concentration immediately below the LOEC. This concentration has no significant effect when compared with the control within a defined exposure time. Hypothesis-based No Observed Effect Concentration (NOEC) and regression-based x% Effective Concentration (EC_x) values are widely used statistical approaches used to outline ecotoxicological effects. The OECD report published in 1998 discussed whether to use NOEC or EC_x (OECD, 1998). A contradiction is reaching consensus on whether EC_{10} or EC_{20} is the most applicable substituent for the NOEC (Iwasaki et al., 2015).

Tugcu and Saçan (2018) summarized the use of low-toxic-effect concentrations in risk assessment. The low-toxic-effect concentrations (NOEC, LOEC, and EC_x) and the acute median effective concentration (EC_{50}) are used to calculate the Acute to Chronic Ratio (ACR). There are two types of ACR in use. One is calculated with the EC_{50} and maximum acceptable toxicant concentration (MATC) values, and the other one is calculated with the EC_{50} and NOEC values. The deficiency of the EC_{50} causes some researchers to steer into the use of EC_{20} or EC_{10} as the chronic value. Chronic value (ChV) in ECOSAR refers to the geometric mean of NOEC and LOEC values of the chemicals, which is also known as MATC (ECOSAR, 2012). Also, the U.S. Environmental Protection Agency (EPA) describes ACR as the ratio of acute LC_{50}/EC_{50} to the chronic MATC value or regression-derived EC_{20} value (Hoff et al., 2010). Additionally, EU Technical Guidance Document (TGD) indicates ACR as the ratio of EC_{50} value to the NOEC value (EU TGD, 2003).

There have been studies that show the correlations between ACRs of aquatic organisms and the properties of substances (May et al., 2016). When environmental risk assessment is mentioned, the

mode of action (MoA) of the chemical should be shown with some general information, since the MoA of a substance may vary among species. Furthermore; correlation of the chronic values for algae to higher organisms like daphnia or fish is complicated, as the type of chronic values (i.e. survival and reproduction as the end-point) depend on taxonomic rank of the organisms. For the substances with no specific mode of action, there are reliable QSAR estimates available for daphnia, fish and algal toxicity. These data can be used to highlight the necessity of the further testing.

3. MATERIALS AND METHODS

3.1. Test Chemicals

Acetamiprid, acetochlor, boscalid, gibberellic acid, ioxynil, and 2,4,5-T were purchased from Sigma Aldrich, while diclofop and diphenamid were purchased from Dr. Ehrenstorfer (Wesel, Germany) with the financial support of Scientific Research Projects (Project No: 13463). All the chemicals were ≥97% pure; therefore, no further purification was performed. Detailed information about purity and source of test chemicals are listed in Appendix B (Table B1). Because all the compounds have low water solubility to prepare the stock solutions in water, the stock solutions were prepared in dimethyl sulfoxide (>99.9 purity) purchased from Merck and Co. Inc. Thus, additional control group with the maximum DMSO concentration 0.1% (v/v) was conducted according to OECD test protocol No.201 (OECD, 2011). In order to observe if there is any significant difference between the growth of algae in control groups with and without DMSO, *t*-test was used (*p* value is set to 0.05).

On 20th January 2009, a new regulation (EC No 1272/2008) on classification, labelling and packaging (CLP Regulation) of substances and mixtures was issued. It aims to protect human health and environment to a great extent. Hazard labelling informs the consumer of the presence of a substance or mixture hazard and warns of exposure risks. The United Nations Globally Harmonised System of Classification and Labelling of Chemicals (UN GHS) enables a uniform worldwide physical, environmental, health and safety information on hazardous chemicals by the blending of classification and labeling criteria. These GHS pictograms of selected test chemicals are given in Table 3.1, with their meanings in Table 3.2.

Table 3.1. The tested chemicals with their CAS numbers and corresponding GHS pictograms.

	Chemical Name	CAS No	GHS Pictograms
1	Acetamiprid	135410-20-7	
2	Acetochlor	34256-82-1	
3	Boscalid	188425-85-6	
4	Diclofop	40843-25-2	
5	Diphenamid	957-51-7	
6	Gibberellic Acid	77-06-5	
7	Ioxynil	1689-83-4	
8	2,4,5 – Trichlorophenoxyacetic acid	93-76-5	

Table 3.2. The meanings of GHS pictograms of test chemicals used in the study.

	GHS06: Toxic		GHS08: Health hazard
	GHS07: Harmful		GHS09: Environmental hazard

3.2. Algal Growth Inhibition Assays Using *Chlorella vulgaris*

Algal growth inhibition assays were carried out in batch cultures in accordance with standard procedures (OECD, 2011) on freshwater alga *Chlorella vulgaris*. The parent cultures of *Chlorella vulgaris* strain (CCAP 211/11B) were purchased from Culture Collection of Algae and Protozoa – (CCAP, The Scottish Association for Marine Science, Scottish Marine Institute, Dunbeg, Argyll, UK).

The inoculum was conducted by harvesting 5-day old *Chlorella vulgaris* cultures in their exponential growth phase, so that the inoculum contained approximately $1.5x10^5$ cells/mL. After the completion of their lag phase, 100 mL test medium with algal culture was transferred into the 500 mL borosilicate Erlenmeyer flasks for further measurements. In the present study, the growth medium of *Chlorella vulgaris* was prepared using 3N BBM+V (Bold Basal Medium with 3-fold Nitrogen and Vitamins) (Bilanovic et al., 2009) (Table 3.3).

Table 3.3. 3N BBM+V growth medium for *Chlorella vulgaris.*

Constituents	**Concentration (mg/L)**
$NaNO_3$	750
$CaCl_2.2H_2O$	25
$MgSO_4.7H_2O$	75
$K_2HPO_4.3H_2O$	75
KH_2PO_4	175
NaCl	25
Na_2EDTA	4.5
$FeCl_3.6H_2O$	0.584
$MnCl_2.4H_2O$	0.246
$ZnCl_2$	0.03
$CoCl_2.6H_2O$	0.012
$Na_2MoO_4.2H_2O$	0.024
Vitamin B1	1.2
Vitamin B2	0.01

Three range finding assays were set up to determine the toxicity range. Then, each chemical was tested in 3 replicates of five concentrations after the range finding assay.

All experiments were carried out with sterile equipment to avoid any contamination. All the glassware were kept in the oven (WiseVen, Daihan Scientific; Korea) for 3 hours at 180℃ and the other equipments (plastic pipette tips, algae medium, filters, etc.) used in the experiment were autoclaved for 15 minutes at 120℃ under 2 atm pressure. All the glassware were cleaned with hexane to remove any organic residue after rinsing the glassware 2-3 times with tap water and washed with Liquinox Phosphate free detergent purchased from Sigma Aldrich. To remove any inorganic residue, all the glassware was rinsed with dilute nitric acid (10% v/v) and rinsed 2-3 times with distilled water. After each use, the spectrophotometer cuvettes were also rinsed twice with distilled water. All tests were performed in a laminar air-flow cabinet reserved for microbiological assays which was cleaned with 70% ethanol, and pre-sterilized with UV light for at least one hour.

Experiments were carried out in a light and temperature controlled growth chamber with cool white fluorescent lights, and continuous illumination (60 μmol photons m^{-2} s^{-1} at the level of test solutions) and at 24±0.5 ℃ (Figure 3.1). Experiments were conducted as static non-renewal, with daily agitation by hand.

Figure 3.1. A light and temperature controlled algal culture growth chamber with continuous illumination.

The pH change in the control vessel was checked at the beginning and end of the test (Appendix C (Table C1)), since it should not change more than ±0.5 between the first and last day of the experiment (OECD, 2011). An increase of 16-factor in algae growth for control groups after 72-h incubation is recommended for the validity of the test according to OECD test protocol No.201 (OECD, 2011). Therefore, the results of algal assays were considered as valid if the algal growth for control groups achieved a 16-fold increase. In addition to the test acceptability criteria stated above, 3,5-dichlorophenol (3,5-DCP) was used as a reference toxicant to test if the response of algae was the same.

The test chemicals were freshly prepared before each experiment. Any decrease in chemical concentration due to volatilization and adsorption on test vessel has been monitored during the algal assay. In this context, to understand whether there was any significant chemical loss (≥20%), chemical concentrations were measured with UV-Vis spectrophotometer (Lasany, LI-2804) on the first and last day of each experiment.

3.2.1. Measurement of Algal Growth

A linear relationship between algal cell numbers and absorbance (at 680 nm) for *Chlorella vulgaris* has already been observed in a previous study (Tugcu et al., 2017). The graph was redrawn with the addition of measured absorbance values and corresponding cell counts. Algal cells were counted in a haemocytometer (Thoma Chamber, 0.1 mm depth) using an optical microscope (Olympus CX41) with phase contrast.

The inhibition of algal growth at different chemical concentrations was defined with respect to the growth in solvent controls. The algal growth was monitored by the measurement of absorbance at 680 nm using a spectrophotometer (Lasany, LI-2804) at 24-h interval during the 96-h test period. The selected wavelength, 680 nm, gives the maximum chlorophyll absorption for *Chlorella vulgaris*.

3.2.2. Average Growth Rate

For a specific period of time, and for each treatment concentration, the average specific growth rate is calculated by the following Equation (3.1):

$$\mu_{i-f} = \frac{ln\, C_f - ln\, C_i}{t_f - t_i} \tag{3.1}$$

where,

μ_{i-f} : average specific growth rate from time *i* to f (day^{-1});

C_i : concentration of biomass at time *i* (cells/mL);

C_f : concentration of biomass at time f (cells/mL).

3.2.3. Percent Growth Inhibition

Percent growth inhibition (Ig (%)) in terms of average growth rate is calculated by the following equation for each treatment concentration (3.2):

$$\text{Ig (\%)} = \frac{\mu_B - \mu_C}{\mu_B} x\ 100 \tag{3.2}$$

where,

μ_B : mean value for average specific growth rate in the control group (day^{-1});

μ_C : average specific growth rate for the treatment concentration (day^{-1}).

3.2.4. Statistical Analysis of Algal Growth Test

Statistical analysis of algal growth test is shown in Figure 3.2 (USEPA, 2002). A nonparametric test, Steel's Many-one Rank Test, and a parametric test, Dunnett's Procedure are used via hypothesis tests in statistical analysis. Requirements of the Dunnett's Procedure which includes normality and homogeneity of variance are tested respectively. Normal or non-normal distribution are described via Shapiro-Wilk's Test. Homogeneity of variance is described by Bartlett's Test. In case of the failure of the tests mentioned above, NOEC and LOEC endpoints are defined by Steel's Many-one Rank Test. The endpoints are defined by the parametric test, in a scenario where the assumptions of Dunnett's procedure are met.

When there are unequal numbers of replicates of the tested concentration levels, a *t*-test with the Bonferroni adjustment which is a parametric method, and the Wilcoxon Rank Sum Test with the Bonferroni adjustment which is a nonparametric alternative can be used.

For each chemical, the average specific growth rate was calculated using Equation 3.1 (OECD, 2011). These data were used as an input for the ToxCalc software v.5.0.32 (Tidepool Scientific, 2009) to derive the endpoints, 96-h IC_{10}, IC_{20}, IC_{50}, NOEC and LOEC values. 96-h IC_{10}, IC_{20}, IC_{50} values (mg/L). All these endpoints were calculated with 95% confidence intervals using linear interpolation with bootstrapping (ICp) method as implemented in ToxCalc software v.5.0.32 (Tidepool Scientific, 2009). NOEC and LOEC values were calculated with parametric tests as implemented in ToxCalc software v.5.0.32 (Tidepool Scientific, 2009) and defined by USEPA (2002) (Figure 3.2).

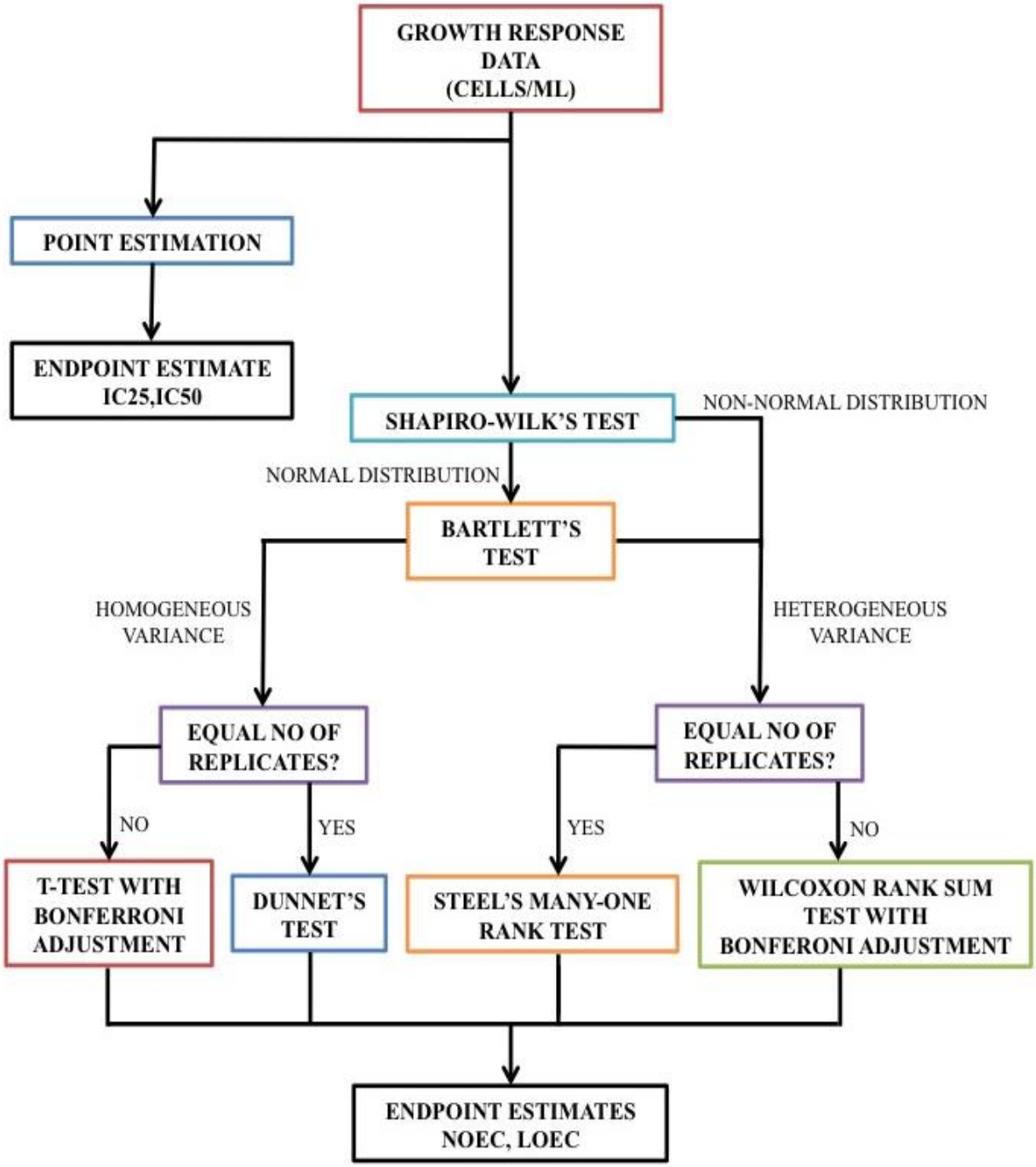


Figure 3.2. Statistical analysis of algal growth test flowchart (USEPA, 2002).

3.3. Prediction of Toxicity Values of Tested Chemicals from the Literature QSAR Models

Four different QSAR models which fulfill the OECD validation criteria mentioned in Section 2.3 were selected from the literature in order to compare the measured and predicted toxicity values of the studied 8 chemicals from these models. Further discussion was held on the predictive ability of these models by using the tested 8 chemicals as the external set.

3.3.1. QSAR Model on the Toxicity of PCPs to *Pseudokirchneriella subcapitata*

Previously published QSAR model on the aquatic toxicity of Personal Care Products (PCPs) to *Pseudokirchneriella subcapitata* was used to predict the algal toxicity of 8 pesticides. The QSAR model was retrieved from the library of models implemented in the software QSARINS v.2.2.2 (Gramatica et al., 2013). The QSAR model including descriptors from PaDEL-Descriptor software (Yap, 2011) was given in Equation 3.3. The model was generated for the prediction of PCPs toxicity to *P. subcapitata* and validated by Gramatica et al., (2016).

$$pEC_{50}(96\text{-h}) = -10.5802(\pm 2.8147) + 21.8813(\pm 4.2274)\ Mp + 13.0453(\pm 2.0252)\ GGI8 \qquad (3.3)$$

Since the software PaDEL-Descriptor was used to calculate descriptors appearing in Equation 3.3, we used the same software for the calculation of the descriptors for the 8 pesticides used in the present study. SMILES strings of each chemical were downloaded from ZINC database (Irwin et al., 2012). Types, chemical meanings and source of descriptors used to predict pEC_{50} values of chemicals were provided in Table 3.4.

Table 3.4. The symbol, type, meaning and source of descriptors appearing in Equation 3.3.

Descriptor	Type	Meaning	Source
Mp	2D	Mean atomic polarizabilities (scaled on carbon atom)	PaDEL
GGI8	2D	Topological charge index of order 8	PaDEL

3.3.2. QSAR Model on the Toxicity of Pharmaceuticals to *Pseudokirchneriella subcapitata*

Previously published QSAR model generated again using the software PaDEL-Descriptor for the prediction of 72-h pharmaceuticals toxicity to *Pseudokirchneriella subcapitata* by Sangion and Gramatica (2016) was used to predict the algal toxicity of 8 pesticides. The QSAR model was retrieved from the library of models implemented in the software QSARINS v.2.2.2 (Gramatica et

al., 2013).

$$pEC_{50}(72\text{-h}) = 0.9344(\pm 0.932) - 4.6149(\pm 1.5269)\ \text{VCH-6} - 0.0944(\pm 0.0525)\ \text{VE3\_Dt} - 3.7194(\pm 1.1743)\ \text{minHother} + 0.4776(\pm 0.2178)\ \text{piPC6} \quad (3.4)$$

Similar to the QSAR model generated for PCPs (Eq. 3.3), the same software, PaDEL-Descriptor (Yap, 2011), was used to calculate the descriptors appearing in the QSAR model generated for pharmaceuticals (Eq. 3.4). We calculated the PaDEL descriptors for the prediction of algal toxicity of 8 pesticides used in the present study. SMILES strings downloaded from ZINC database (Irwin et al., 2012) were used as an input for the software PaDEL to calculate the descriptors. Types, chemical meanings and source of descriptors used to calculate toxicity values of chemicals were provided in Table 3.5.

Table 3.5. The symbol, type, meaning and source of descriptors. Appearing in Equation 3.4.

Descriptor	Type	Meaning	Source
VCH-6	2D	Valence chain, order 6	PaDEL
VE3_Dt	2D	Logarithmic coefficient sum of the last eigenvector from detour matrix	PaDEL
minHother		Minimum atom-type H E-State: H on aaCH, dCH2 or dsCH	PaDEL
piPC6	2D	Conventional bond order ID number of order 6 (ln(1+x)	PaDEL

3.3.3. QSTR Model on the Toxicity of Phenolic Chemicals to *Chlorella vulgaris*

Tugcu et al. (2017) developed and validated a QSTR model for the 96-h toxicity of 46 phenolic chemicals to *Chlorella vulgaris* with Equation 3.5.

$$pIC_{50}(96\text{-h}) = 12.7013(\pm 6.1788) + 0.2015(\pm 0.1748)\ \log P - 2.7482(\pm 1.3286)\ \text{Hardness} \quad (3.5)$$

In this model, descriptors were calculated from the software SPARTAN 10 (Wavefunction Inc., 2011). Molecule drawing, conformer distribution and geometry optimization of the molecules were performed in SPARTAN 10 software. All the geometry optimization calculations were done with the semi-empirical PM6 method, and gaseous phase energy (E), aqueous phase energy (E_{aq}), highest occupied molecular orbital energy (E_{HOMO}), lowest unoccupied molecular orbital energy (E_{LUMO}) and hardness were calculated as molecular descriptors. Hardness which is a quantum chemical descriptor is calculated as half of the E_{LUMO}-E_{HOMO} (Tugcu et al., 2017). Hardness provides an interpretation on molecule reactivity/stability, where high values of hardness are generally associated to the stability of a molecule (Todeschini and Consonni, 2009). The other

descriptor, log P values which refer to hydrophobicity for each chemical were obtained from Danish (Q)SAR Database (2015).

3.3.4. QSTR Model on the 72-h Toxicity of Industrial Chemicals and Pharmaceuticals

A QSTR model equation derived on 389 diverse chemicals to a variety of freshwater algae species by Önlü and Saçan (2017) is given in Equation 3.6.

$$pEC_{50}(72\text{-h}) = 5.1396(\pm 0.9302) + 3.4836(\pm 1.3968)\,SPAM - 1.9237(\pm 0.4941)\,Mor31p + 0.237(\pm 0.1151)\,NdsCH - 0.4385(\pm 0.1825)\,CATS2D\_02\_AP + 0.9503(\pm 0.296)\,B05[C\text{-}S] + 0.1498(\pm 0.0297)\,F03[C\text{-}N] + 0.0978(\pm 0.0141)\,MLOGP2 - 0.7645(\pm 0.1451)\,Hardness \quad (3.6)$$

Descriptors appearing in this model were obtained from SPARTAN 10 and DRAGON 6.4 (Talete, 2010) software packages. The calculation of SPARTAN descriptors was similar to Equation 3.5. Each conformer with the lowest aquous enerji (E_{aq}) for each chemical was obtained from SPARTAN 10 sofware. The input files for DRAGON software were prepared in .mol2 files by SPARTAN. And .mol2 files were transferred to DRAGON 6.4 software (Talete, 2010) to calculate DRAGON descriptors. QSARINS 2.2.1 software was used to predict the algal toxicities of the studied chemicals. The type, meaning and source of descriptors used to calculate 72-h algal toxicity of chemicals were provided in Table 3.6.

Table 3.6. The symbol, type, meaning and source of descriptors appearing in Equation 3.6.

Descriptor	Type	Meaning	Source
SPAM	Geometrical descriptors	Average span R	DRAGON
Mor31p	3D-MoRSE descriptors	Signal 31/weighted by polarizability	DRAGON
NdsCH	Atom–type E–state indices	Number of atoms of type dsCH	DRAGON
CATS2D_02_AP	CATS 2D	CATS2D acceptor-positive at lag 02	DRAGON
B05[C-S]	2D Atom Pairs	Presence/absence of C–S at topological distance 5	DRAGON
F03[C-N]	2D Atom Pairs	Frequency of C–N at topological distance 3	DRAGON
MLOGP2	Molecular properties	Squared Moriguchi octanol–water partition coefficient	DRAGON
Hardness	Quantum chemical (energy)	Half of the energy difference between the lowest unoccupied and highest occupied molecular orbitals	SPARTAN-based

3.4. Prediction of Toxicity of Chemicals from a QTTR Model

Tugcu et al. (2017) derived an inter-species QTTR model using toxicity values of chemicals reported for *C. vulgaris* and *P. subcapitata*. The inter-species QTTR model is given in equation (3.7):

$$pT_{C.vulgaris} = 1.04(\pm 0.16)\, pT_{P.subcapita} - 0.21(\pm 0.19) \quad (3.7)$$

Two different pT values for the 8 chemicals on *P. subcapitata* were calculated from the EC_{50} values retrieved from Danish (Q)SAR Database (2015) were applied in the QTTR equation to predict pT value for *C. vulgaris*. One of the EC_{50} value for *P. subcapitata* was predicted from the Leadscope model, the other EC_{50} value for *P. subcapitata* was predicted from the SciQSAR model. The AD of QTTR model was defined by leverage approach (standardized residuals versus hat values). The reported critical hat value is 0.38. The descriptor range of the model in terms of pT value (mM) of *P. subcapitata* was between −0.32 and 1.94.

3.5. Global Half-Life and Persistence, Bioaccumulation and Toxicity Indices

The GHLI model generated for the POP screening and validated by Gramatica and Papa (2007) was used to calculate the half-life index of 8 pesticides. The GHLI model is given in equation (3.8).

$$GHLI = -0.566(\pm 0.2326) + 0.0117(\pm 0.0013)\, MW - 0.1506(\pm 0.0225)\, maxHBa - 0.0475(\pm 0.0151)\, nBondsS2 - 0.4297(\pm 0.1347)\, nHBDon\_Lipinski + 0.7472(\pm 0.229)\, minsCl \quad (3.8)$$

As stated in section 3.3.1. PaDEL-Descriptor software was used to calculate descriptors for the GHLI model similar to the other QSAR models generated by the same researchers (Gramatica et al., 2013, 2014, 2016). PaDeL descriptors were calculated similarly for the 8 pesticides used in the present study. The symbol, type, meaning and source of descriptors appearing in GHLI model were provided in Table 3.7.

Table 3.7. The symbol, type, meaning and source of descriptors appearing in Equation 3.8.

Descriptor	Type	Chemical meaning	Source
MW	2D	Molecular weight	PaDEL
maxHBa	2D	Maximum E-States for (strong) Hydrogen Bond acceptors	PaDEL
nBondsS2	2D	Total number of single bonds (including bonds to hydrogens, excluding aromatic bonds)	PaDEL
nHBDon_Lipinski	2D	number of hydrogen bond donors, using Lipinski's definition	PaDEL
minsCl	2D	Minimum atom-type E-State: -Cl	PaDEL

Gramatica et al., (2015) developed Persistent Bioaccumulation and Toxicity (PBT) Index model to evaluate PBT behavior of chemicals directly from their structural features. The following equation (3.9) gives the equation of PBT index derived and validated by (Gramatica et al., 2015) with respect to the OECD principles for QSAR (OECD, 2007).

$$\text{PBT Index} = -1.4607 \pm 0.1876 + (0.642 \pm 0.0497)\ \text{nX} + (0.2161 \pm 0.0175)\ \text{nBondsM} - (0.062 \pm 0.0617)\ \text{MAXDP2} - (0.3894 \pm 0.125)\ \text{nHBDon\_Lipinski} \quad (3.9)$$

PaDEL-Descriptor software was used to calculate descriptors in the PBT model. For the 8 pesticides, PaDEL descriptors were calculated using SMILES strings downloaded from ZINC database (Irwin et al., 2012). The symbol, type, meaning and source of descriptors used to calculate PBT Index of chemicals were provided in Table 3.8.

Table 3.8. The symbol, type, meaning and source of descriptors appearing in Equation 3.9.

Descriptor	Type	Meaning	Source
nX	2D	number of Halogen atoms	PaDEL
nBondsM	2D	number of multiple bonds	PaDEL
nHBDon_Lipinski	2D	number of hydrogen bond donors, using Lipinski's definition	PaDEL
MAXDP2	2D	maximal electrotopological positive variation	PaDEL

3.6. Correlation of *C. vulgaris* Toxicity with Hydrophobicity

The correlation between algal toxicity and hydrophobicity was searched for the studied chemicals. SPARTAN 10 software (Wavefunction Inc., 2011) was used to calculate log P values of chemicals. They were labeled as log P_S. The other log P values were compiled from Danish (Q)SAR Database (2015). They were labeled as log P_D. pH dependent hydrophobicity parameter named as log D reported for pH 6 was retrieved again from Danish (Q)SAR Database (2015) for each chemical. Since the algal assay was carried out at pH around 6, log D values were compiled for pH=6. The linear correlation was tested with the Pearson correlation coefficient, *R*. The

statistical significance was set at 0.05.

3.7. Risk Assessment Perspective

Risk factor can be calculated by dividing predicted/measured environmental concentration (PEC) to predicted no effect concentration (PNEC) as given in the following equation (3.10)

$$\text{Risk factor} = \frac{\text{PEC}}{\text{PNEC}} \tag{3.10}$$

Calculation of PNEC is required for each environmental compartment (e.g., water, sediment, soil, air, etc.). For fresh water, PNEC is chosen from the lowest value of EC_{50}, LC_{50} or NOEC divided by the appropriate assessment factor for each aquatic organism. In other words, the PNEC value of the data belongs to the most sensitive organism is chosen. Common assessment factors (AF) for PNEC calculation using freshwater data are given in Table 3.9. To calculate the PNEC value, the following two equations are used depending on the toxicity data available:

$$\text{PNEC} = \frac{EC_{50} \text{ or } LC_{50}}{\text{AF}} \tag{3.11}$$

$$\text{PNEC} = \frac{\text{NOEC}}{\text{AF}} \tag{3.12}$$

To summarize, the 1000 factor (to be used on a short-term toxicity) is a protective factor and is used to identify the substances that may cause adverse effects in the assessment of effect. If a NOEC value is established for the trophic level with the lowest EC_{50} value in short-term tests, 100 is applied as a factor to a single long-term NOEC data (for fish/daphnia). If there is long-term toxicity information (NOEC) for both trophic levels (algae, daphnia or fish), a factor of 50 is used for calculations from these two trophic levels. A factor of 10 can normally be applied only when there are at least three species with NOECs present in three trophic levels (for fish, daphnia and algae or a nonstandard species) (EU TGD, 2003).

Table 3.9. Assessment factors for $PNEC_{freshwater}$ calculation (Hansen, 2007).

Available data	**Assessment factor**
At minimum, one acute assay at one trophic level: (Algae, Daphnia or Fish)	1000
One long-term, chronic toxicity assay (NOEC): with Fish or Daphnia	100
Two long-term, chronic toxicity assays (NOEC) at two trophic levels: Algae and/or Daphnia and/or Fish	50
Three long-term, chronic toxicity assay at three species (NOEC): Algae, Daphnia and Fish (three trophical levels)	10

3.7.1. Acute to Chronic Ratio

Acute to Chronic Ratio (ACR) can be calculated in two different ways. The U.S. Environmental Protection Agency (EPA) describes ACR as in the following equation (Hoff et al., 2010):

$$MATC = \sqrt{(NOEC)(LOEC)} \tag{3.13}$$

$$ACR_{MATC} = \frac{LC_{50}}{MATC} \tag{3.14}$$

Whereas, EU Technical Guidance Document (EU TGD, 2003) and Hoff et al. (2010) state ACR based on NOEC (Eq. 3.15) and IC_{20} (Eq. 3.16), respectively.

$$ACR_{NOEC} = \frac{EC_{50}}{NOEC} \tag{3.15}$$

$$ACR_{IC20} = \frac{EC_{50}}{IC_{20}} \tag{3.16}$$

4. RESULTS AND DISCUSSION

4.1. Algal Assay

The linear relationship between the absorbance and the number of cells/mL with R^2=0.973 was shown in Figure 4.1. The plot was optimized using a total of 64 data points together with the previous data (Tugcu et al., 2017). The equation of fitted line is y = 0.0019x, where y is the absorbance (at 680 nm) and x is the number of *Chlorella vulgaris* (x10^5 cells/mL).

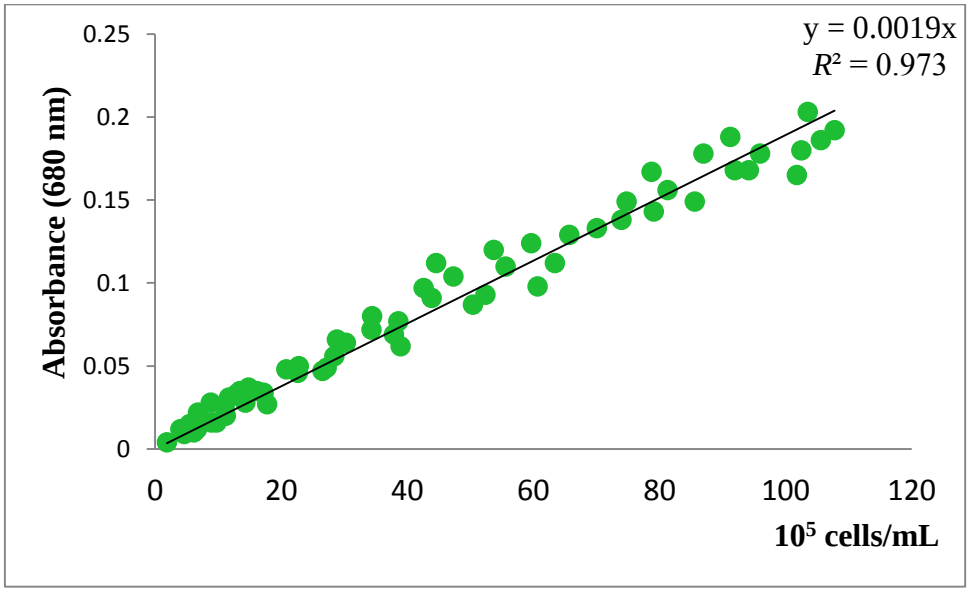


Figure 4.1. A plot of absorbance versus the number of cells/mL for *Chlorella vulgaris*

The average of the 38 data for blank samples during the each test run (day 0, day 1, day 2, day 3, and day 4) was taken and the increase in the number of cells during the test duration (96-h) is shown in Figure 4.2. At least 16-fold increase in the number of algal cells/mL was obtained at the end of 96-h test period. This increase in the number of algal cells in the control samples ensures that the algal assay is valid (OECD, 2011). The equation for the exponential fit of the trend line is $y = 0.821e^{0.775x}$ with $R^2 = 0.977$, where y is the number of *Chlorella vulgaris* cells/mL and x is the test duration (day).

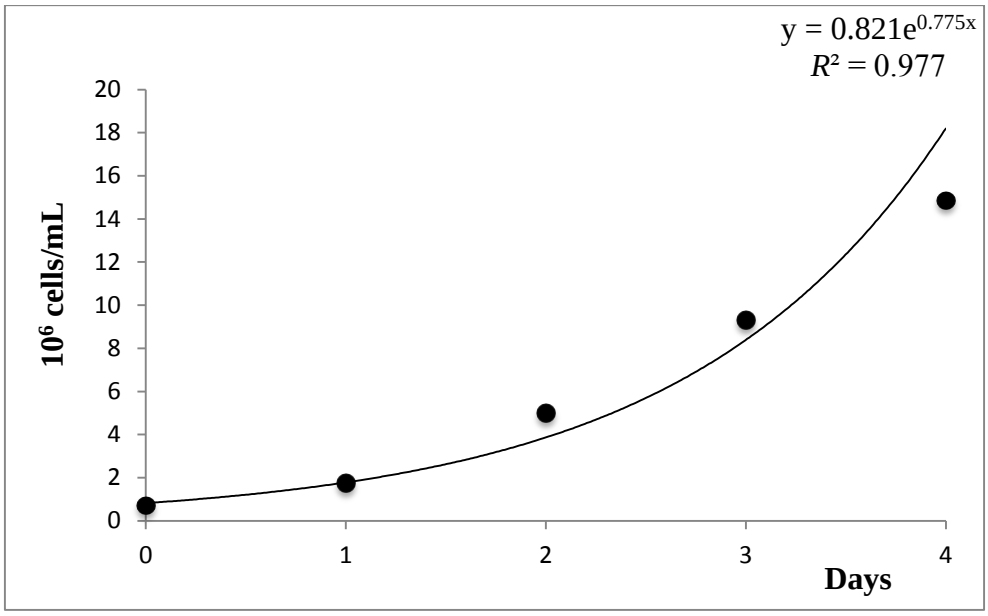


Figure 4.2. Exponential growth curve of average number of cells/mL per day collected from blank samples.

The results of all the "Freshwater Alga Growth Inhibition Tests" showed compliance with the OECD (2011) criteria. Also, 3,5-DCP was used as a reference toxicant to check the test procedure. 96-h algal toxicity of 3,5-DCP was determined as 3.00 mg/L ($\pm$ 0.33) which is in line with the previous findings from our laboratory and international standards (ISO 8692, 2012).

For each run, the pH value of the control assay was almost the same regarding the first and the last days of the test Appendix C (Table C1). The pH was 6.24 ($\pm$ 0.18) during the test period, this ensured that the increase in *p*H between the beginning and end of the test did not exceed 1.5 units (OECD, 2011).

4.2. Toxicity of Selected Chemicals to Freshwater Alga *Chlorella vulgaris*

In this study, 8 chemicals selected from the list of chemicals with no ecotoxicological data recently announced by TUBITAK-ARDEB (SU0303, 2015) are listed in Table 4.1 together with their CAS registry number and the information on the lack of data relevant to the environmental compartments. All of the 8 chemicals have no ecotoxicological data for biota, where one chemical has no data for both water and biota, and 3 of the chemicals have no data for the three compartments; water, biota and sediment.

Table 4.1. The tested chemicals with their CAS numbers and the environmental compartments where they are lack of data.

	Chemical Name	CAS No	Water	Biota	Sediment
1	Acetamiprid	135410-20-7		**X**	
2	Acetochlor	34256-82-1		**X**	
3	Boscalid	188425-85-6		**X**	
4	Diclofop	40843-25-2	**X**	**X**	**X**
5	Diphenamid	957-51-7	**X**	**X**	**X**
6	Gibberellic Acid	77-06-5	**X**	**X**	**X**
7	Ioxynil	1689-83-4	**X**	**X**	
8	2,4,5 - T	93-76-5		**X**	

UV-Vis spectrum of each chemical was taken between 200 and 500 nm during experiments. The UV-Vis spectrum of chemicals was given in Appendix D (Table D1-D4).

There was no significant chemical loss (>20 %) during the test period (96-h), regarding the UV-Vis spectrum of each test substance, except gibberellic acid. Therefore, calculations were done by the nominal concentrations as stated by the OECD (2011). For gibberellic acid there was about 4-fold increase in the intensity of the peak appeared around 270 nm. It is the only chemical ionized in the test medium.

Physico-chemical properties of tested chemicals were taken from Danish (Q)SAR Database (2015) and given in Table 4.2. Experimental values were shown in bold. Stock solution for each chemical was prepared in DMSO, because of their low water solubiliy. The result of *t*-test was $p=0.16$ indicating that there was no significant difference ($p>0.05$) between the growth of algae in control groups with and without DMSO. The type of the tests and concentrations of each chemical were listed in Table 4.3. For the compounds with IC_{50} value >100 ppm in their range finding assays were not subjected to further testing. OECD states that if no inhibition is observed at this concentration the chemical is not toxic to test species OECD (2011). Of the tested chemicals, boscalid showed no toxic effects up to its solubility limit (~7 mg/L). For the rest of the chemicals, experiments with 5 different concentration were conducted following the range finding assays (Table 4.3).

Table 4.2. Physicochemical properties of test substances taken from Danish (Q)SAR Database (2015).

Chemical Name	**CAS No**	**Molecular weight (g/mol)**	**Water solubility (mg/L)**	**Vapor pressure (mmHg)**	**Log D (at pH=6)**	**Log P**
Acetamiprid	135410-20-7	222.67	**4200***	4.36E-005	1.04	2.55
Acetochlor	34256-82-1	269.77	**223**	2.8E-005	3.01	**3.03**
Boscalid	188425-85-6	343.21	20.19	6.89E-011	4.79	**2.96**
Diclofop	40843-25-2	327.17	**453**	1.67E-007	1.77	**4.58**
Diphenamid	957-51-7	239.3	**260**	**3.00E-08**	2.79	2.86
Gibberellic Acid	77-06-5	346.37	**5000**	1.34E-013	-1.09	**0.24**
Ioxynil	1689-83-4	370.91	**50**	1.38E-007	1.91	**3.43**
2,4,5-T	93-76-5	255.48	**278**	**3.75E-05**	0.43	**3.31**

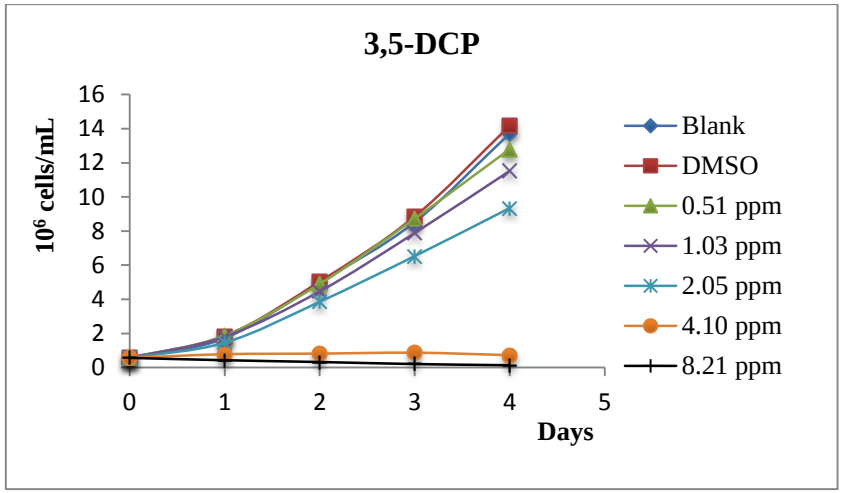
* ***Bold values are experimental.***

Table 4.3. Test type and tested concentrations for each chemicals. The solvent is DMSO.

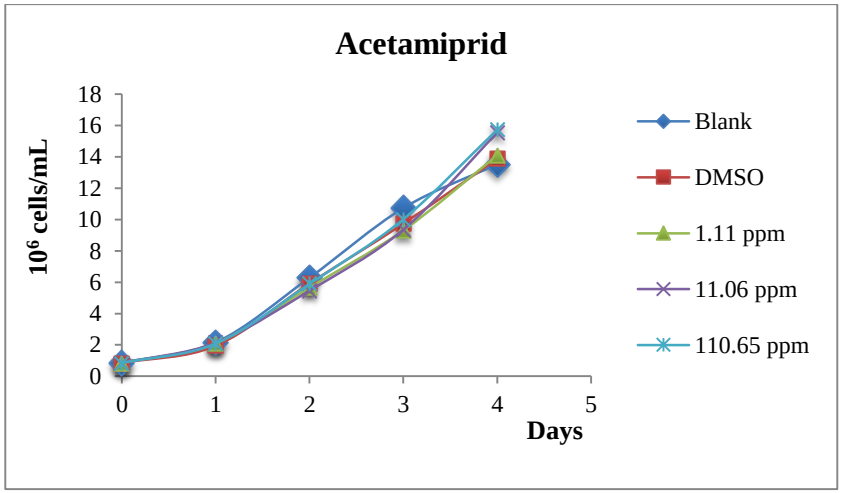
Chemical	Test type	Tested Concentrations (mg/L)
3,5-DCP	Range finding	0.95-1.9-3.8
3,5-DCP	Experiment	0.51-1.03-2.05-4.1-8.21
Acetamiprid	Range finding	1.11-11.06-110.65
Acetochlor	Range finding	0.93-9.26-92.59
Acetochlor	Experiment	5.79-11.57-23.15-46.30-92.59
Boscalid	Range finding	1.3-5.6-7.53
Diclofop	Range finding	0.92-9.23-92.31
Diclofop	Experiment	11.71-23.42-46.85-76.46-93.4
Diphenamid	Range finding	1.16-11.65-116.48
Gibberellic acid	Range finding	1.04-10.41-104.13
Ioxynil	Range finding	10.81-21.62-43.24
Ioxynil	Experiment	3.90-7.79-15.58-31.16-62.33
2,4,5-T	Range finding	10.24-30.72-61.44
2,4,5-T	Experiment	12.14-24.28-48.57-82.69-97.14

Algal response to each chemical and to the reference toxicant were depicted in Figure 4.3 and Figure 4.4 by plotting the number of cells/mL against the test duration. The cell numbers of algae decreased with increasing concentrations of 3,5-DCP, acetochlor, diclofop, ioxynil, and 2,4,5-T, whereas acetamiprid, boscalid, diphenamid, and gibberellic acid did not show a meaningful inhibitory effect on algal cells at tested concentrations.

Figure 4.5 and Figure 4.6 show the percent growh inhibition (Ig (%)) of algal species exposed to studied chemicals. A slight stimulatory effect can be noted in Ig (%) versus concentration graphs of diclofop and gibberellic acid for the lowest concentrations, however, since this effect is very small in percentages (<20%), it can be considered within the experimental error. On the other hand, 3,5-DCP, acetochlor, diclofop, and 2,4,5-T showed 100% inhibition in the growth of algal culture at the highest test concentrations. Ioxynil caused 77.76% inhibition in the growth of algal culture at its highest tested concentration. Acetamiprid, boscalid, diphenamid, and gibberellic acid did not show a meaningful inhibitory effect on the growth of algal culture at the tested concentrations. The graphs in Figure 4.5 and Figure 4.6 are consistent with the graphs in the Figure 4.3 and Figure 4.4, respectively.



(a)



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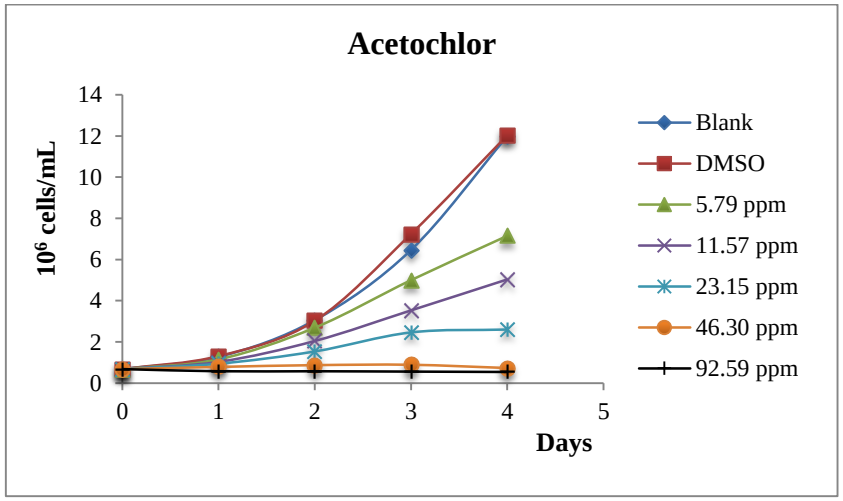
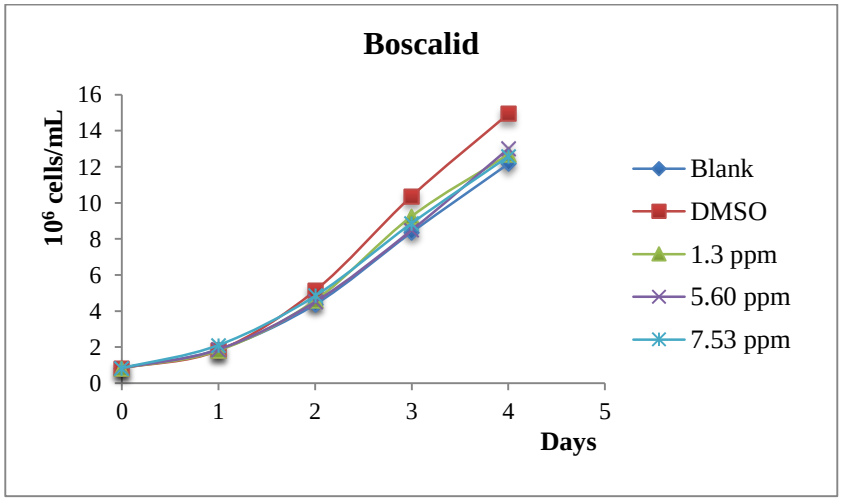


(c)



(d)

Figure 4.3. Response of *C. vulgaris* exposed to (a) 3,5-DCP, (b) acetamiprid, (c) acetochlor, (d) boscalid under the test conditions during 96-h.

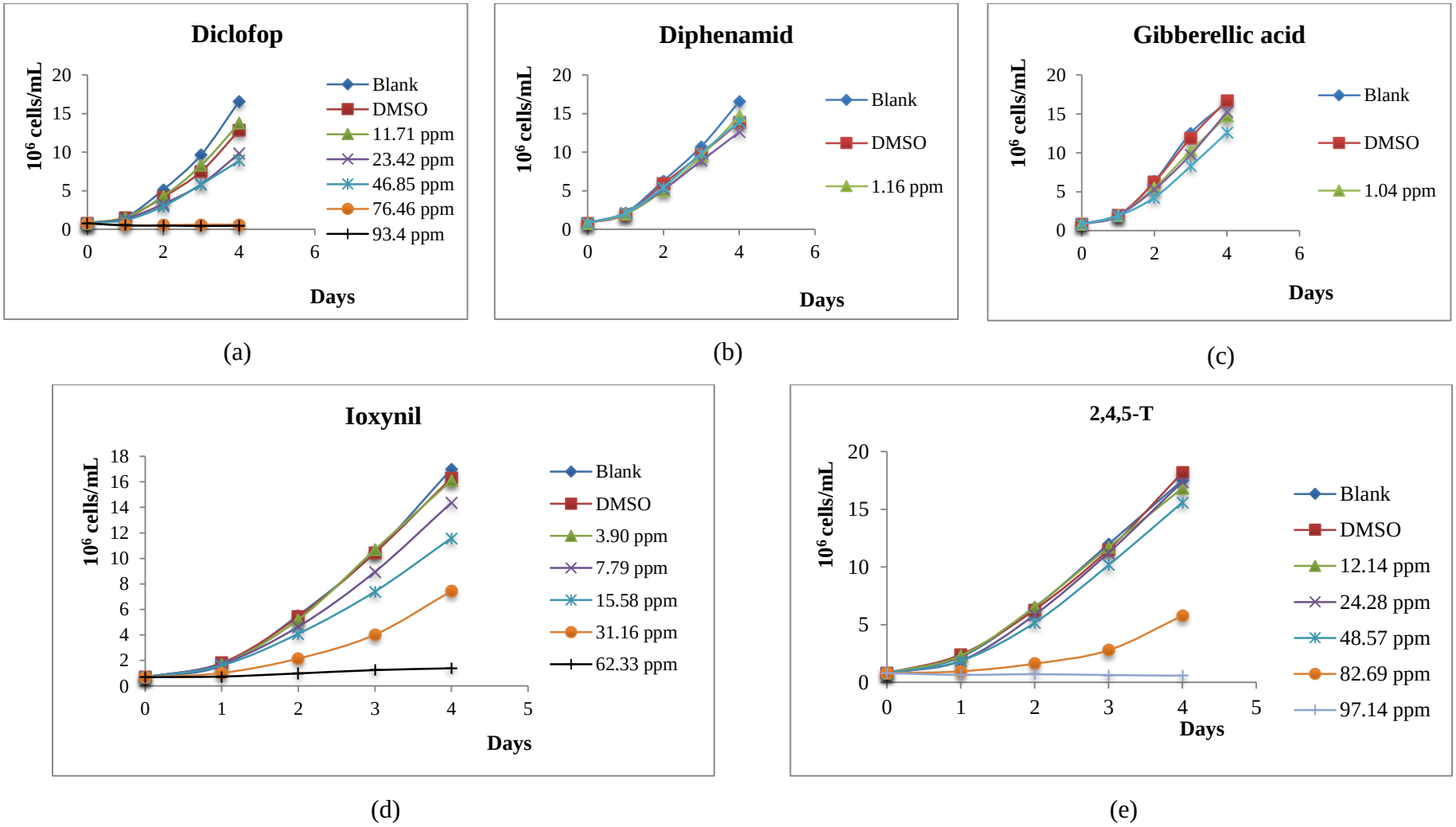
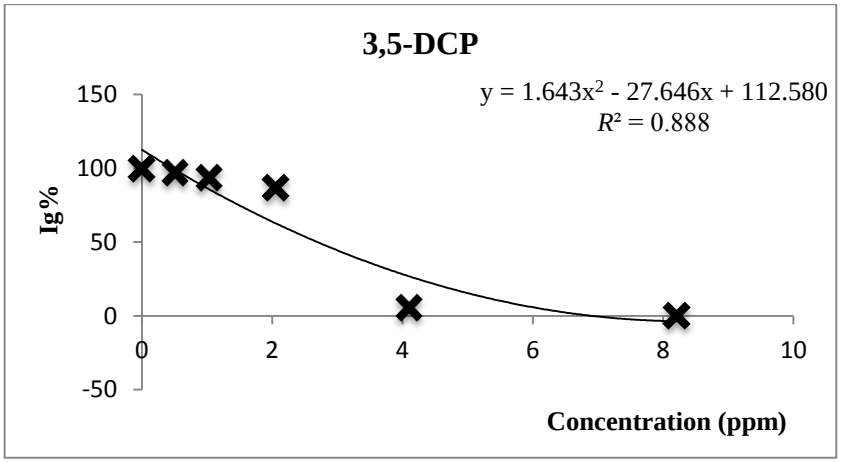
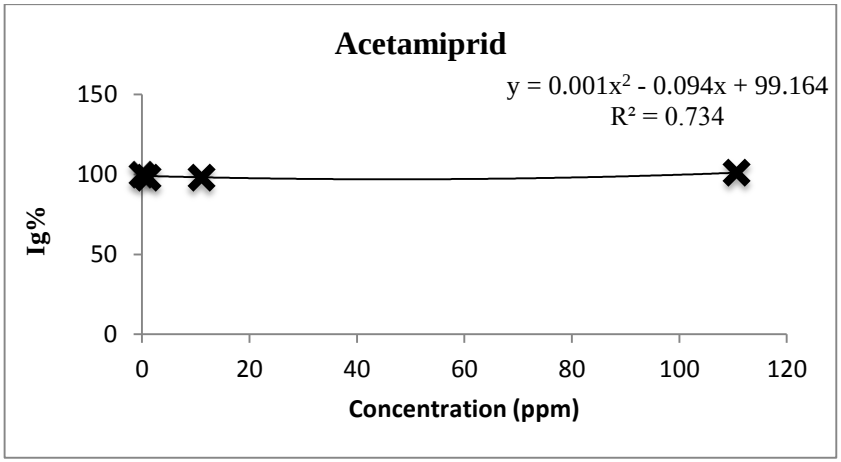


Figure 4.4. Response of *C. vulgaris* exposed to (a) diclofop, (b) diphenamid, (c) gibberellic acid, (d) ioxynil, (e) 2,4,5-T under the test conditions during 96-h.



(a)



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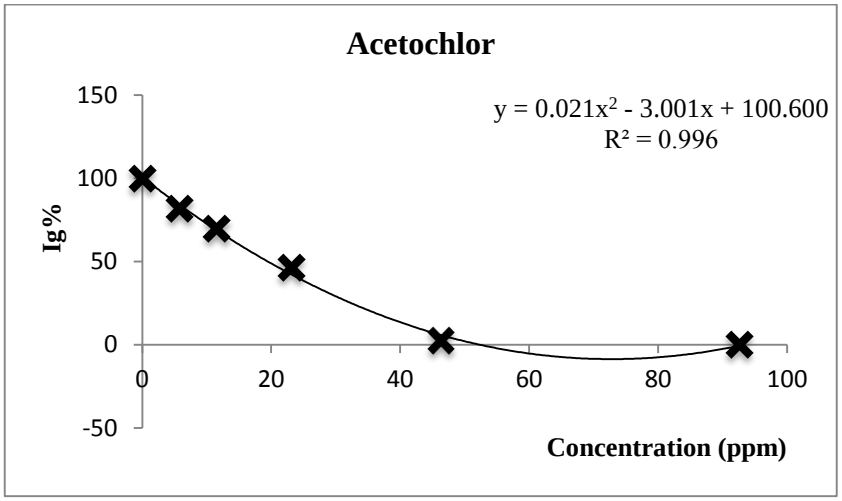
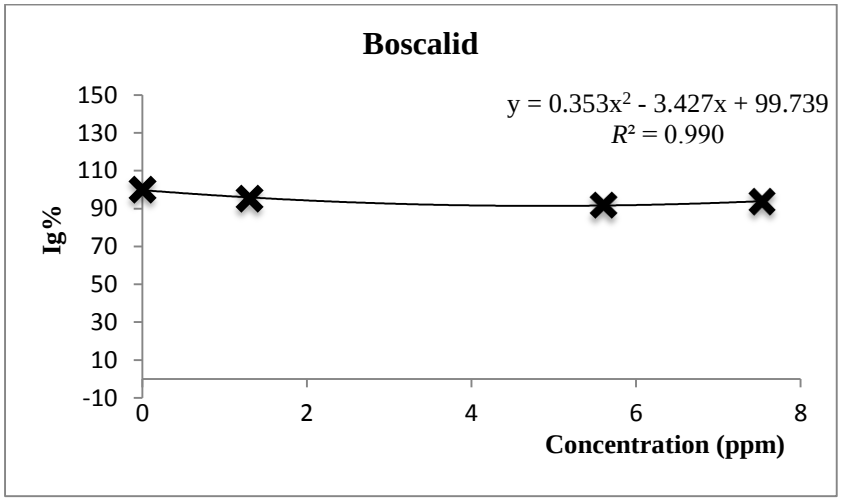
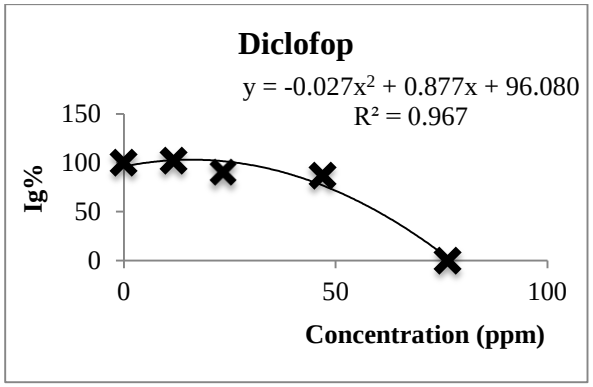


(c)

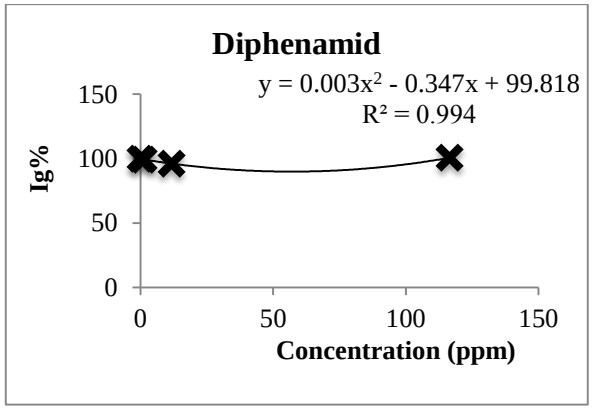


(d)

Figure 4.5. Percent growth inhibition of *C. vulgaris* exposed to (a) 3,5-DCP, (b) acetamiprid, (c) acetochlor, (d) boscalid under the test conditions during 96-h.



(a)



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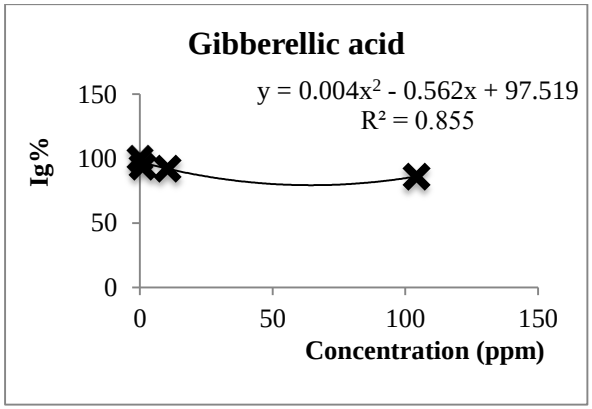

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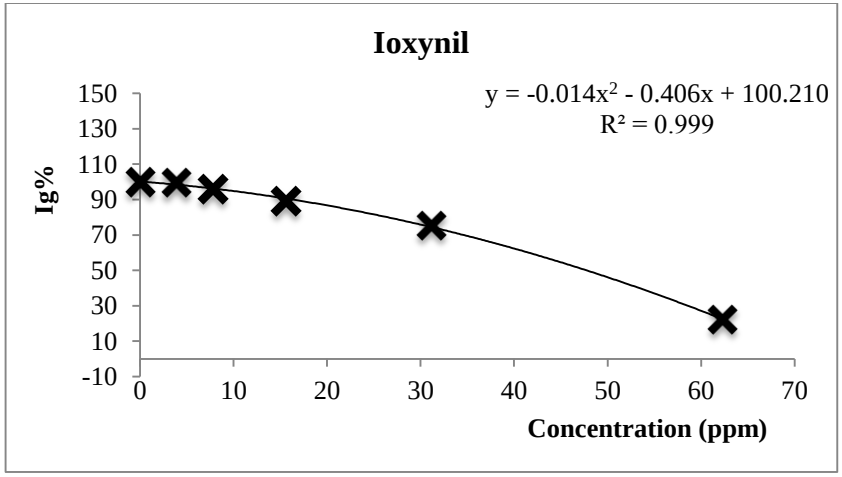
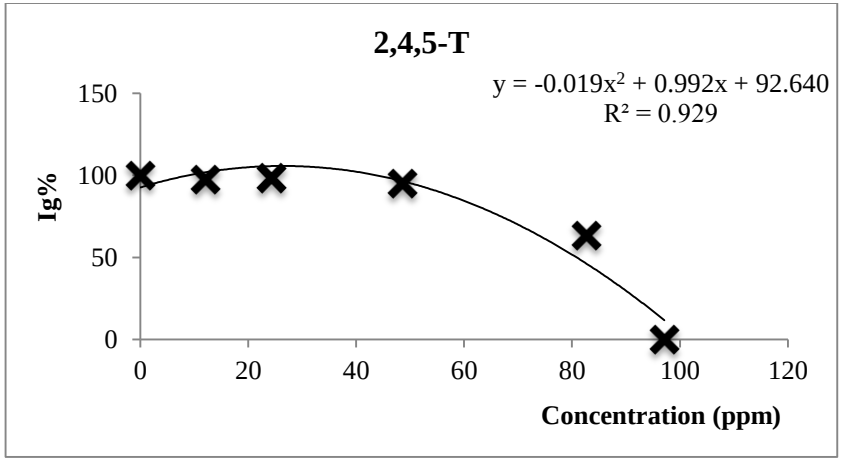


(d)



(e)

Figure 4.6. Percent growth inhibition (Ig %) of *C. vulgaris* exposed to (a) diclofop, (b) diphenamid, (c) gibberellic acid, (d) ioxynil, (e) 2,4,5-T under the test conditions during 96-h.

The experimental 96-h IC_{50}, IC_{20}, IC_{10}, NOEC and LOEC values obtained for the studied pesticides are listed in Table 4.4 Among the 8 tested pesticides, acetamiprid, diphenamid, and gibberellic acid were determined as non-toxic with IC_{50}>100 mg/L. Boscalid showed no toxic effect in its solubility limits. Acetochlor showed the most toxic effect with IC_{50} value of 21.47 mg/L.

Table 4.4. Chemicals tested in the present study and their 96-h 50%, 20%, and 10% inhibitory concentrations (IC_x) with their 95% confidence intervals, NOEC and LOEC values (mg/L).

Chemical	**IC_{50}**	**IC_{20}**	**IC_{10}**	**NOEC**	**LOEC**
3,5-DCP	3.00 (2.77-3.33)*	2.23 (2.05-2.41)	1.59 (0.50-2.60)	1.03	2.05
Acetamiprid	>110.65	>110.65	>110.65	110.65	>110.65
Acetochlor	21.47 (19.32-23.57)	6.64 (3.70-9.17)	3.18 (2.00-4.48)	<5.79	<5.79
Boscalid	>7.53	>7.53	>7.53	>1.3	1.3
Diclofop	59.32 (58.12-60.41)	48.88 (46.97-50.67)	22.82 (17.66-44.52)	11.71	23.42
Diphenamid	>116.48	>116.48	>116.48	116.48	>116.48
Gibberellic acid	>104.13	>104.13	>104.13	10.41	104.13
Ioxynil	46.02 (44.19-47.37)	25.91 (20.52-29.36)	14.72 (8.50-18.76)	7.79	15.58
2,4,5-T	85.74 (84.59-86.80)	64.76 (60.68-69.45)	53.98 (48.48-59.41)	24.28	48.57

* *Numbers within the parenthesis are the 95% confidence intervals.*

4.3. Prediction of Algal Toxicity Values of Tested Chemicals from the Literature QSAR Models

4.3.1. QSAR Model on the Prediction of Algal Toxicity for Personal Care Products (PCPs)

The first QSAR model used for prediction of algal toxicity values of tested chemicals was generated by Gramatica et al. (2016). The algal toxicity data of personal care products used in this QSAR model belongs to freshwater green algae *Pseudokirchneriella subcapitata*. The two-descriptor QSAR model (Equation 3.3) was developed on 20 personal care products (PCP). The descriptors appearing in Equation 3.3 are GGI8 and Mp. The meaning of these descriptors are given in Table 3.4.

The applicability domain of this model was defined by the leverage approach. The dependent variable, pEC_{50} (M), ranges from 2.16 to 7.93. The critical hat value (h^*) is reported as 0.45. The calculated descriptor values for the studied 8 chemicals using the software PaDEL-Descriptor v.2.21 are given in Table 4.5 together with the predicted algal toxicity and hat values.

Table 4.5. Chemicals used in this study, their descriptor and hat values, and the predicted pT values by Equation 3.3. for *Pseudokirchneriella subcapitata.*

Chemical / Range	**Mp**	**GGI8**	**pT by Eq. 3.3 (M)**	**Hat values (h^*=0.45)**
Acetamiprid	0.71	0.06	5.60	0.11
Acetochlor	0.66	0.02	4.03	0.08
Boscalid	0.78	0.19	**8.93****	**0.53****
Diclofop	0.74	0.10	6.84	0.22
Diphenamide	0.68	0	4.38	0.10
Gibberellic acid	0.65	0	3.70	0.10
Ioxynil	**1.12***	0	**14.03****	**4.72****
2,4,5-T	0.81	0	7.10	**0.55****
Range of Descriptor	0.59 - 0.82	0 - 0.30		

* ***Descriptors "Out of the descriptor space"***
** ***Values "Out of the response range"***

The calculated value of Mp descriptor for ioxynil was out of the descriptor interval reported for the model. The calculated descriptor values of other chemicals were within the specified descriptor space. For the descriptor range, the compound with a descriptor value out of the descriptor space defined for a QSAR model can be considered out of the model's applicability domain (AD). In other words, if a chemical has a hat (leverage) value greater than the cut-off value (h^*) of a QSAR model, it is considered as out of the descriptor space of model. Predicted pT values for ioxynil and boscalid were out of the model's AD. Although the hat value of 2,4,5-T (h=0.55) is greater that the critical hat value (h^*=0.45), its predicted pT value is within the response range of the model. Therefore; the predicted pT value of 2,4,5-T can be reliable. Insubria graph shows the extrapolated toxicity values of these chemiclas from the model (Eq. 3.3) (Figure 4.7). The predicted pT values of the rest of the chemicals were interpolated from the model. Therefore, these values can be considered as reliable.

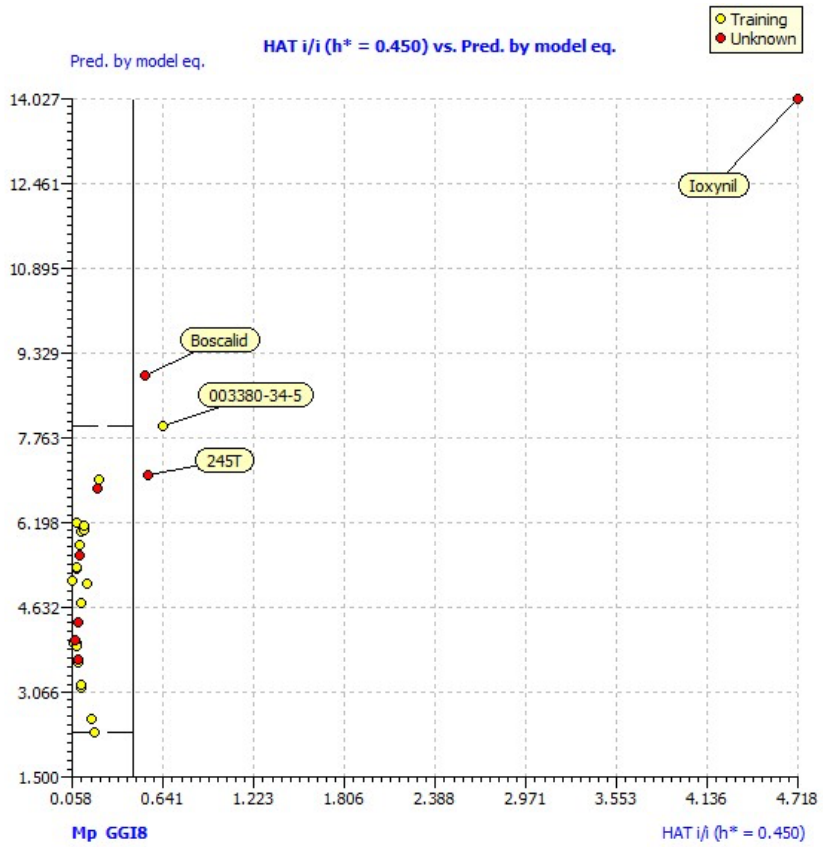


Figure 4.7. Insubria graph of Eq. 3.3: The predicted toxicity and hat values of the 8 chemicals (red marks) together with the training set chemicals (yellow marks).

4.3.2. QSAR Model on the Prediction of Algal Toxicity for Pharmaceuticals

The second QSAR model used for the prediction of algal toxicity values of tested chemicals was again generated by Sangion and Gramatica (2016). The algal toxicity data of pharmaceuticals used in this QSAR model belongs to freshwater green algae *Pseudokirchneriella subcapitata*. The four-descriptor QSAR model (Eq. 3.4) was developed on 45 pharmaceutically active chemicals. The descriptors appearing in Equation 3.4 are minHother, VCH6, piPC6, and VE3_Dt. The meaning of these descriptors was given in Table 3.5.

The applicability domain of this model (Eq. 3.4) was defined by the leverage approach. The dependent variable, pEC_{50} (mM), ranges from -0.61 to 5.57. The ranges of descriptors was given in Table 4.6. The critical hat value (h^*) is reported as 0.33. The calculated descriptor values for the studied 8 chemicals using the software PaDEL-Descriptor v.2.21 are given in Table 4.6 together with the predicted algal toxicity and hat values.

The calculated value of desciptor, VE3_Dt, for diphenamide was out of the descriptor interval reported for the model (Eq. 3.4). The calculated descriptor values of other chemicals were within the specified descriptor space. The predicted pT value of diphenamide by Equation 3.4 is obviously out of the model's AD in terms of both the descriptor space and response range. Insubria Graph shows the extrapolated toxicity values of this chemical from the model (Eq. 3.4) (Figure 4.8.). Hat value of gibberellic acid is slightly higher (h=0.36) than the cut-off value (h^*=0.33) of the model. However, the predicted toxicity value of this chemical for *P. subcapitata* can be accepted as reliable. The predicted pT values of the rest of the chemicals were interpolated from the model. Therefore, these values can be considered as reliable.

Table 4.6. Chemicals used in this study, their descriptor and hat values, and the predicted pT values by Equation 3.4. for *Pseudokirchneriella subcapitata*.

Chemical / Range	**VCH-6**	**VE3_Dt**	**minHother**	**piPC6**	**Predicted pT by Eq. 3.4 (mM)**	**Hat values (h^*=0.33)**
Acetamiprid	0.02	-1.96	0.47	4.47	1.42	0.05
Acetochlor	0.02	-2.32	0.43	4.92	1.80	0.06
Boscalid	0.08	-6.78	0.44	6.16	2.51	0.05
Diclofop	0.05	-3.71	0.45	5.43	1.95	0.05
Diphenamide	0.06	**-60.92**[*]	0.46	5.25	**7.20**[**]	**4.76**[**]
Gibberellic acid	0.57	-5.18	0.41	5.96	0.09	**0.36**[**]
Ioxynil	0.02	-3.39	0.53	4.85	1.51	0.04
2,4,5-T	0.02	-3.66	0.44	4.75	1.80	0.04
Range of decriptor	0-0.65	(-19.45) - (-1.64)	0-0.82	0-6.42		

[*] ***Descriptors with "Out of the descriptor space"***
[**] ***Values with "Out of the response range"***

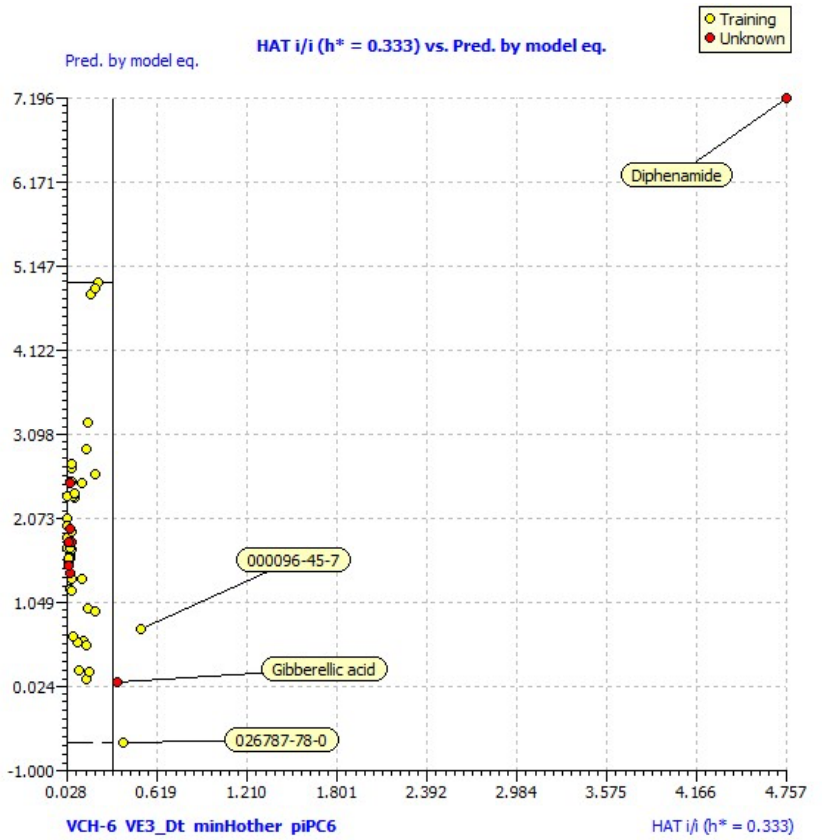


Figure 4.8. Insubria graph of Eq. 3.4: The predicted toxicity and hat values of the 8 chemicals (red marks) together with the training set chemicals (yellow marks).

2-imidazolidinethione and amoxicillin appeared in the graph with their CAS numbers were also reported as the structural outlier during the model development by Sangion and Gramatica (2016).

4.3.3. QSTR Model on the Toxicity of Phenolic Chemicals to *Chlorella vulgaris*

The third QSAR/QSTR model used for the prediction of algal toxicity values of tested chemicals was generated by Tugcu et al., (2017). The algal toxicity data of phenolic chemicals used in this QSTR model belongs to freshwater green algae *Chlorella vulgaris*. The two-descriptor QSTR model (Eq. 3.5) was developed on 46 phenol derivatives. The descriptors appearing in Equation 3.5 are log P and hardness. The meaning and source of these descriptors were mentioned in Section 3.3.3.

The applicability domain of this model (Eq. 3.5) was also defined by the leverage approach. The dependent variable, pIC_{50} (mM), ranges from -0.60 to 2.34. The ranges of descriptors are given in Table 4.7. The critical hat value (h^*) reported for this model is 0.26. The log P values of the

studied 8 chemicals were compiled from Danish (Q)SAR Database (2015), whereas hardness value was calculated as half of the difference between E_{LUMO} and E_{HOMO} values (eV) retrieved from the SPARTAN 10 software. The descriptor values of chemicals are given in Table 4.7 together with the predicted *Chlorella vulgaris* toxicity and hat values.

The compiled log P values for gibberellic acid, and the calculated value of descriptor, hardness, for boscalid, diphenamid, gibberellic acid, and ioxynil were out of the limit set for the descriptor intervals reported for the model. The calculated descriptor values of other chemicals were within the specified descriptor space. The predicted pT values of boscalid, gibberellic acid, and ioxynil by Equation 3.5 are obviously out of the model's AD in terms of both the descriptor space and response range. Insubria Graph shows the extrapolated toxicity values of these chemicals from the model (Eq. 3.5) (Figure 4.9.). Although the hardness descriptor value of diphenamid is out of the descriptor range of the model its predicted toxicity value is within the model's AD (Figure 4.9). The pT values of acetamiprid, acetochlor, diclofop and 2,4,5-T were interpolated from Eq. 3.5. Therefore, these values can be considered as reliable.

Table 4.7. Chemicals used in this study, their descriptor and hat values, and the predicted pT values by Equation 3.5 for *Chlorella vulgaris*.

Chemical / **Range**	**Log P**	**Hardness**	**pT by Eq. 3.5 (mM)**	**Hat values (h^*=0.26)**
Acetamiprid	2.55	4.25	1.54	0.11
Acetochlor	3.03	4,69	0.44	0.18
Boscalid	2.96	**3.91***	**2.55***	**0.58****
Diclofop	4.58	4.18	2.14	0.16
Diphenamid	2.86	**4.76***	0.21	0.25
Gibberellic acid	**0.24***	**5.24***	**-1.64****	**0.94****
Ioxynil	3.43	**3.97***	**2.50****	**0.42****
2,4,5-T	3.31	4.28	1.62	0.07
Range of decriptors	0.59 - 5.12	4.13 - 4.73		

* ***Descriptors with "Out of the descriptor space"***
** ***Values with "Out of the response range"***

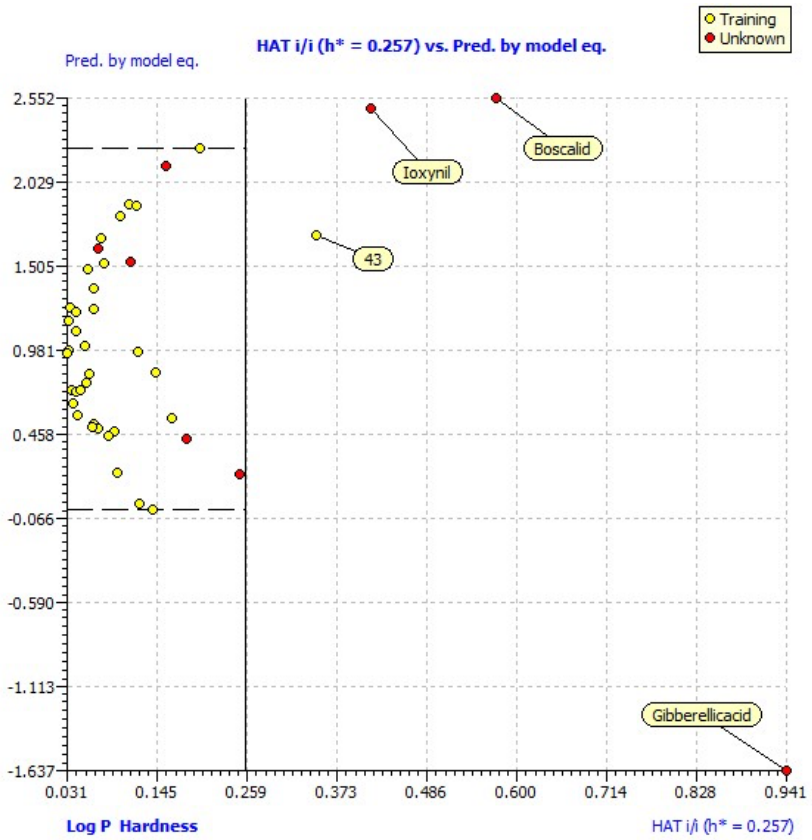


Figure 4.9. Insubria graph of Eq. 3.5: The predicted toxicity and hat values of the 8 chemicals (red marks) together with the training set chemicals (yellow marks).

4.3.4. QSTR Model on the 72-h Algal Toxicity of Industrial Chemicals and Pharmaceuticals

The fourth QSAR/QSTR model used for the prediction of algal toxicity values of tested chemicals was generated by Önlü and Saçan (2017). The algal toxicity data of industrial chemicals and pharmaceuticals used in this QSAR model are belong to mixed algal species. The eight-descriptor QSTR model (Eq. 3.6) was developed on 389 diverse chemicals. The descriptors appearing in Equation 3.6 are SPAM, Mor31p, NdsCH, CATS2D_02_AP, B05[C-S], F03[C-N], MLOGP2, and hardness. The meaning and source of these descriptors are given in Table 3.6.

The applicability domain of this model (Eq. 3.6) was also defined by the leverage approach. The dependent variable, pT (mM), ranges from 0.96 to 8.04. The ranges of descriptors are given in Table 4.8. The critical hat value (h^*) reported for this model is 0.07. All of the descriptor values for the 8 chemicals were retrived from DRAGON 6.4, except hardness. The same steps used in section 4.3.3 were applied for the calculation of hardness value of the 8 chemicals. The descriptor values of chemicals are given in Table 4.8 together with the predicted algal toxicity and hat values.

The calculated descriptor values of all chemicals were within the specified descriptor space. The predicted pT values for all the chemicals were within the model's AD. Hat values of all chemicals were also smaller than the cut-off value (h^*=0.07). Insubria Graph shows the interpolated toxicity values of all chemicals from the model (Eq. 3.6) (Figure 4.10). Therefore, the predicted algal toxicity values from this model can be reliable.

Table 4.8. Chemicals used in this study, their descriptor and hat values, and the predicted pT values by Equation 3.6.

Chemical / **Range**	**SPAM**	**Mor31p**	**NdsCH**	**CATS2D_02_AP**	**B05[C-S]**	**F03[C-N]**	**MLOGP2**	**Hardness**	**pT by Eq. 3.6 (M)**	**Hat values (h^*=0.07)**
Acetamiprid	0.46	0.24	0	0	0	8	2.68	4.25	5.41	0.04
Acetochlor	0.38	0.23	0	0	0	5	10.13	4.69	5.05	0.01
Boscalid	0.41	-0.12	0	0	0	7	15.23	3.91	5.90	0.03
Diclofop	0.46	-0.13	0	0	0	0	11.72	4.18	4.45	0.01
Diphenamid	0.41	0.11	0	1	0	2	8.06	4.76	3.79	0.02
Gibberellic acid	0.34	0.45	2	0	0	0	3.72	5.24	4.05	0.04
Ioxynil	0.60	-0.33	0	0	0	2	8.63	3.97	4.71	0.03
2,4,5-T	0.52	-0.10	0	0	0	0	8.49	4.28	4.33	0.01
Range of descriptors	0.34 - 0.65	-0.33 - 0.92	0 - 4	0 - 4	0 - 1	0 - 13	0 - 27.61	3.44 - 7.51		

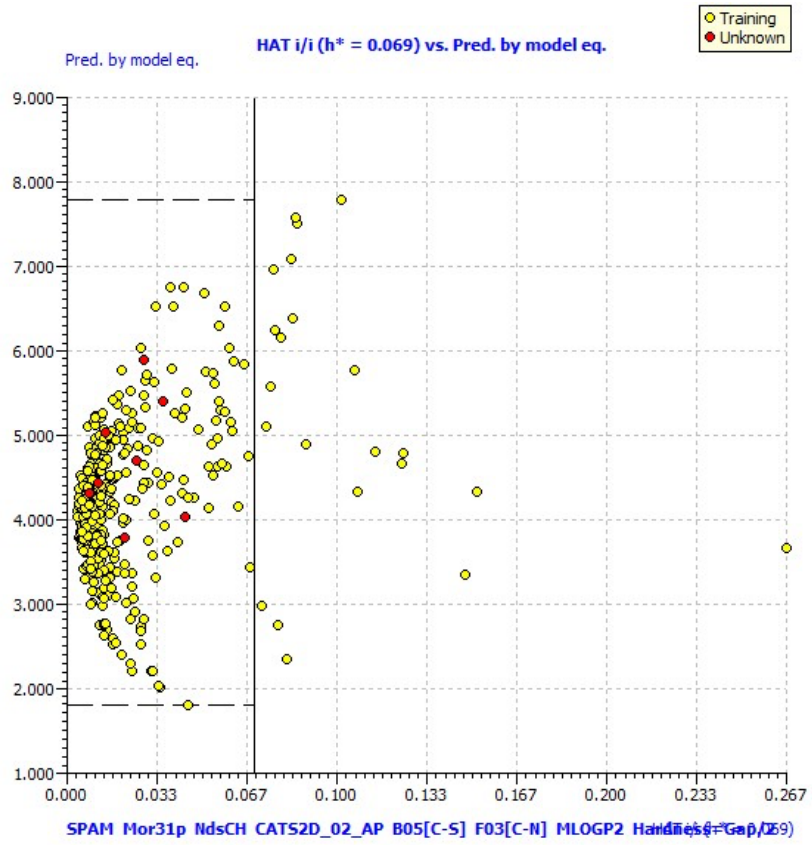


Figure 4.10. Insubria graph of Eq. 3.6: The predicted toxicity and hat values of the 8 chemicals (red marks) together with the training set chemicals (yellow marks).

4.4. Prediction of Algal Toxicity of Chemicals from a QTTR Model

The QTTR model used for prediction of algal toxicity values of tested chemicals was generated by Tugcu et al. (2017). The algal toxicity data of 23 chemicals were used in this QTTR model. It was an algae-algae, namely *C. vulgaris* and *P. subcapitata*, relationship study. The descriptor in the QTTR model (Eq. 3.7) was the 72-h predicted toxicity values of chemicals for *P. subcapitata*. 72-h algal *(P.subcapitata*) toxicity data ($pT_{P.subcapitata}$=-log EC_{50} (mM)) were taken from Aruoja et al. (2011). However, in the present study, since there are no experimental algal toxicity values for these 8 chemicals, the predicted algal toxicity values for *P.subcapitata* were used. The predicted EC_{50} values as mg/L for the test chemicals were compiled from Danish (Q)SAR Database (2015). They were converted to mmol/L (mM) and then to $pT_{P.subcapitata}$ (pT=−log EC_{50} (mM)) values and listed in Table 4.9. Danish (Q)SAR Database (2015) reports the predicted toxicity values from the Leadscope and SciQSAR models. Therefore, 72-h predicted algal toxicity ($pT_{P.subcapitata}$) values from these two models were given in Table 4.9.

The applicability domain of this model (Eq. 3.7) was also defined by the leverage approach. The dependent variable range, $pT_{C.vulgaris}$, was between -0.60 and 1.86 (as mM), whereas the independent variable range, $pT_{P.subcapitata}$, was between -0.32 and 1.94 (as mM). The critical hat value (h^*) reported for this model is 0.38. The 72-h predicted toxicity values of the 8 chemicals for *C. vulgaris* are given in Table 4.10 together with the hat values. The predicted values of acetamiprid and gibberellic acid were not listed in Table 4.9 and Table 4.10 since they were definitely out of the AD of the QTTR model.

The predicted $pT_{C.vulgaris}$ values of acetochlor, boscalid and diclofop based on the reported Leadscope $pT_{P.subcapitata}$ values and derived from the QTTR model were out of the model's AD (Figure 4.11(a)). On the other hand, the predicted $pT_{C.vulgaris}$ values of only ioxynil and 2,4,5-T based on the reported SciQSAR $pT_{P.subcapitata}$ values and derived from the QTTR model were within the model's AD (Figure 4.11(b)). These findings can also be seen from Table 4.9, Table 4.10 besides Figure 4.11.

Table 4.9. 72-h predicted EC_{50} (mg/L) values from Danish (Q)SAR Database (2015) and corresponding EC_{50} (mM) and pT values for *P. subcapitata*.

	***Pseudokirchneriella s.* 72-h toxicity values**					
Chemical	**EC_{50}[a] (mg/L)**	**EC_{50}[b] (mg/L)**	**EC_{50}[a] (mM)**	**EC_{50}[b] (mM)**	**pT^a (mM)**	**pT^b (mM)**
Acetochlor	$2.92x10^{-3}$	0.32	$1.08x10^{-5}$	$1.17x10^{-3}$	4.97	2.93
Boscalid	2.87	0.03	0.01	$2.62x10^{-2}$	2.08	4.12
Diclofop	0.01	0.58	$3.34x10^{-5}$	$1.7x10^{-3}$	4.48	2.75
Diphenamid	42.02	0.80	0.18	$3.36x10^{-3}$	0.76	2.47
Ioxynil	24.48	178.48	0.07	0.48	1.18	0.32
2,4,5-T	7.91	5.57	0.03	0.02	1.51	1.66

[a] *Leadscope predictions*
[b] *SciQSAR predictions*

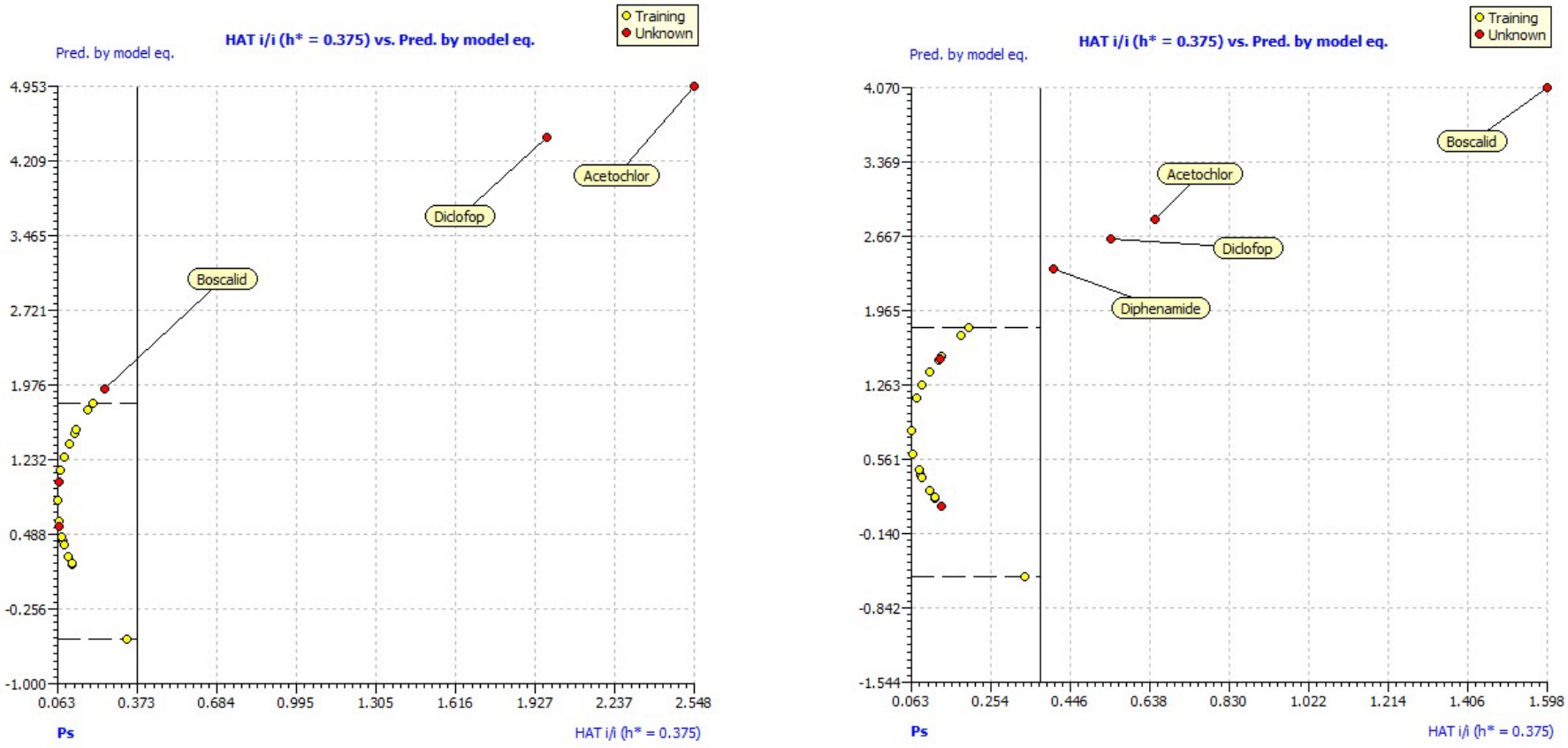
Table 4.10. 72-h predicted pT values of the 8 tested chemicals by the QTTR model for *C. vulgaris*.

Name	Predicted pT by Eq. 3.7 [a] (mM)	Hat values (h^*=0.38)	Predicted pT by Eq. 3.7 [b] (mM)	Hat values (h^*=0.38)
Acetochlor	**4.95**[*]	**2.55**[**]	**2.84**[*]	**0.65**[**]
Boscalid	**1.95**[*]	0.25	**4.07**[*]	**1.60**[**]
Diclofop	**4.44**[*]	**1.97**[**]	**2.65**[*]	**0.55**[**]
Diphenamide	0.57	0.07	**2.36**[*]	**0.41**[**]
Ioxynil	1.02	0.07	0.12	0.14
2,4,5-T	1.36	0.10	1.52	0.13

[a] *Values from Leadscope predictions*
[b] *Values from SciQSAR predictions*
[*] ***Values with "Out of the response range"***
[**] ***hat values >h**[*]

4.5. Prediction of Global Half-Life and Persistance, Bioaccumulation and Toxicity Indices for the Studied Chemicals

The five-descriptor Global Half-Life Index (GHLI) model (Eq. 3.8) was previously developed by Principal Component Analysis (PCA) from half-life data (Gramatica and Papa, 2007). The half-life data were obtained by studying the transformation of a set of 250 organic POP-type chemicals in air, water, sediment and soil. The descriptors appearing in Equation 3.8 are MW, maxHBA, nBondsS2, nHBDon_Lipinski, and minsCl. The meaning and source of these descriptors are shown in Table 3.7. Descriptors were calculated for the 8 chemicals by PaDEL-Descriptor 2.21 software. The dependent variable in the GHLI model refers to the negative logarithm of half-life values (hours) of chemicals in the data set. Table 4.11 shows the value of descriptors for each test chemical required for the prediction of half-life index. The applicability domain of this model (Eq. 3.8) was also defined by the leverage approach. The dependent variable, GHLI (h), ranges from -3.13 to 4.98. The ranges of descriptors are given in Table 4.11 together with the predicted half-life index and hat values. The critical hat value (h^*) reported for this model is 0.07.



(a) (b)

Figure 4.11. Insubria graphs of the the inter-species QTTR model for *C. vulgaris*; the predicted toxicity values of *P. subcapitata* taken from (a) Leadscope and (b) SciQSAR.

Table 4.11. Chemicals used in this study, their descriptor and hat values, and the predicted GHLI values by Equation 3.8. for *Pseudokirchneriella subcapitata*.

Chemical / Range	**MW**	**maxHBa**	**nBondsS2**	**nHBDon_Lipinski**	**minsCl**	**GHLI by Eq. 3.8 (-log(hours))**	**Hat values (h^*=0.07)**
Acetamiprid	222.07	1.93	18	0	0.80	1.50	0.01
Acetochlor	269.12	12.13	31	0	0.70	-0.18	0.05
Boscalid	342.03	12.55	17	1	0.55	0.74	0.03
Diclofop	326.01	10.73	21	1	0.75	0.78	0.03
Diphenamide	239.13	12.37	23	0	0	-0.71	0.03
Gibberellic acid	346.14	12.69	**47***	3	0	-1.93	**0.17***
Ioxynil	370.83	9.44	7	1	0	1.61	**0.08***
2,4,5-T	253.93	10.34	12	1	0.72	0.40	0.02
Range of descriptors	32.03 - 493.69	0 - 12.84	3 / 37	0 - 4	0 - 1.38		

* ***Descriptors "Out of the descriptor space".***

The calculated descriptor values of six chemicals were within the specified descriptor space of the GHLI model. Although the hat values of gibberellic acid and ioxynil were greater than the cut-off value (h^*=0.07), the predicted GHLI values for all chemicals were within the model's response range. Insubria Graph shows the predicted GHLI values of all chemicals from the model (Eq. 3.8) together with the hat values (Figure 4.12). Benzidine, decachlorobiphenyl and n-dodecane appearing in the Insubria Graph with the CAS numbers 92-87-5, 2051-24-3 and 112-40-3, respectively, were the structural outliers from the training set in the GHLI model (Gramatica and Papa, 2007). The order of half-life index is ioxynil<acetamiprid<diclofop<boscalid<2,4,5-T<acetochlor<diphenamide<gibberellic acid.

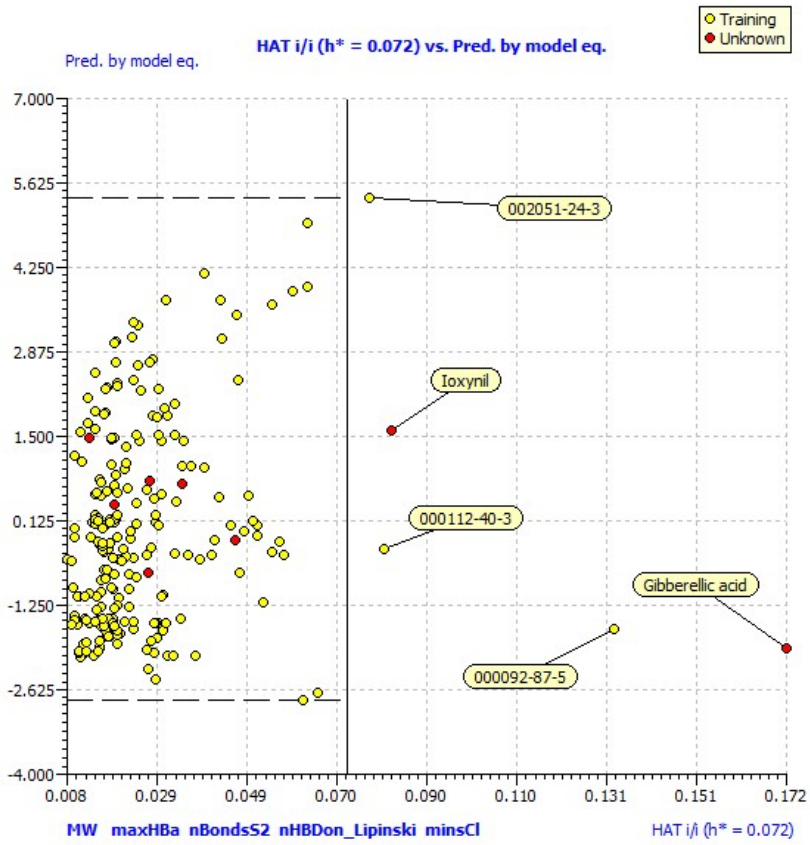


Figure 4.12. Insubria graph of Eq. 3.8: the predicted GHLI and hat values of the 8 chemicals (red marks) together with the training set chemicals (yellow marks).

The four-descriptor PBTI model (Eq. 3.9) was previously developed using Principal Component Analysis (PCA) from half-life, Bioconcentration Factor (BCF) and *P.promelas* toxicity for a set of 180 heterogeneous organic chemicals (Papa and Gramatica, 2010). The descriptors appearing in Equation 3.9 are nX, nBondsM, MAXDP2, and nHBDon_Lipinski. The meaning and source of these descriptors are given in Table 3.7. Descriptors were calculated for the studied chemicals by PaDEL-Descriptor 2.21 software. They were used to calculate PBTI values of the 8

tested chemicals. Calculated molecular descriptors used in the model calculation for each test chemical are listed in Table 4.12.

The applicability domain of this model (Eq. 3.9) was also defined by the leverage approach. The dependent variable, PBT Index, ranges from -3.08 to 5.02. The ranges of descriptors are given in Table 4.12 together with the predicted PBT Index and hat values. The critical hat value (h^*) reported for this model is 0.08. The calculated values of nBondsM and MAXDP2 for boscalid, the calculated value of MAXDP2 for diphenamid and the calculated values of MAXDP2 and HBDon_Lipinski for gibberellic acid were out of the descriptor space of the PBTI model.

Although the hat values of boscalid and gibberellic acid were greater than the cut-off value (h^*=0.08), the predicted PBTI values for all chemicals were within the model's response range. Probably the structure of boscalid and gibberellic acid are different from the chemicals in the training set of the PBTI model. Insubria Graph shows the predicted PBTI values of all chemicals from the model (Eq. 3.9) together with the hat values (Figure 4.13). The limit value of PBT Index for PBT and vPvB compounds are set to 1.5 by Papa and Gramatica (2010). Therefore, the chemicals with a PBT index value greater than 1.5 were written in bold in Table 4.12. Of the 8 chemicals, boscalid and diclofop were highlighted as Persistent, Bioaccumulative and Toxic chemicals based on their predicted PBTI values by Equation 3.9. Therefore, it is likely that further testing is required for these chemicals, since boscalid is found in many bee matrices, also in aquatic environment, and at high concentrations around 36 μg/L in main coastal estuaries in California (Qian et al., 2018). Also, it is found in more than 50% of the samples collected from the fishponds in the Lorraine Region, north-eastern France (Gaillard et al., 2016). Also, it is recorded that the total use of diclofop methyl in the USA was approximately 340,200 kg of active substance per year between 1987 and 1996, and the use in 2006 was 1 to 5 kilograms in China, while in Canada, the total use in 1986 was over one million kilograms (Ye et al., 2013). The order of PBT index is gibberellic acid<acetochlor<ioxynil<acetamiprid<diphenamide<2,4,5-T<diclofop<boscalid.

Table 4.12. Chemicals used in this study, their descriptor and hat values, and the predicted PBT index values by Equation 3.9.

Chemical / Range	nX	nBondsM	MAXDP2	nHBDon_Lipinski	PBT Index by Eq. 3.9	Hat values (h^*=0.08)
Acetamiprid	1	8	2.39	0	0.76	0.01
Acetochlor	1	7	4.95	0	0.39	0.04
Boscalid	2	**20***	**5.42***	1	**3.42**	**0.11****
Diclofop	2	13	3.70	1	**2.01**	0.04
Diphenamide	0	13	**5.37***	0	1.02	0.07
Gibberellic acid	0	4	**5.69***	3*	-2.12	**0.13****
Ioxynil	2	7	3.30	1	0.74	0.02
2,4,5-T	3	7	3.21	1	1.39	0.03
Range of Descriptors	0 - 6	0 - 16	0 - 5.24	0 - 2		

* ***Descriptors "Out of the descriptor space".***
** ***Values with "Out of the response range"***

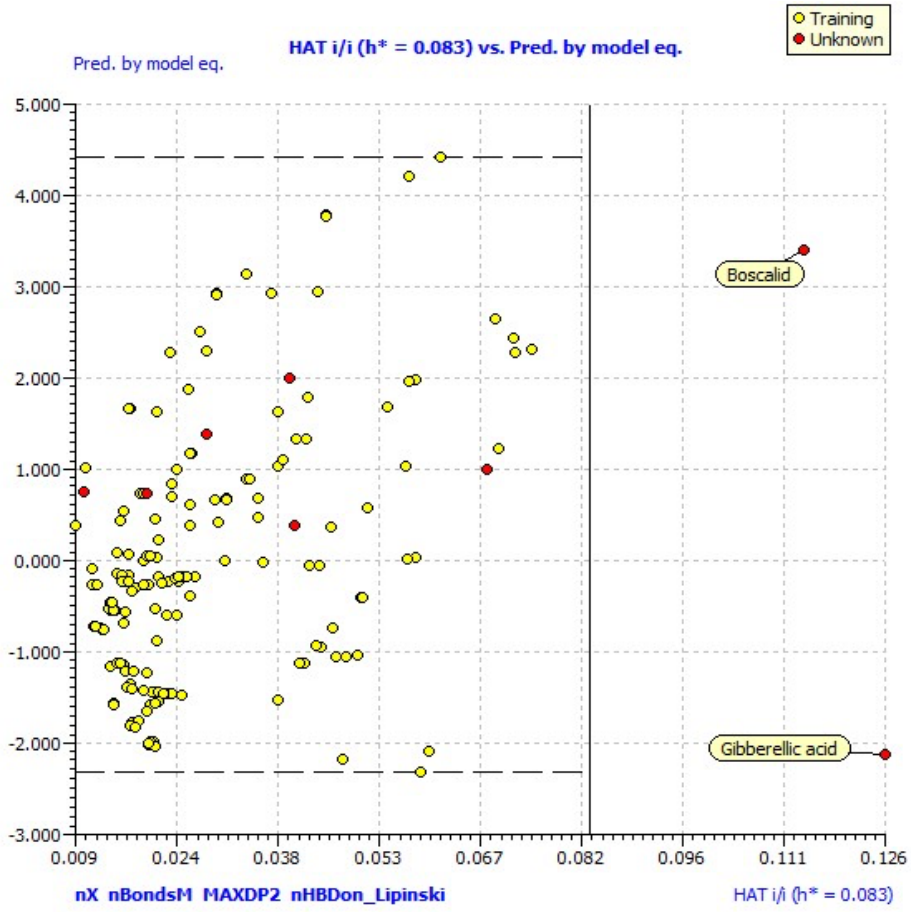


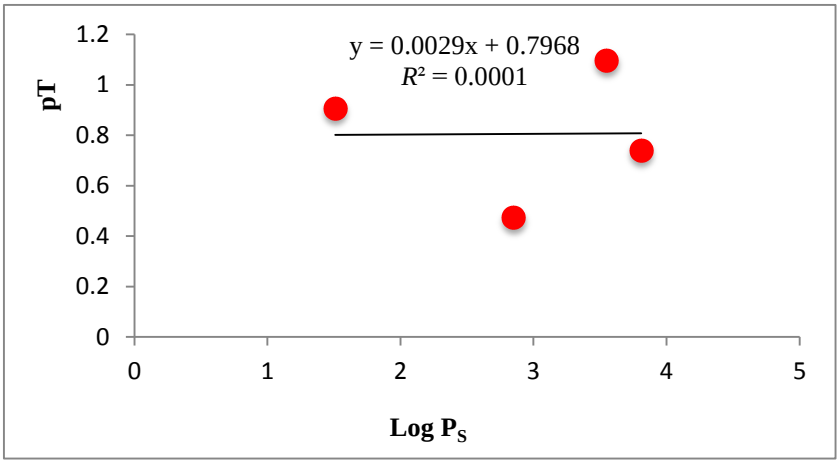
Figure 4.13. Insubria graph of Eq. 3.9: the predicted PBT index and hat values of the 8 chemicals (red marks) together with the training set chemicals (yellow marks).

4.6. Relationship Between Hydrophobicity and Algal Toxicity

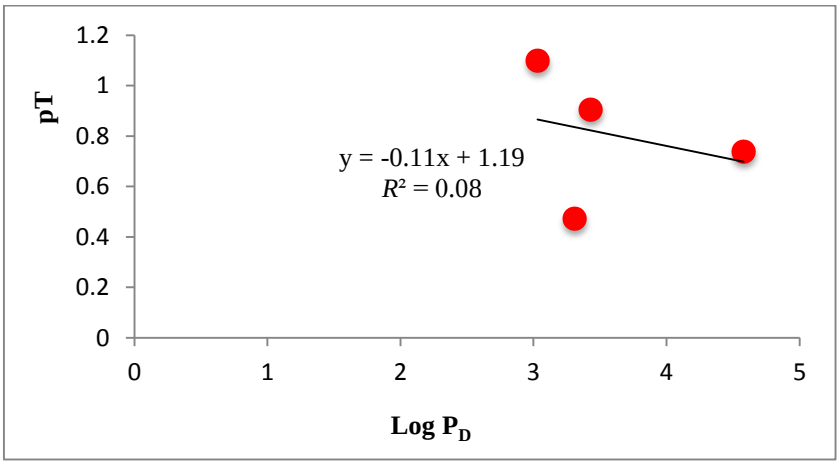
Hydrophobicity is one of the most important parameters which correlate with toxicity. Therefore, in an attempt to see the relationships between the experimental algal toxicity values and hydrophobicity, several correlation analyses were carried out. Of the 8 chemicals, the experimental pT value of only 4 chemicals, namely acetochlor, diclofop, ioxynil, 2,4,5-T can be exactly obtained from the algal assay (Table 4.4). Since it is well-known that for the same chemical, not all calculated and/or experimental log P values are equal, in the present study, the calculated log P values from the SPARTAN software (log P_S) and the experimental log P values (log P_D) and pH-dependent hydrophobicity parameter, log D (pH=6), from Danish (Q)SAR Database (2015) were used in correlation analysis. With the smallest data points, there is no relationship between pT values and either experimental or calculated log P values (Figure 4.13 (a) and Figure 4.13 (b)). It is also well-known that chemicals with baseline toxicity give a better correlation with log P. The low correlation between pT and log P values reflects that the chemicals have toxicity other than baseline toxicity.

The correlation is much better between the experimental pT values and log D (Figure 4.14 (c)). This is in line with the findings reported in a previous study (Ertürk and Saçan, 2013). The most prevailing reason for this can be explained by the significance of ionization on partitioning for the tested chemicals.

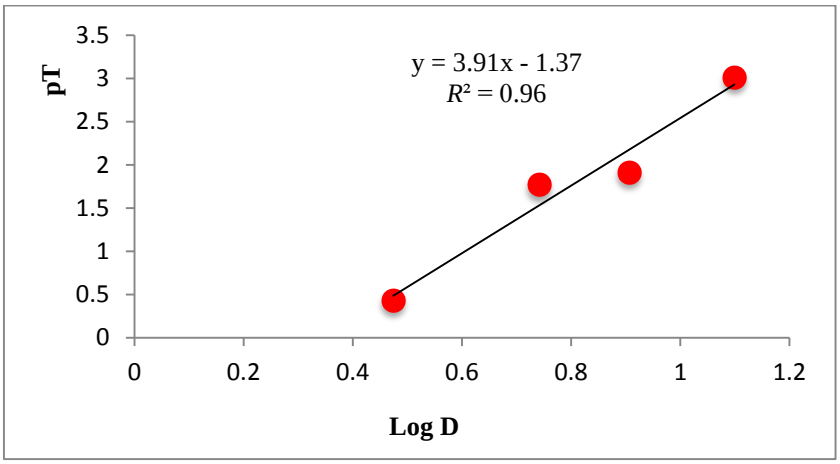
Usually pesticides are regarded as acetylcholine esterase inhibitors. In the present study chemicals were selected from pesticide groups. We have seen that there is no correlation between algal toxicity and log P. Therefore, the expected mode of action was searched and listed in Table 4.13. Consideration of the information in Table 4.13 shows that these chemicals do not have a common mode of action.



(a)



(b)



(c)

Figure 4.14. The relationship between experimental pT and (a) log P_S, (b) log P_D (c) log D.

Acetamiprid is the only insecticide among the 8 test chemicals acting as an acetylcholine receptor agonist. Nicotinic acetylcholine receptors (nAChRs) exist in insect nervous tissue in high

densities and are targeted by neonicotinoid insecticides (Brown et al., 2006). Diclofop, ioxynil and 2,4,5-T belong to the herbicides group with different modes of action, except for acetochlor and diphenamid which are both inhibitors of Very Long Chain Fatty Acids (VLCFAs). Boscalid is a fungicide based on the succinate dehydrogenase enzyme inhibition, capable of blocking the mitochondrial respiration chain and causes spore germination, germ-tube elongation, mycelial growth and sporulation of pathogenic fungi on the leaf surface of spacious variety of crops (Stammler et al., 2007; Stammler et al., 2007). Gibberellic acid is a plant growing hormone which enhances the synthesis of o α-Amylase.

Table 4.13. Chemicals tested in the present study and their expected mode of actions (MoAs).

Chemical Name	MoA
Acetamiprid	Acetylcholine receptor (nAChR) agonists [a]
Acetochlor	Inhibition of VLCFA (inhibition of cell division) [b]
Boscalid	Succinate dehydrogenase inhibitor [a]
Diclofop	acetyl CoA carboxylase (ACCase) inhibitor [a]
Diphenamid	Inhibition of VLCFA (inhibition of cell division) [b]
Gibberellic acid	Inhibition of α-Amylase development [c]
Ioxynil	Inhibits photosynthesis (photosynthesis II) [a]
2,4,5-T	Synthetic auxins [a]

[a] *Guide to pesticide modes of action http://www.dropdata.org/RPU/pesticides_MoA.htm (Last Access: 22.07.2018)*
[b] *The Pesticide Properties Database https://sitem.herts.ac.uk/aeru/ppdb/en/index.htm (Last Access: 22.07.2018)*
[c] *(Lewak and Khan, 1977)*

4.7. Comparison of Experimental and Predicted pT Values

In an attempt to compare the experimental algal toxicity values obtained in this study with the predicted toxicity values from different QSAR/QSTR/QTTR models available in the literature we listed all pT values in Table 4.14. All the 50% inhibitory concentration values were converted to pT values with the same unit (M) for convenience. Acetochlor was the most toxic chemical among 8 tested chemicals with pT=4.10. QSAR model with Equation 3.3 has the most accurate prediction on acetochlor with pT value 4.03. Also, experimental toxicity value of acetochlor was found in the range between the minimum and the maximum values of model predictions. Acetamiprid, diphenamide and gibberellic acid are the least toxic three chemicals with no definite results. Only two models predicted pT values of gibberellic acid and boscalid. The experimental algal toxicity value for boscalid could not be determined due to its low solubility. Of the six models only Eq. 3.4 and Eq. 3.6 predicted high toxicity values for this chemical as pT=5.51 and pT=5.90, respectively. In all models predicted pT values of acetamiprid were higher than the experimental result. Ioxynil gave the second highest experimental toxicity, and the model Equation 3.7 predicted the toxicity value of this chemical close to experimental results. The difference between experimental and

predicted values is only 0.11 log unit. Experimentally both diclofop and 2,4,5-T gave lower toxicity results, than predicted toxicity values. Although the pT values are different when the experimental and the predicted pT values from Eq. 3.6 are compared, the order of toxicity is the same for the chemicals with experimental pT values (acetochlor>ioxynil>diclofop>2,4,5-T). The predicted values of this chemicals are more conservative which is also very good from the environmental point of view. Another outcome of this finding is the use of QSAR/QSTR modeling is very important for the screening prioritization and further testing of chemicals with no data.

Table 4.14. Experimental and predicted pT (M) values of the 8 chemicals.

Name	**Experimental**	**Predicted pT (M)**						
	pT (M)	**pT by Eq. 3.3**	**pT by Eq. 3.4**	**pT by Eq. 3.5**	**pT by Eq. 3.6**	**pT by Eq. 3.7[a]**	**pT by Eq. 3.7[b]**	**Status***
Acetamiprid	<3.3	5.60	4.42	4.54	5.41	NA	NA	OUT
Acetochlor	4.10 (4.06 / 4.14)	4.03	4.80	3.44	5.05	NA	NA	IN
Boscalid	<4.66	NA	5.51	NA	5.90	NA	NA	OUT
Diclofop	3.74 (3.73 / 3. 75)	6.84	4.95	5.14	4.45	NA	NA	OUT
Diphenamid	<3.31	4.38	NA	3.21	3.79	3.57	NA	IN
Gibberellic Acid	<3.52	3.70	NA	NA	4.05	NA	NA	OUT
Ioxynil	3.91 (3.89 / 3.92)	NA	4.51	NA	4.71	4.02	3.12	IN
2,4,5-T	3.47 (3.47 / 3.48)	NA	4.80	4.62	4.33	4.36	4.52	OUT

**OUT indicates that the experimental value is out of the range of minimum and maximum predicted pT values; IN indicates that the predicted value is in the range of minimum and maximum predicted pT values.*

NA=Not applied indicates that the predicted value is out of the corresponding model's AD (unreliable prediction).

4.8. Risk Assessment

4.8.1. Case Study for the Risk Assessment

A ratio of the predicted/measured environmental concentration (PEC) and the predicted no-effect concentration (PNEC) usually is derived to decide whether risk-reduction measures must be taken for industrial chemicals/contaminants. The PEC is determined from monitoring data or from modeling. The PNEC is derived from acute or chronic data using assessment factors, which are meant to extrapolate the results of monospecies laboratory tests to a multispecies ecosystem.

In the present study, the PNEC values for only 4 chemicals were calculated. Assessment factor 1000 was used for the calculation of $PNEC_{freshwater}$, since the 4 test chemicals were taken from the list of no ecotoxicological data. This means that there is no aquatic toxicity data for biota (fish, algae and daphnid) for these chemicals. According to Section 3.8.1, the most suitable model was selected as equation 3.11. The calculated $PNEC_{freshwater}$ values of the test chemicals by equation 3.11 which includes the assessment factor as 1000 is listed in Table 4.15.

Table 4.15. Experimental toxicity values and related calculated $PNEC_{freshwater}$ values.

Chemical	**IC_{50}**	**PNEC calculated from IC_{50}**
Acetochlor	21.47 (19.32-23.57)*	0.021
Diclofop	59.32 (58.12-60.41)	0.059
Ioxynil	46.02 (44.19-47.37)	0.046
2,4,5-T	85.74 (84.59-86.80)	0.086

* *Numbers within the parenthesis are the confidence intervals.*

We used this PNEC values to evaluate the risk status of these chemicals. For this purpose, as a case study, local concentrations of only acetochlor and diclofop were collected from the literature (Table 4.16). To the best of our knowledge, no relevant data on the environmental concentrations of ioxynil and 2,4,5-T was found in the literature. Therefore, only the the risk status of acetochlor and diclofop was evaluated using the calculated PNEC values. Calculated risk status using the PEC data from the literature and PNEC values are reported in Table 4.16.

When the PEC/PNEC value of a chemical is more than 1, it means there is a risk for that substance. The risk factors were calculated for water compartment as a case study using available

literature data for acetochlor and diclofop and listed in Table 4.16. The fact that the literature data for PEC are out of date reduces the reliability of the results. Since there is no recent literature data for the calculation of risk factor, these results are not up-to-date and there is definetly a data gap for these chemicals to be used in risk assessment. Recalling Table 4.1, there is a big data gap in the ecotoxicological data for the studied chemicals in biota compartment, for diclofop, diphenamid, gibberellic acid and ioxynil in water, and for diclofop, diphenamid and gibberellic acid in sediment. Current measurements should be taken to ensure a more precise risk assessment, and calculations should be made in the direction of these measurements. Furthermore, in the present study acetochlor and boscalid were found to be a PBT compound from the previous predictions (equation 3.9) with a PBT index of 2.01 and 3.55, respectively (PBT index>1.5 are considered to be PBT compounds). We suggest that measures should be taken and further testings are required for these chemicals.

Table 4.16. Local concentrations and risk status for acetochlor and diclofop. A case study for risk assessment for water compartments.

Chemical	**Concentration (μg/L)**	**Compartment**	**Place**	**Risk Factor (PEC/PNEC)**	**Risk**
Acetochlor	1	Surface water in corn-growing area site E8	South Africa[a]	0.05	N[d]
	2.5	Rain water	Midwestern United States[b]	0.12	N
Diclofop	0.03–0.04	Wetlands	Northern praire landscapes[c]	$5.90\ 10^{-4}$	N
	0.46	Shallow groundwater	Northern praire landscapes[c]	$7.75\ 10^{-3}$	N

[a,b,c] *Data taken from Du Preez et al., 2005; Kolpin et al., 1996; Donald et al., 2001*
[d] *N: No Risk (PEC/PNEC<1)*

4.9. Acute to Chronic Ratio Calculation (ACR)

Three types of ACR were used for the comparison with the literature: ACR_{MATC}= IC_{50}/MATC ACR_{20}= IC_{50}/IC_{20} and ACR_{NOEC}= IC_{50}/NOEC (EC, 2003) (Table 4.17). The present study had an average NOEC as 13.54 (mg/L) for four chemicals, whereas the average of NOEC was found as 16.55 for *Chlorella pyrenoidosa* exposed to 11 narcotic chemicals particularly phenol and aniline derivatives (Ramos et al., 1999). The average calculated ACR in terms of MATC, IC_{20}, and NOEC for the three chemiclas studied are 3.42, 2.35 and 4.83, respectively.

Tugcu and Saçan (2018) studied the toxicity of 60 phenol and aniline derivatives on *C. vulgaris* and reported an average ACR_{NOEC} of 5.34. In their study, the reported average ACR_{NOEC} of

chemicals with the mode of actions such as respiratory uncouplers and soft electrophiles are 4.59 and 4.89, respectively. The average ACR of organic chemicals for algae reported in ECOSAR Methodology Document is 4 (ECOSAR, 2012). This value is obtained using 72/96-h EC_{50} and ChV of their data set. The present data has an average ACR_{MATC} of 3.42. The reported average ACR_{MATC} is 3.75 in the study of Tugcu and Sacan (2018). The average ACR reported in EU TGD for algae is 5.4. All these values are considered to be in general agreement. In general, for each trophic level, the lowest valid effect value from both acute and chronic tests are considered for the conservation of aquatic species. The average ACR_{NOEC} for algae is less than a factor of 10 suggest that regulations are conservative for this species. Ahlers et al., (2006) suggest an aquatic ecosystem ACR (ACR_{eco}) by dividing the lowest acute value over the lowest chronic value, irrespective of the species. It is also very important to note that only industrial chemicals are considered, and bioactive agents, such as pesticides, are not taken into account for the assessment of ACR. Besides risk assessment, classification and labeling are areas for which the ACR also is important.

Table 4.17. Chemicals, their NOEC, LOEC, MATC values and relevant ACR values.

Chemical / Average	**IC_{50}(mg/L)**	**NOEC**	**LOEC**	**MATC**	**ACR_{MATC}**	**ACR_{IC20}**	**ACR_{NOEC}**
Diclofop	59.32 (58.12-60.41)*	11.71	23.42	16.56	3.58	2.60	5.07
Gibberellic acid	>104.13	10.41	104.13	32.92			
Ioxynil	46.02 (44.19-47.37)	7.79	15.58	11.02	4.18	3.13	5.91
2,4,5-T	85.74 (84.59-86.80)	24.28	48.57	34.34	2.50	1.32	3.53
Average		13.55			3.42	2.35	4.83

* *Numbers within the parenthesis are the confidence intervals.*

4.10. Predicted Environmental Distribution of Chemicals from Level III Fugacity Model

Level III fugacity model simulates environmental compartments and predicts partitioning of chemicals in air, soil, sediment, and water under steady state conditions. Predicted environmental distribution of the studied chemicals from Level III fugacity model were taken from Danish (Q)SAR Database (2015). Pie charts display the partitioning of chemicals in each compartment (mass %) in Figure 4.15 and Figure 4.16.

One of the most noticeable points when pie charts are examined is that boscalid has the smallest water partitioning among the 8 chemicals. It is because of the low water solubility of boscalid (water, 6 mg/L at 20 °C (US EPA, 2003)). The exact algal toxicity of this chemical couldn't be measured due to its low water solubility. Another remarkable point is that gibberellic

acid has the highest water partitioning. Gibberellic acid as its name implies it is an acid and it dissociates in water, hence its solubility increases. Therefore, its partitioning in water is more than the other chemicals.

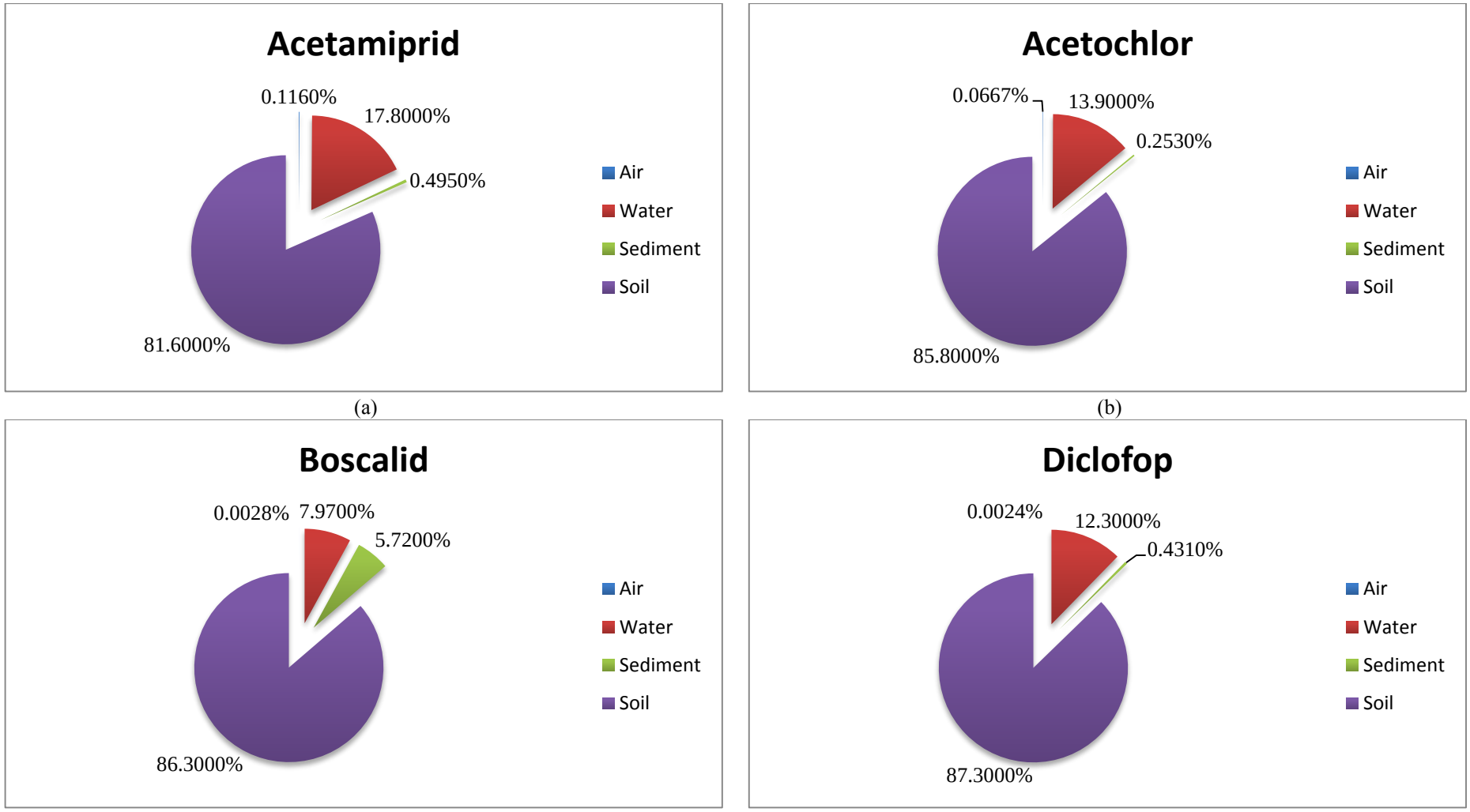


Figure 4.15. Predicted Level III Fugacity Mass Amount of (a) acetamiprid, (b) acetochlor, (c) boscalid, (d) diclofop according to Danish (Q)SAR Database (2015).

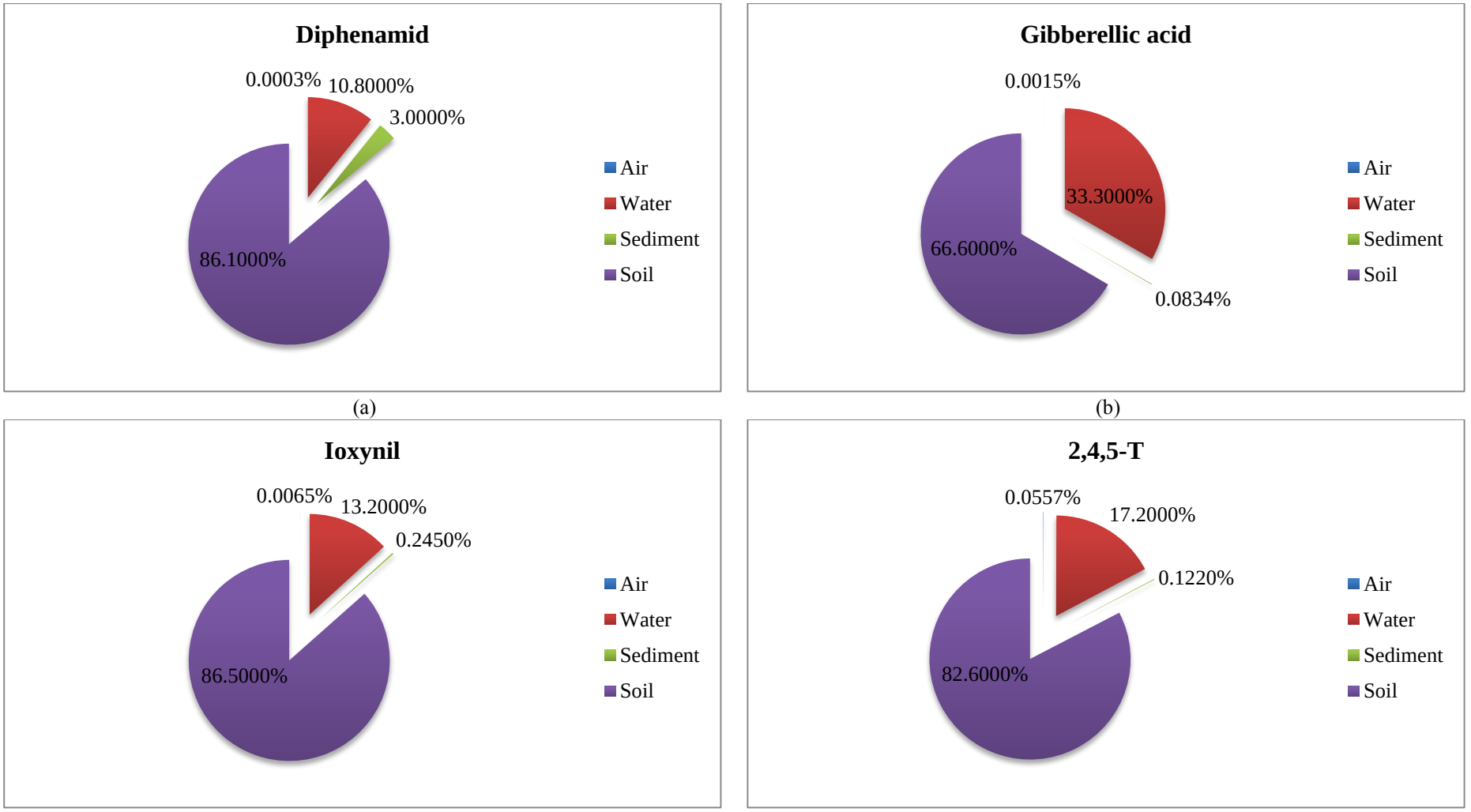


Figure 4.16. Predicted Level III Fugacity Mass Amount of (a) diphenamid, (b) gibberellic acid, (c) ioxynil, (d) 2,4,5-T according to Danish (Q)SAR Database (2015).

5. CONCLUSION

In the presented study, the 96-h toxicity values of 8 pesticides (acetamiprid, acetochlor, boscalid, diclofop, diphenamid, gibberellic acid, ioxynil, and 2,4,5-T) selected from the list of chemicals with no ecotoxicological data were investigated according to the standard procedures for growth inhibition test using freshwater alga, *Chlorella vulgaris* as a test organism for the first time. Among the 8 test chemicals, the most toxic compound was acetochlor with an IC_{50} value of 21.47 mg/L, while the toxicity of acetamiprid, diphenamid, and gibberellic acid could not be clearly defined. The endpoints for these chemicals should be further tested up to their solubility limits to make a contribution to risk assessment. Boscalid showed no toxic effect in its solubility limits.

Moreover, from literature QSTRs which shows a complience with the OECD guidelines which require the models should have (1) a defined endpoint, (2) unambiguous algorithm, (3) a defined domain of applicability, (4) appropriate measures of goodness of fit, robustness and predictivity, (5) a mechanistic interpretation (if possible) were retrieved. These models were used to compare the experimental toxicity values of 8 chemicals and the respective predicted toxicity values. There is not a particular model that gave accurate predictions for all the chemicals. However, all the chemicals were in the AD of QSTR model generated for the 72-h toxicity of industrial chemicals and pharmaceuticals to a variety of freshwater algae species with a wide applicability potential (Eq. 3.6).

Algae-algae QTTR model was also retrieved from literature. Two toxicity datasets for 8 chemicals on *P. subcapitata* predicted from two different sources were implemented in the model to calculate toxicity value for *C. vulgaris*. The QTTR model which has the toxicity of chemicals to *P. subcapitata* as the independent variable from LeadScope and toxicity data on *C. vulgaris* as the dependent variable revealed a good correlation, while SciQSAR data revealed some distinction. Additionaly it is necessary to investigate the toxic effects of these chemicals to different aquatic organisms, in addition to different algae species, to provide more detailed information on mode of action and inter-species toxicity relationships.

The toxicities of the chemicals to *C. vulgaris* and their hydrophobicity relationship were investigated. Although no definite interpretations could be made because the data set was small, a better linear relationship between toxicity and log D was found than between toxicity and log P.

The lack of a linear relationship between toxicity and log P implies that the chemicals are neither non-polar nor polar narcotics and their mode of action is different than the baseline toxicity.

The risk factors were calculated for water compartment as a case study using available literature data for acetochlor and diclofop. Risk factors of acetochlor and diclofop were found to be less than 1. Since there is no recent literature data for the calculation of risk factor, these results are not up-to-date and there is definetly a data gap for these chemicals.

GHLI and PBT index values were calculated by validated QSAR models retrieved from the literature. The order of half-life index is ioxynil<acetamiprid<diclofop<boscalid<2,4,5-T<acetochlor<diphenamide<gibberellic acid. However, boscalid and diclofop were screened as PBT chemicals among all the chemicals studied. The order of PBT index is gibberellic acid<acetochlor<ioxynil<acetamiprid<diphenamide<2,4,5-T<diclofop<boscalid. The order of half-life and PBT indices are not compatible. Therefore, a holistic approach should be applied to understand the fate of the chemicals in the environment. On the other hand, although the fate of these chemicals seems to be particularly the soil compartment based on the fugacity model level III, the outcome of this study suggests that these chemicals are a major concern for all environmental compartments.

The calculated low-toxic-effect concentrations (LOEC, NOEC and IC_{20}) were used in risk assessment and Acute to Chronic Ratio (ACR) were evaluated to control if the ACR values are within the safe level of chemicals set for algae. The average NOEC was found 13.54 (mg/L) for four chemicals. The average calculated ACR in terms of MATC, IC_{20}, and NOEC for the three chemicals studied are found as 3.42, 2.35 and 4.83, respectively. These ACR values were found within the proposed safety level of chemicals for algae.

Further studies should be carried on to establish Carcinogenic, Mutagenic, Reprotoxic (CMR) effects of these chemicals to fulfill the data gaps under REACH regulation.

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APPENDIX A

Table A1. Strains shown to be suitable for the Algal growth inhibition test.

Type	Species	Strains
Green algae	*Pseudokirchneriella subcapitata*, (formerly known as *Selenastrum capricornutum*)	ATCC 22662 CCAP 278/4 61.81 SAG
	Desmodesmus subspicatus (formerly known as *Scenedesmus subspicatus*)	86.81 SAG
Diatoms	*Navicula pelliculosa*	UTEX 664
Cyanobacteria	*Anabaena flos-aquae*	UTEX 1444 ATCC 29413 CCAP 1403/13A
	Synechococcus leopoliensis	UTEX 625 CCAP 1405/1

APPENDIX B

Table B1. Name, CAS no. and relative purity and source of test chemicals

Chemical	CAS No	Purity (%)	Source
Acetamiprid	135410-20-7	≤100	SIGMA-ALDRICH
Acetochlor	34256-82-1	≤100	SIGMA-ALDRICH
Boscalid	188425-85-6	≤100	SIGMA-ALDRICH
Diclofop	40843-25-2	98.83	EHRENSTORFER
Diphenamid	957-51-7	98.5	EHRENSTORFER
Gibberellic acid	77-06-5	≤100	SIGMA-ALDRICH
Ioxynil	1689-83-4	≤100	SIGMA-ALDRICH
2,4,5-T	93-76-5	≤100	SIGMA-ALDRICH

APPENDIX C

Table C1. pH values in the first and last days of the bioassays conducted by corresponding chemicals.

Chemical Name	pH_i	pH_f
3,5-DCP	6.05	6.35
Acetamiprid	6.07	6.30
Acetochlor	6.06	6.44
Boscalid	6.07	6.40
Diclofop	6.04	6.50
Diphenamid	6.06	6.42
Gibberellic Acid	6.09	6.40
Ioxynil	6.07	6.42
2,4,5-T	6.05	6.47

APPENDIX D

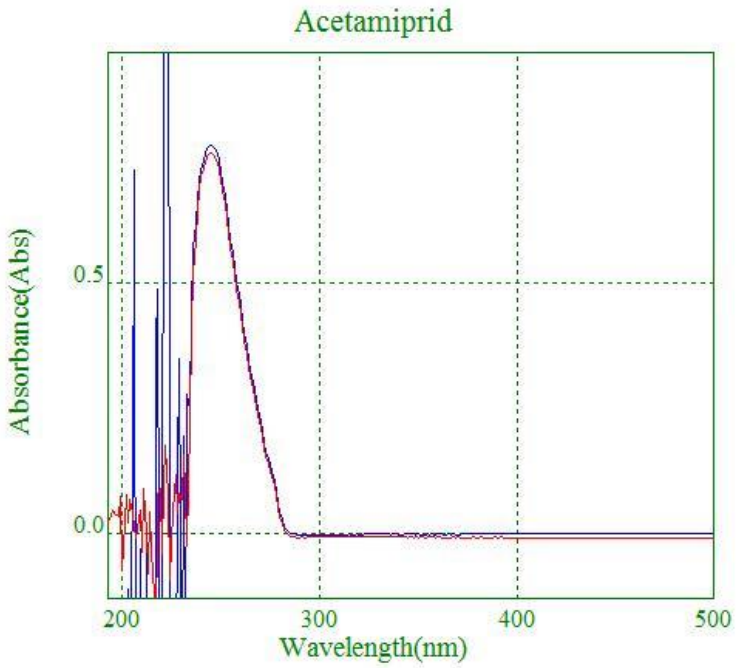
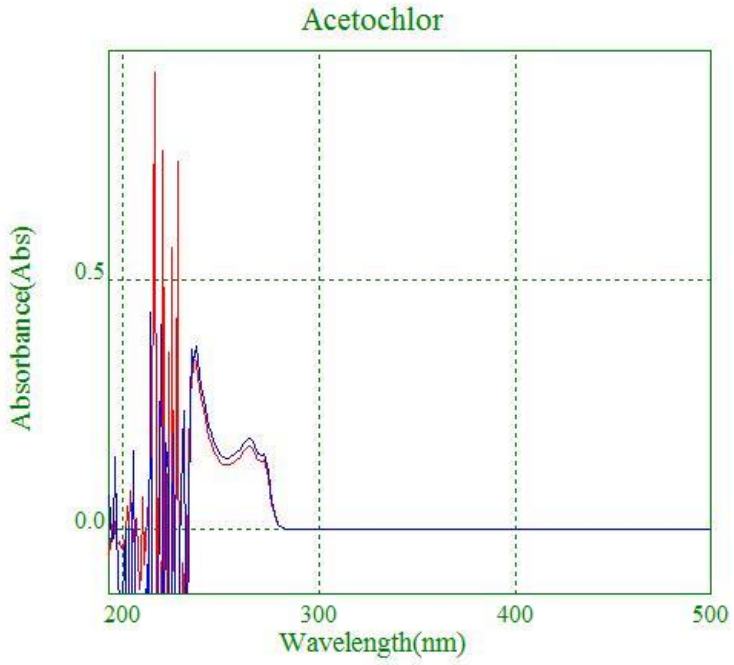
Chemical	Spectrum
Acetamiprid	
Acetochlor	

Figure D1. UV-Vis spectroscopy results of test chemicals.

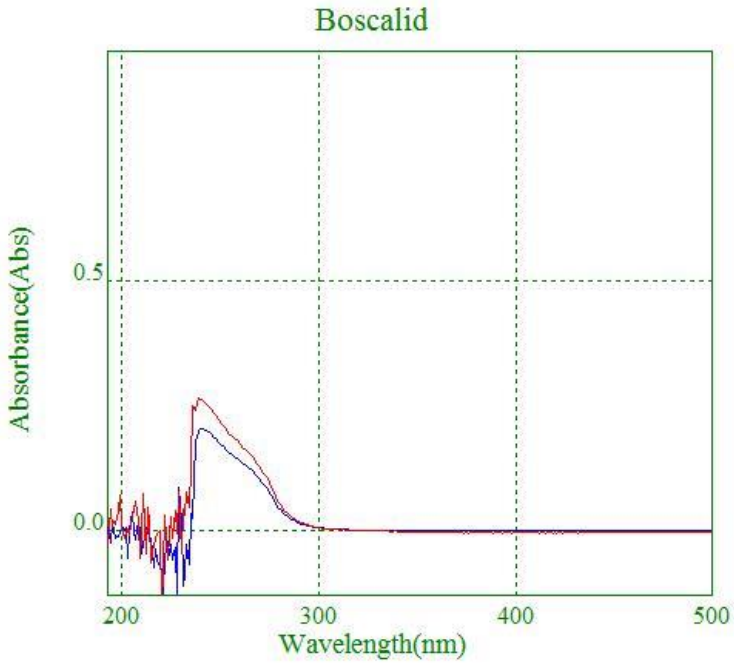
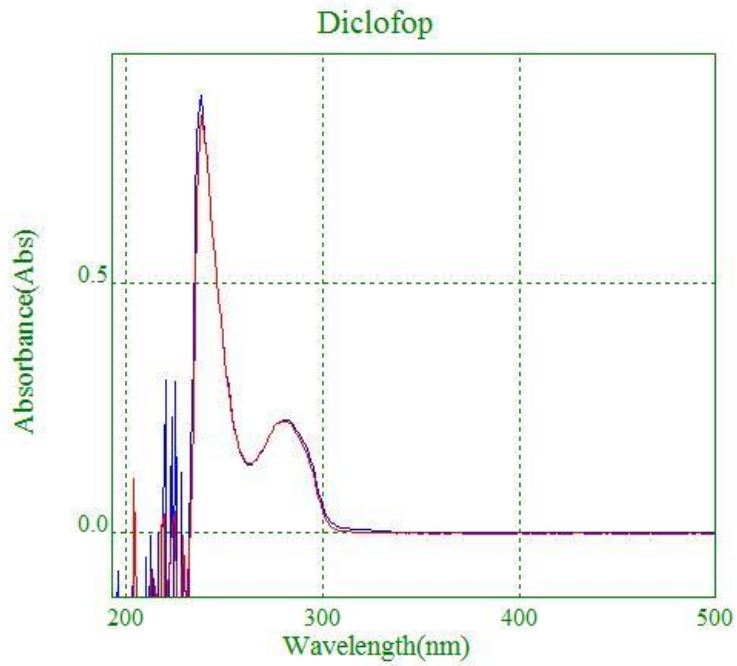
Chemical	Spectrum
Boscalid	
Diclofop	

Figure D2. UV-Vis spectroscopy results of test chemicals.

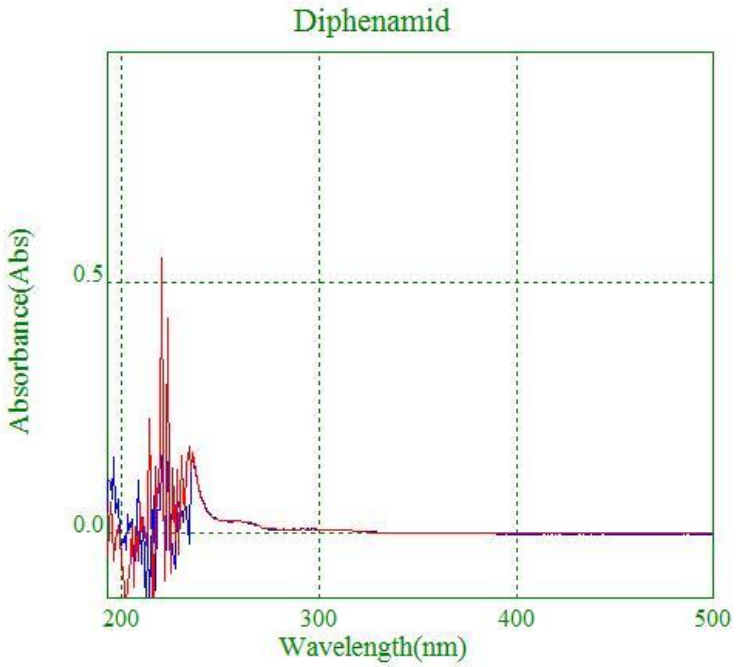
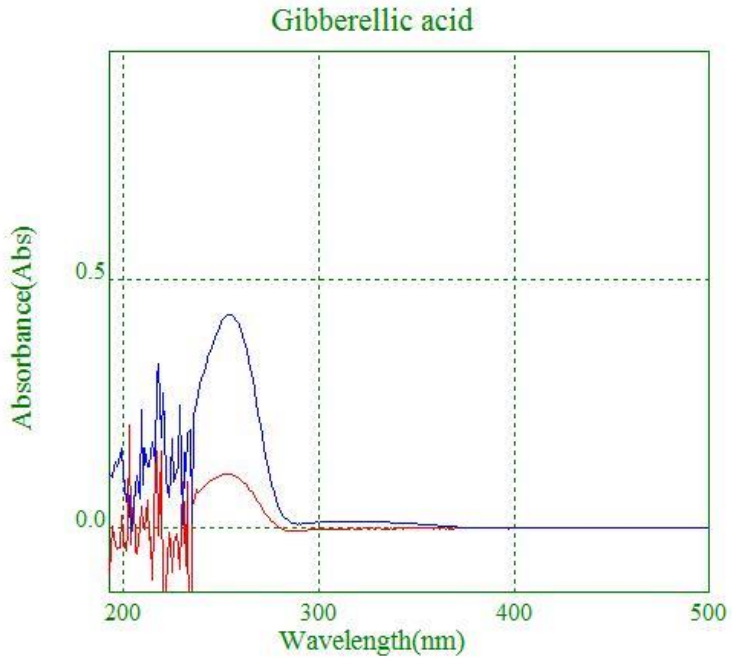
Chemical	Spectrum
Diphenamid	
Gibberellic acid	

Figure D3. UV-Vis spectroscopy results of test chemicals.

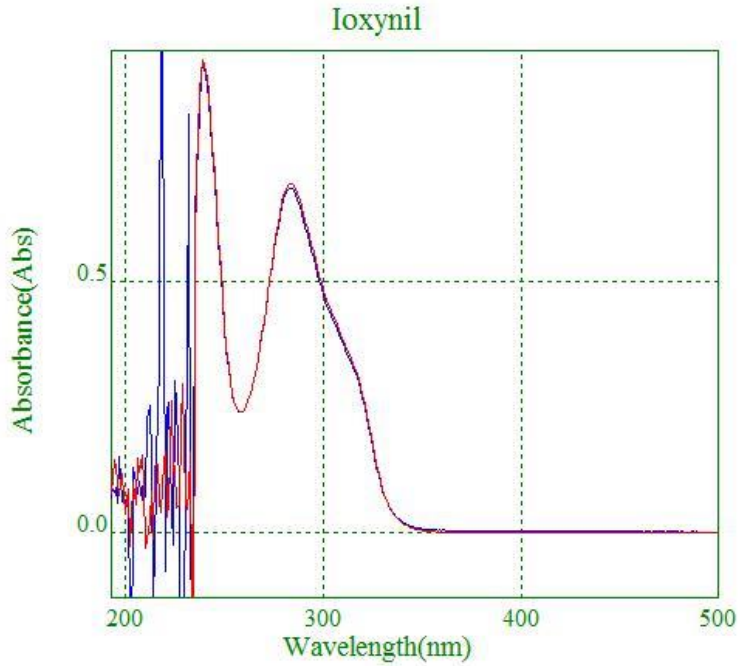
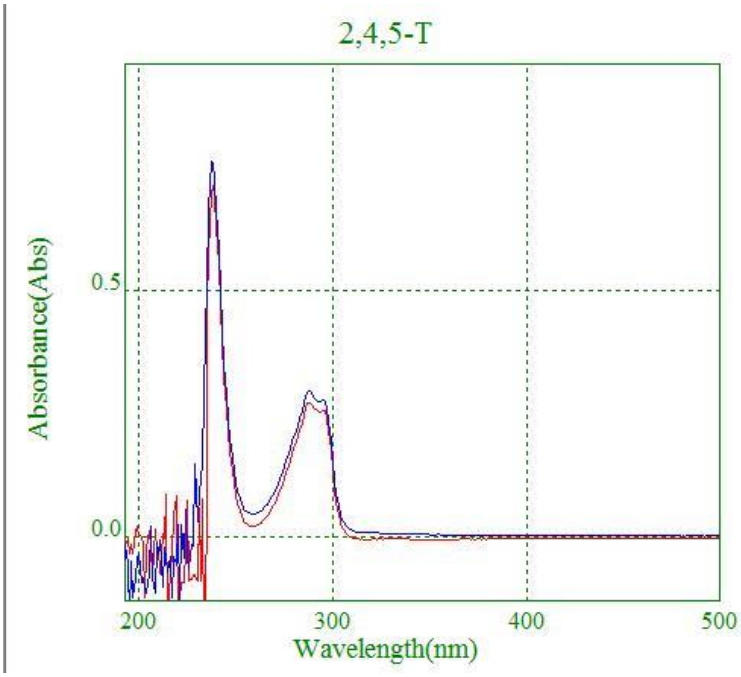
Chemical	Spectrum
Ioxynil	
2,4,5-Triphenoxyacetic acid	

Figure D4. UV-Vis spectroscopy results of test chemicals.