

RAMIPRIL, SULFAMETHOXAZOLE, SELENITE, AND SELENATE:
INDIVIDUAL AND COMBINED EFFECTS ON FRESHWATER ALGAE,
Chlorella vulgaris

by

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Chlorella vulgaris

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“Do you know how a man makes his way here? By brilliant genius or by skillful corruption. You must either cut your way through these masses of men like a cannon ball or steal among them like a plague. Men bow before the power of genius; but at first, they hate it, and try to slander it, because genius does not divide the spoil; but if genius persists, they bow before it. To sum it all up in a phrase, if they fail to smother genius in the mud, they fall on their knees and worship it”.

BALZAC

To my mother,

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ABSTRACT

RAMIPRIL, SULFAMETHOXAZOLE, SELENITE, AND SELENATE: INDIVIDUAL AND COMBINED EFFECTS ON FRESHWATER ALGAE, *Chlorella vulgaris*

Freshwater ecosystems have been exposed to various chemicals from industrial, domestic, and agricultural activities for an extended period. The existence of chemicals in water bodies poses potential adverse outcomes for aquatic organisms. In this study, considering the increasing amount and omnipresence of them in the environment, laboratory experimentations were conducted to analyze the impacts of the single and binary mixtures of ramipril (RAM), sulfamethoxazole (SMX), selenite (SeO_3^{2-}) and selenate (SeO_4^{2-}) on the growth of freshwater algae *Chlorella vulgaris* (*C. vulgaris*). At the end of the test duration, the growth of algae was inhibited by single test chemicals. The toxicity order of the chemicals was $\text{SeO}_4^{2-} > \text{SeO}_3^{2-} > \text{SMX}$. The algal growth was proved to be unaffected by RAM. When binarily mixed, all the test chemicals showed higher toxicity. The Toxic Unit (TU) method was employed to determine whether the mixed chemicals have a synergistic, antagonistic, or additive effect on algal growth. The test results of the study for the binary mixtures were compared with those obtained from predictive models Concentration Addition (CA), Independent Action (IA), and Combination Index (CI)-isobologram. The predictive power of the CA, IA, and CI methods was assessed by model deviation ratios (MDRs). Among all, CI-isobologram is the best method for the mixture toxicity prediction. The risk assessment of test chemicals was done by using predicted no-effect concentrations (PNEC) and measured environmental concentrations (MECs). Accordingly, acute-to-chronic ratios (ACR) were calculated to check whether the ACRs remained within the chemical safety threshold established for algae.

ÖZET

RAMİPRİL, SÜLFAMETOKSAZOL, SELENİT VE SELANAT: TATLI SU ALGI *Chlorella vulgaris* ÜZERİNDE TEK BAŞINA VE İKİLİ KARIŞIM HALİNDEKİ ETKİLERİ

Tatlı su ekosistemleri uzun süredir endüstriyel, evsel ve tarımsal faaliyetlerden kaynaklanan çeşitli kimyasallara maruz kalmaktadır. Kimyasalların sucul ortamlardaki varlığı, buralarda yaşayan canlılar için olumsuz sonuçlar doğurmakla birlikte yaşam alanlarını da tehdit etmektedir. Bu çalışmada, kimyasalların çevrede artan miktarı ve her yerde bulunması dikkate alınarak, ramipril (RAM), sülfametoksazol (SMX) selenit (SeO_3^{2-}) ve selenat (SeO_4^{2-})'ın tek başına ve ikili karışımlarının tatlı su alglerinden olan *Chlorella vulgaris* (*C. vulgaris*) üzerindeki etkilerini analiz etmek için laboratuvar deneyleri yapılmıştır. 96 saatlik test süreci sonunda seçilen kimyasallar alglere toksisite göstermiştir. Kimyasalların toksisite sırası $\text{SeO}_4^{2-} > \text{SeO}_3^{2-} > \text{SMX}$ olarak tespit edilmiştir. RAM ise *C. vulgaris*'e toksik etki göstermemiştir. Kimyasalların ikili kombinasyonunda, RAM dahil tüm test kimyasallarının alglere olan toksisitesi artmıştır. Toksik Birim (TU) yaklaşımı, ikili kombinasyonda kimyasalların alg büyümesi üzerinde sinerjistik, antagonistik veya katkı etkisine sahip olup olmadığını belirlemek için kullanılmıştır.

İkili karışımlar için çalışmanın deneysel sonuçları Konsantrasyon İlavesi (CA), Bağımsız Etki (IA) ve Kombinasyon İndeksi (CI)-izobologram tahmin modellerinden elde edilen sonuçlar ile karşılaştırılmıştır. CA, IA ve CI yöntemlerinin tahmin gücü ise model sapma oranları (MDR'ler) ile belirlenmiştir. Sonuç olarak, karışım halindeki kimyasalların toksisitesinin en iyi şekilde CI-izobologram metodu ile tahmin edilebileceği saptanmıştır. Kullanılan kimyasallarının risk analizi, kimyasalların tahmin edilmiş etkisiz konsantrasyonları (PNEC) ve çevrede ölçülmüş konsantrasyonları (MECs) kullanılarak yapılmıştır. Kimyasalların konsantrasyonlarının alg için belirlenmiş olan güvenli seviyede olup olmadığını değerlendirmek için akut kronik oranları (ACR) hesaplanmıştır.

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LIST OF SYMBOLS/ABBREVIATIONS

Symbols	Explanation	Units
CO ₂	Carbon dioxide	
Se	Selenium	
SeO ₃ ²⁻	Selenite Ion	
SeO ₄ ²⁻	Selenate Ion	
Na ₂ SeO ₃	Sodium Selenite	
Na ₂ SeO ₄	Sodium Selenate	
R^2	Coefficient of Determination	
μg	Microgram	
μm	Micrometer	

Abbreviations	Explanation	Units
3,5-DCP	3,5-dichlorophenol	
ACEI	Angiotensin-Converting-Enzyme Inhibitor	
ACE2	Angiotensin Converting Enzyme II	
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	
AF	Assessment Factor	
AMR	Antimicrobial Resistance	
Ang I	Angiotensin I	
Ang II	Angiotensin II	
ARB	Angiotensin-II Receptor Blockers	
ARG	Antimicrobial Resistance Gene	
CA	Concentration Addition	
ChV	Chronic Value	
CI	Combination Index	
CLP	Classification, Labelling and Packaging	
CMR	Carcinogenic, Mutagenic, and Reproductive	

Abbreviations	Explanation	Units
COVID-19	Coronavirus Disease 2019	
<i>C. pyrenoidosa</i>	<i>Chlorella pyrenoidosa</i>	
<i>C. vulgaris</i>	<i>Chlorella vulgaris</i>	
d	Day	
DMSO	Dimethyl Sulfoxide	
ECHA	European Chemicals Agency	
ECOSAR	Ecological Structure-Activity Relationship	
EDC	Endocrine Disrupting Chemical	
EU	European Union	
EPA	Environmental Protection Agency	
EC ₅₀	50% Effective Concentration	mg L ⁻¹
f _a	Fraction Affected	
GHS	Globally Harmonized System of Classification and Labelling of Chemicals	
h	Hour	
HAB	Harmful Algal Bloom	
IA	Independent Action	
IC ₂₀	20% Inhibitory Concentration	mg L ⁻¹
IC ₅₀	50% Inhibitory Concentration	mg L ⁻¹
ICp	Linear Interpolation Combined with Bootstrapping	
Ig	Growth inhibition	
LC ₅₀	50% Lethal Concentration	mg L ⁻¹
LD ₅₀	50% Lethal Dose	mg L ⁻¹
LOEC	Lowest Observed Effective Concentration	
MEC	Measured Environmental Concentration	
MDR	Model Deviation Ratio	
mg	Milligram	
MoA	Mechanism of Action	
MTD	Minimum Therapeutic Dose	

Abbreviations	Explanation	Units
<i>N. oceanica</i>	<i>Nannochloropsis oceanica</i>	
ng	Nanogram	
nm	Nanometer	
NOEC	No Observed Effective Concentration	
OECD	Organization for Economic Co-operation and Development	
OD	Optical Density	
PBT	Persistence, Bioaccumulation, and Ecotoxicology	
PEC	Predicted Environmental Concentration	
pH	Potential Hydrogen	
PhACs	Pharmaceutically Active Compounds	
PKA	Protein Kinase A	
PNEC	Predicted No-Effect Concentration	
PPCP	Pharmaceuticals and Personal Care Product	
<i>P. subcapitata</i>	<i>Pseudokirchneriella subcapitata</i>	
QSAR	Quantitative Structure-Activity Relationship	
QSTR	Quantitative Structure-Toxicity Relationship	
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus-2	
SVHCs	Substances of Very High Concern	
SMX	Sulfamethoxazole	
<i>S. capricornutum</i>	<i>Selenastrum capricornutum</i>	
<i>S. quadricauda</i>	<i>Scenedesmus quadricauda</i>	
SSRI	Selective Serotonin Reuptake Inhibitor	
SSD	Species Sensitivity Distribution	
<i>R. subcapitata</i>	<i>Raphidocelis subcapitata</i>	
RAM	Ramipril	
REACH	Registration, Evaluation, Authorization, and Restriction of Chemicals	
RF	Risk Factor	

Abbreviations	Explanation	Units
TU	Toxic Unit	
UN	United Nations	
US	United States	
UV-light	Ultraviolet Light	
WHO	World Health Organization	
WWTP	Wastewater Treatment Plant	



1. INTRODUCTION

Humanity is almost leaving behind the first quarter of the 21st century, marked by environmental problems. It is now an undoubtful fact that these problems, which have reached a crisis dimension, are caused by human activities, in other words, by anthropogenic reasons. Among these problems, chemical pollution is an alerting one. It is alerting because chemical production has increased 50-fold since 1950 globally, and by 2050, it is expected to triple from its 2010 level (European Environment Agency, 2018). Based on the market sales of chemicals and pharmaceuticals, the United Nations (UN) environment report indicates that the chemical industry is the second-largest manufacturing industry in the world (UN, 2019). Over time, most of those chemicals can potentially be toxic for living organisms and adversely affect ecosystems. Aquatic ecosystems are the ones that have been exposed to chemicals through either intentional or unintentional discharges for a long period. Therefore, assessing the effects of chemicals is necessary to safeguard the ecosystem and guarantee the well-being and survival of diverse species.

In this context, the European Union (EU) established (EC) No 1907/2006, the Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH) regulation, which entered into force in 2007, to better safeguard human and the environment from the damaging effects of chemicals (EC, 2006). The regulation is based on a precautionary principle that substances produced or imported in quantities totaling over one ton per year must be evaluated regarding risks they pose, risk management, and how to be used safely before registration to the European Chemicals Agency (ECHA). It is required to assess environmental and human hazards in terms of PBT properties, which stand for Persistence, Bioaccumulation, and Ecotoxicology, as well as CMR properties, which stand for Carcinogenic, Mutagenic, and Reproductive toxicity. Moreover, authorization is required for the substances of very high concern (SVHCs), and authorities can restrict or ban the use of some substances if the risks are unmanageable. Following the EU, REACH became a world standard in most countries, to name a few; Canada, Korea, Japan, China, and Turkey revised their chemical legislation and adopted a series of REACH-like regulations. Such amendments indicate a forthcoming increase in the demand for assessing chemicals' PBT and CMR properties. Along with REACH, the European Commission adopted an international standard called "Globally Harmonized System of Classification and Labelling of Chemicals (GHS)" by law in 2009 through the Regulation Directive EC 1272/2008 (EC, 2008). The scope of the system is harmonizing information on the hazards and toxicity of chemicals during the handling, transportation, and consumption of chemical compounds. GHS aims to determine the intrinsic hazards of chemicals, classify them regarding environmental,

physical, and human health hazards, and decide hazard communication tools by the type of hazard (toxic, harmful, dangerous, etc.) for labeling (UN Economic Commission for Europe, 2023).

Although there are some legislative regulations, there is still a threat to the well-being of living organisms due to continuous discharges of chemical compounds. Among the chemical industry products, Pharmaceuticals and Personal Care Products (PPCPs) have been considered emerging contaminants owing to their threats to the environment and adverse effects on human health over the last 20 years (Zuccato et al., 2000; Wilkinson et al., 2016; Ebele et al., 2017; Keerthanan et al., 2021; Wang et al., 2021; Kock et al., 2023). Despite the considerable research on the risks that they pose, PPCPs keep threatening natural habitats because of their ubiquitous and increasing use. If just the pharmaceuticals are considered, in 2019, it has been estimated that there are 4,000 pharmaceuticals on the global market with an annual consumption of 100,000 tons (Patel et al., 2019). Among pharmaceuticals, cardiovascular drugs, and antibiotics take attention due to their high and widespread use. As cardiovascular diseases such as stroke, hypertension and myocardial infarction are highly prevalent all around the world, the consumption of these drugs is also very high, making them among the most prescribed drugs worldwide (Mojiri et al., 2022). For instance, 24.5% of the top 200 most commonly prescribed drugs in the United States were cardiovascular drugs (Namara et al., 2019). When it comes to antibiotics, between 2000 and 2015, global antibiotic consumption has risen 65%, and by 2030, it is predicted to be 200% higher if the policies do not change (Klein et al., 2021). In 2015, 63,151 tons of antibiotics were used for livestock, and by 2030, that number is predicted to rise by 15% (Mojiri et al., 2022). Moreover, during the COVID-19 pandemic, due to misuse, the consumption of antibiotics increased globally; for instance, an estimated 75% of patients with SARS-CoV-2 receive antibiotics prescriptions, even though less than 10% of the patients are diagnosed with a secondary bacterial infection requiring antibiotics (Nandi et al., 2023). Unfortunately, from production to consumption, aquatic environments serve as terminal stations where all those pharmaceuticals reach or are intentionally discharged; hence, numerous of them can be found in surface water, lakes, groundwater, and so on (Jiang et al., 2023).

Since the therapeutic effects of pharmaceuticals come from their biological activity on organisms, their mode of action can affect non-target organisms adversely; for instance, they and their metabolites can cause both acute and chronic toxicities, which results in growth inhibition, immobilization, behavioral changes, endocrine disruption, reproduction disorders and so on (Hejna et al., 2023). However, the lack of official regulatory standards and firm legal regulations on the production, consumption, and discharge of pharmaceuticals into aquatic environments shows that aquatic organisms will continue to be exposed to pharmaceuticals for a long time (Hejna et al., 2023).

Together with pharmaceuticals, considering the huge production and consumption of other chemicals, a cocktail of chemicals threatens aquatic ecosystems all around the world.

Apart from pharmaceuticals, some micronutrients in aquatic environments have attracted attention due to their paradoxical situation of being essential or toxic. Selenium (Se) is one of the micronutrients that can be beneficial or toxic depending on the concentration (Ekumah et al., 2021). This trace element is present in many foods and dietary supplements, and in nature, it is mainly found in coal, crustal rock, and phosphate soil (Ekumah et al., 2021; Zhao et al., 2022). Se pollution in aquatic bodies mainly originate from mining for phosphates and metals, metal smelting, oil refining, fly ash from coal mining and combustion, agricultural drainage, animal husbandry on Se-containing soils, disposal of unused or expired supplements by flushing, and sewage sludge (Lemly, 2004). The global production of Se in the market is estimated at 2500–2800 tons per year, and it exists in freshwater bodies mainly as its inorganic forms of selenate (SeO_4^{2-}), and selenite (SeO_3^{2-}) when discharged without treatment (Mehdi et al., 2013).

As aforementioned, lots of chemicals coexist with other chemicals, their metabolites, and other contaminants in aquatic ecosystems. The chemical legislations and REACH-like regulations aim to safeguard human, other species, and the environment, but the global chemical inventories show that the trend is mostly on the assessment of the impact of single chemicals and rarely based on the joint effects of chemicals. Moreover, even though many EU regulations direct attention to the combined and complex effects of chemical mixtures, toxicity studies are commonly based on the assessment of single chemicals contrary to more realistic scenarios such as combined effects of chemicals (Bopp et al., 2019; Silva et al., 2022). Therefore, there is an increasing concern to assess the mixture toxicity of chemicals.

For the water quality and toxicity assessment of chemicals in aquatic environments, algae have been proposed as an indicator around the world (Stevenson, 2014). Algae as a primary producer in the food chain has a significant role in sustaining biogeochemical cycles and biodiversity. Unfortunately, like other aquatic organisms, they can simultaneously be non-target species of chemicals. Changes in algal populations may have an impact on the entire aquatic ecosystem. Algae biomass exceeding certain levels, or plenitude of specific species can significantly impact water quality. For instance, in the case of algal over productivity, water transparency decreases and oxygen consumption in bottom waters increases. Insufficient transparency can impact the movement of fish populations and the aptitude of higher-order vascular aquatic plants to grow and survive. As a result, stimulation and inhibition effects on algae are both significant environmental phenomena. Protecting

aquatic ecosystems requires an understanding of the impacts of chemicals and their mixtures on algae, given the potentially harmful implications of changes in the algal population.

In this thesis, considering their widespread use and environmental importance, the effect of single and binary mixtures of four chemicals were on freshwater algae *Chlorella vulgaris* (*C. vulgaris*) were evaluated based on the data attained from the laboratory experiments. These chemicals included two pharmaceuticals - ramipril (RAM) and sulfamethoxazole (SMX) -, as well as inorganic forms of selenium (Se) - selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}) -.

1.1. Aim of the Study

The study aimed to investigate:

- The individual effects of each chemical belonging to different classes (RAM, SMX, SeO_4^{2-} , and SeO_3^{2-}) on algal growth based on standardized OECD Test No. 201 (OECD, 2011)
- The impacts of binary mixtures of the compounds on the growth of *C. vulgaris* and the behaviors, such as antagonistic, synergistic, or additive, that each mixture exhibits in the background using Toxic Unit (TU) approach.
- The predictability of combined toxicity using Concentration Addition (CA), Independent Action (IA), Combination Index (CI)-Isobologram methods.
- Analyzing the risk of the selected chemicals using measured environmental concentrations (MEC), predicted no- effect concentration (PNEC), and acute-to-chronic ratios (ACR).

2. LITERATURE REVIEW

2.1. Pharmaceuticals

Pharmaceuticals are chemicals that have a certain biological activity generating a therapeutic effect on patients suffering from various diseases. The pharmaceutical industry has developed various types of medications, experiencing rapid growth from the past to the present. It has traveled a long way in the treatment of various diseases and paved the way for a healthy and high standard of modern life. However, the increased production, consumption, and applications of pharmaceuticals created heavy pollution in the environment and serious health effects over time. Pharmaceuticals and their active metabolites present a potential hazard, especially to the aquatic environments, because, from the manufacture through the point of disposal, pharmaceuticals from various sources migrate to water bodies at the end. The existence of pharmaceuticals in aquatic bodies can stem from both point sources and non-point sources. The point source is a single, noticeable source such as an industrial wastewater discharge pipe, a sewage treatment plant, or a hospital effluent (Li, 2014). On the other hand, non-point sources are nonidentifiable sources due to not having a specific discharge point; some examples are agricultural, rural, city, and suburban runoff, rainfall and snowmelt runs over land or through the ground, and leakage from waste treatment systems and plants (Ravikumar et al., 2022). Due to the scarcity of advanced effluent treatment systems, pharmaceuticals and personal care products (PPCPs) blend from the effluents mentioned above into the surface and groundwater, and it is known that they remain active after being released into the environment (Kümmerer, 2010). In addition to PPCPs, their active metabolites (modified or transformed form of the parent compound) are mostly found in aquatic bodies, which can be more toxic than the parent compound to aquatic biota (Celiz et al., 2009).

In an extensive review by Adeleye et al. (2022), based on the collected data, it's been reported that concentrations of common pharmaceuticals such as antibiotics and analgesics are higher in freshwater than in groundwater and marine. In the same study, the detected concentrations of (PPCPs) have been compared to several experimental toxicity studies. The results show that environmental concentrations of PPCPs in wastewater treatment plants (WWTP) effluents and other aquatic bodies were on average six-fold lower than the reported LC_{50} (half maximal lethal concentration) and EC_{50} (half maximal effective concentration) for fish, amphibians, algae, and rotifers. The review also indicates cases where the detected concentrations of pharmaceuticals in the surrounding environment are lower than those found in aquatic organisms' tissues. Finding higher concentrations of

pharmaceuticals in body tissues is a sign of the bioaccumulation factor. Due to continuous discharge and migration into aquatic systems, pharmaceuticals have been observed to be persistent in aquatic environments. As a result, aquatic biota are subjected to chronic pharmaceutical exposure (Zenker et al., 2014; Xie et al., 2017). These pharmaceuticals can accumulate in the body of aquatic organisms with an increasing concentration compared to that in the surrounding water (bioaccumulation), and consequently, their concentration can increase along a food chain (biomagnification) (Yarsan & Yipe, 2013). Thus, not just pharmaceuticals, the presence of all xenobiotic substances in aquatic environments poses a threat to all living organisms in a bottom-up food web, and also to humans consuming aquatic organisms.

On the other hand, the environmental concentration of many pharmaceuticals, such as paracetamol, ibuprofen, naproxen, carbamazepine, fluoxetine, and SMX in the aquatic environment is found considerably higher than the ecotoxicity endpoints, namely, no effect concentration (NOEC) and lowest effect concentration (LOEC) (Fekadu et al., 2019). Furthermore, the measured concentrations of pharmaceuticals, in some cases, reach the lowest clinically effective dose or even exceed therapeutic levels (Brand et al., 2015). As a result, ecotoxicological studies done in the laboratory may or may not be consistent with the measured concentrations of toxicants in the environment, and the lower concentrations of the toxicants will not cause mortality or any adverse effects to aquatic organisms directly. However, sub-lethal effects may occur even at concentrations of ng L^{-1} . For instance, exposure to $1\text{--}10 \text{ ng L}^{-1}$ of fluoxetine (Selective Serotonin Reuptake Inhibitor (SSRI), an antidepressant) can elicit phototactic responses in amphipods (*Gammarus pulex*). Similarly, seven days of exposure to propranolol (β -blocker, an antihypertensive) at concentrations of 0.3 ng L^{-1} can lead to a considerable reduction of cyclic adenosine monophosphate (cAMP) levels and protein kinase A (PKA) activities in the digestive gland in *Mytilus galloprovincialis* (Mezzelani & Regoli, 2022). Many other adverse effects for other aquatic organisms such as change in behavior, anomalies in reproduction, sex alteration, decrease in sperm motility and counts, growth inhibition, reduction in activity causing reduced feeding and body weight, etc. can occur at very low concentrations of pharmaceuticals (Nentwig, 2007; Brodin et al., 2014; Zhang et al., 2020; Mezzelani & Regoli, 2022). Thus, more than comparing absolute levels of environmental concentrations of pharmaceuticals with the experimental ecotoxicity values is required to monitor them thoroughly. In addition, considering numerous chemicals, there is a significant knowledge gap regarding combined effect scenarios. Individual pharmaceuticals or other chemicals may not show considerable adverse effects, but synergetic interactions can elicit significant toxicities (Ebele et al., 2017; Cunha et al., 2023; Li et al., 2023; Ricky & Shanthakumar, 2023).

As aforementioned, humans are also exposed to pharmaceuticals through their dietary patterns. Besides, they can be exposed to pharmaceuticals in the environment through drinking water because conventional drinking-water treatment plants are not specially designed to remove pharmaceuticals and their active metabolites from the water (Watts et al., 2007). Compared to freshwater, wastewater, and groundwater, the reported concentration of pharmaceuticals in drinking/tap water is low (mostly of ng L^{-1}) (Hawash et al., 2023). According to the World Health Organization (WHO) those detected ng L^{-1} concentrations are generally far below the minimum therapeutic dose (MTD) (or the “lowest clinically effective dose”), so severe impacts on the human body are very unlikely (WHO, 2022). However, the organization also highlighted the knowledge gap in the risk assessments of long-term exposure and potential combined effects of pharmaceuticals coexisting in drinking water. Since one of the main courses of pharmaceuticals in freshwater is drinking water, precautions will be important in terms of accessing safely managed water. Case studies from different countries in an OECD workshop show that there is a trend towards effect-based monitoring of pharmaceuticals (more specifically for the endocrine-disrupting chemicals (EDCs)) and mitigation of pharmaceutical load in freshwater (OECD, 2021).

One another threat arising from pharmaceutical pollution in the environment, which actually should be addressed under a separate heading, is antimicrobial resistance (AMR). AMR occurs when several microorganisms such as bacteria, fungi, parasites, and viruses evolve to develop some defense mechanisms to alter the antimicrobial (including antibiotics, antivirals, antifungals and antiparasitic) components and evade their effects (Aslam et al., 2018). Widespread and indiscriminate use of antimicrobials and their discharge from such as human and animal excrete, industrial and domestic effluents, etc. into the environment generate suitable conditions for pathogens to develop resistance (Biyela et al., 2004). Aquatic environments mostly serve as natural reservoirs of microbes containing AMR genes owing to being the main discharge points of wastewater, industrial, and hospital effluents (Suzuki et al., 2017). These microbes are capable of spreading and being transmitted to numerous species due to intricate interconnections throughout nature. Dispersal of AMR-resistant pathogens is a public health concern because they no longer respond to medicines used for the treatment of various diseases (WHO, 2023). WHO (2023) considers AMR as one of the top ten threats to human and animal health globally; in 2019, 4.95 million deaths were directly associated with bacterial AMR, and by 2050, it is estimated that those numbers will increase to 10 million per year (UN, 2023). From an ecological point of view, pharmaceuticals in the environment threaten natural microbial communities because they can change the structure and functions of the communities and increase the community's tolerance to them by recruiting antimicrobial resistance genes (ARGs) via mobile genetics elements (Brandt et al., 2015). Microbiome changes may have adverse effects on the balance of broader

ecosystem services such as biogeochemical cycles. Furthermore, microbial diversity may serve as a natural barrier to impede the expansion of AMR; on the other hand, using antibiotics creates a risk of reducing microbial diversity (UN, 2023).

2.1.1. Sulfamethoxazole

SMX is an antibiotic for the treatment of human and animal diseases that stem from pathogenic bacteria and protozoa (Xiong et al., 2020). It regulates the dihydrofolate synthetase enzyme and inhibits the conversion of *p*-aminobenzoic acid into dihydrofolic acid; this is where its bactericidal effect comes from (Romero et al., 2012).

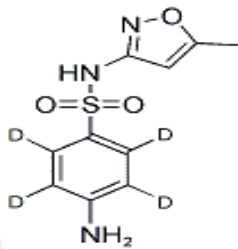
Although SMX is an old sulfonamide antibiotic, it has still been extensively used and available in most countries (Osorio et al., 2016; Straub, 2016; Li et al., 2021; Zhang et al., 2022). For instance, among the other antibiotics, SMX is the most predominant in the African aquatic samples, and Liaodong Peninsula, China (Fekadu et al., 2019; Xie et al., 2019). According to Katsikaros and Chrysikopoulos (2021), among the reported 286 pharmaceuticals present in the lakes around the world, caffeine, carbamazepine, and SMX were found in almost every continent. Sulfonamides are still widely used in treating human and animal diseases owing to their broad bactericidal spectrum, and compared to other antibiotics, it is argued that their relatively low cost contributes to being used extensively (Fekadu et al., 2019; Iftikhar et al., 2023). However, besides being consumed in large amounts, SMX predominance is attributed to its resistance to hydrolysis and low biodegradation in water bodies (Chen et al., 2023; Xie et al., 2019). The maximum concentrations of SMX detected in surface water of several countries range from 0.1 ng L⁻¹ to 142,600 ng L⁻¹ (Kumar et al., 2019; Xie et al., 2019; Kovalakova et al., 2020; Katsikaros & Chrysikopoulos, 2021; Adeleye et al., 2022; Duan et al., 2022). The half-lives of SMX under light, non-sterile, and sterile water-sediment systems in the WWTP have been reported to be 5 h 35 d, 4.90 ± 0.2 d, and 47.3 ± 0.1 d, respectively (Straub, 2016). Hence, SMX attenuation in aquatic environments is immensely affected by several parameters, such as the presence or absence of light, humic acid, methanol occurrence, and microbial diversity (Alexy et al., 2004; Benotti & Brownawell, 2009; Xu et al., 2011).

The physicochemical properties of SMX are given in Table 2.1. Based on those properties, the predicted distribution of sulfamethoxazole in air, soil, water and sediment is estimated by the EPI Suite Level III Fugacity Model.

To date, a considerable number of studies have been done on the ecotoxicological impacts of SMX on aquatic organisms by both individual and mixture toxicity assessments (Liu et al., 2014; Melvin et al., 2014; Xiong et al., 2019a; Drzymała & Kalka, 2020; Narciso et al., 2023; Pinos-Vélez et al., 2023). The results of the studies show that in most cases, SMX shows more toxicity with the presence of another chemical compared to the single form. As an example, 96 h EC₅₀ values of SMX, erythromycin (ERY), and their mixture for *Raphidocelis subcapitata* (a green microalga) are 0.49 mg L⁻¹, 0.044 mg L⁻¹, and 0.06 mg L⁻¹, respectively (Zhang et al., 2021). Xiong et al. (2019a) calculated 96 h EC₅₀ values of sulfamethazine, SMX, and their mixture for *Scenedesmus obliquus* (a green microalga) and the results were 1.23 mg L⁻¹, 0.12 mg L⁻¹, and 0.15 mg L⁻¹, respectively. Apart from growth inhibition in microalgae, SMX changes carbon source metabolization of the bacterial part of the periphyton community and has led to a general decrease in carbon source utilization (Johansson et al., 2014). Also, the environmental concentrations of SMX, such as 0.1 µg L⁻¹, have evoked inflammation among young zebrafish and have adversely affected the host immune defense mechanism (Liu et al., 2020).

In this study, single and mixture toxicity of SMX with RAM, SeO₃²⁻, and SeO₄²⁻ on the green freshwater alga *C. vulgaris*, which does not exist in the literature, were investigated. On the other hand, Baran et al. (2006) reported a 48 h EC₅₀ of 6.2×10^{-3} mM (1.57 µg L⁻¹) for *C. vulgaris* exposed to SMX. Borecka et al. (2016) obtained 0.98 and 1.51 mg L⁻¹ EC₅₀ values for *C. vulgaris* exposed to SMX at the end of the 48 h and 72 h test periods, respectively. Dong et al. (2020) observed nearly 40% growth inhibition (IC₄₀) at a concentration of 200 mg L⁻¹ at the end of 72 h for SMX using *C. vulgaris* as a test organism. Aderemi et al. (2021) reported an EC₅₀ of 47.9 mg L⁻¹ at the end of 96-h for *C. vulgaris* exposed to SMX, while Zhang, T. et al. (2023) detected a close EC₅₀ value of 48.36 mg L⁻¹ for it.

Table 2.1. Estimated chemical properties and environmental mass amounts of SMX, retrieved from the Danish (Q)SAR Database (2021)

Chemical Properties of Sulfamethoxazole	
Chemical class	Antibiotic
Cas No	723-46-6
Chemical Formula	C ₁₀ H ₁₁ N ₃ O ₃ S
Molecular Weight	253.28
2D structure	
Mode of Action	A structural analog of para-aminobenzoic acid
Log K _{ow} [*]	0.48
Log K _{oc} ^{**}	2.4121
Log K _{oa} ^{***}	11.298
BCF [kg L ⁻¹ wet-wt.] [‡]	3.162
pK _a ^{‡‡}	9
Water Solubility from K _{ow} [mg L ⁻¹]	3942
Vapor Pressure [Pa, 25 °C]	1.733E-005
Removal in WW Treatment [%]	1.88
Predicted Mass Amount [%]	
Air	9.31E-006
Water	13.8
Soil	86
Sediment	0.203

^{*}: Logarithm of octanol-water partition coefficient; ^{**}: Logarithm of soil adsorption coefficient of organic compounds; ^{***}: Logarithm of octanol-air partition coefficient; [‡]: Bioconcentration factor; ^{‡‡}: Negative logarithm of the acid dissociation constant (K_a).

2.1.2. Ramipril

RAM is a dicarboxylate-containing angiotensin-converting-enzyme inhibitor (ACEI) that has been used for the treatment of hypertension, to decrease the risk of heart failure and death following myocardial infarction, and other ischemic situations (Warner & Perry, 2002; Ajmera et al., 2021).

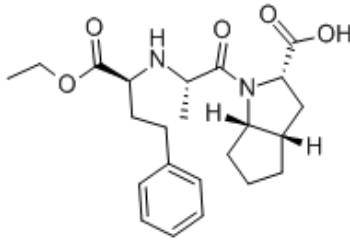
ACEIs and angiotensin-II receptor blockers (ARBs) were controversial medications mainly at the beginning of the COVID-19 pandemic due to the dichotomy between having inhibitory or facilitatory effects against the dissemination of SARS-CoV-2 in the human body (Leclézio et al., 2020; Onweni et al., 2020; Yang et al., 2020; Najafi et al., 2022). The research done during the pandemic showed that SARS-CoV-2 viruses are very likely to introduce the body by binding angiotensin converting enzyme II (ACE2), which catalyzes the transformation of angiotensin II (Ang II) to angiotensin I (Ang I) (Kannan et al., 2020; Najafi et al., 2022). When SARS-CoV-2 viruses enter the body via binding ACE2 enzymes, they downregulate ACE2 and reduce the available amount of it (Najafi et al., 2022). As a result of the decreased amount of ACE2, hydrolysis of Ang II to Ang I diminishes, and the increased Ang II levels induce high blood pressure, and lung injury (Najafi et al., 2022; Onweni et al., 2020). For the treatment of the hypertensive effects owing to the viral entrance to the body, ACEIs and ARBs could be used to block Ang II signals and to promote the expression of the decreased ACE2 (Yang et al., 2020). In that regard, clinical trials were conducted on animals, and the results show that ACEIs have a regulatory effect on the distracted functions of ACE2 (Leclézio et al., 2020). Additionally, ACEIs have the potential to relieve severe symptoms of respiratory infections and pneumonia (Sun et al., 2020). However, increased levels of ACE2 by ACEIs could have increased pathogenic inflammation at the same time by allowing more viral entry into the body, especially into the lungs, heart, and kidneys. (Singh et al., 2020; Yang et al., 2020). Concerns arose around using ACEIs and ARBs, which may increase viral entry into the body, and may be a threat, especially for patients with cardiovascular diseases. Clinical studies conducted to address these concerns have shown no evidence indicating the relationship between their use and increased viral entry (Mancia et al., 2020; Reynolds et al., 2020; Yang et al., 2020). Neither ACEIs were used as a primary medication to treat SARS-CoV-2 infections, nor were enough studies done to prove their pathogenicity. Most medical authorities said that patients who use ACEIs could continue to take these drugs since they have the potential to treat SARS-CoV-2-induced lung injury (Onweni et al., 2020; Sriram & Insel, 2020; Ratha et al., 2021). All these discussions caught the attention of environmental scientists because the probability of the consumption of ACEIs for alleviating lung injury symptoms due to SARS-CoV-2 would increase the environmental concentrations of ACEIs. In such a situation, scientists should have focused on the adverse effects and environmental risk assessments of ACEIs and ARBs, especially those lacking ecotoxicological data.

In 2019, 4 million RAM had been prescribed in the US alone, and an estimated 854,000 patients had been treated with it (Regulska et al., 2023). 3,930 kg of RAM had been sold in Poland in 2013, and 144 ng L⁻¹ of RAM was predicted to be in Vistula River, Poland (Giebułtowicz et al., 2016). Kot-Wasik et al. (2016) measured the environmental concentration of pharmaceuticals in the WWTP

effluents of the city of Gdansk and reported an average of $0.06 \mu\text{g L}^{-1}$ for RAM. 92.6 ng L^{-1} and 114.3 ng L^{-1} of RAM were detected in WWTPs effluents and influents, respectively, in Dresden, Germany (Gurke et al., 2015). Cardoso et al. (2014) collected data from the sewage treatment plant (STP) effluents of various regions across the world and reported an average $100 \mu\text{g L}^{-1}$ concentration for RAM. In addition, an average of 102 ng L^{-1} of RAM was found in WWTPs based on the data taken from the 16 WWTPs across Canada (Ghoshdastidar et al., 2015). To make a parenthesis here, actually, RAM was among the top 20 prescribed pharmaceuticals in Canada in 2008, but its environmental concentrations are considerably low compared to other cardiovascular drugs (Ghoshdastidar et al., 2015). RAM in itself is an inert compound, but within the body, it quickly metabolizes and transforms to ramiprilat; as a result, only 2% of RAM is excreted unchanged from the body, and the unmetabolized RAM is mainly excreted in the form of ramiprilat (Van Griensven et al., 1995). Thus, one reason why RAM is being detected in low amounts in the environment can be attributed to its pharmacokinetics. In a more recent study, environmental concentrations of RAM had been detected less than $6.05 \mu\text{g L}^{-1}$ in Canadian STPs. (Greenham et al., 2019). Lastly, an average of 2.5 ng L^{-1} RAM was found in drinking water in Poland in 2016 (Hawash et al., 2023). The physicochemical properties of RAM are given in Table 2.2. Based on those properties, the predicted distribution of RAM in air, soil, water and sediment is estimated by the EPI Suite Level III Fugacity Model.

Low or high, a sufficient amount of pharmaceuticals in the tissue of aquatic organisms can affect them adversely. Although present in deficient concentrations in the environment, RAM has been reported among the 15 most concerning pharmaceuticals in the Sava River in Croatia due to being among the most detected pharmaceuticals in the plasma of wild fish (Malev et al., 2020). The study indicates that together with other pharmaceutically active compounds (PhACs), RAM is potentially hazardous to wild fish due to possible additive or synergetic interactions and continuous exposure. 96 h dose-dependent exposure of zebrafish (*Danio rerio*) to RAM caused a reduction in the heartbeat rate at concentrations ranging from 0.1 to $10 \mu\text{g L}^{-1}$, while the mixture of amiodarone, RAM, simvastatin, and verapamil at concentrations of 4 to $40 \mu\text{g L}^{-1}$ 100% of mortality (Matijevic et al., 2023). Another study on the toxicity of RAM to zebrafish revealed that $25 \mu\text{g L}^{-1}$ of RAM decreases locomotor activity (Crilly et al., 2022). Further studies need to be done on the toxicity of RAM to aquatic organisms because a considerable knowledge gap exists in the literature. In addition, as seen in the case of zebrafish, the simultaneous coexistence of pharmaceuticals in aquatic environments, besides sub-lethal effects, can cause even mortality. This study aims to close this gap by evaluating the impacts of both single and binary mixtures of RAM on *C. vulgaris* with dose-dependent exposures.

Table 2.2. Estimated chemical properties and environmental mass amounts of RAM, retrieved from the Danish (Q)SAR Database (2021).

Chemical properties of Ramipril	
Chemical Class	Non-sulphydryl ACE inhibitor
Cas No	87333-19-5
Chemical Formula	C ₂₃ H ₃₂ N ₂ O ₅
Molecular Weight	416.52
2D structure	
Mode of Action	Inhibit the actions of angiotensin converting enzyme
Log K _{ow} [*]	3.32
Log K _{oc} ^{**}	2.6665
Log K _{OA} ^{***}	17.172
BCF [kg L ⁻¹ wet-wt.] [†]	3.162
pK _a ^{‡‡}	3.1
Water Solubility from K _{ow} [mg L ⁻¹]	3.551
Vapor Pressure [Pa, 25 °C]	5.066E-009
Removal in WW Treatment [%]	9.54
Predicted Mass Amount [%]	
Air	8.03E-008
Water	12.6
Soil	87
Sediment	0.325

^{*}: Logarithm of octanol-water partition coefficient; ^{**}: Logarithm of soil adsorption coefficient of organic compounds; ^{***}: Logarithm of octanol-air partition coefficient; [†]: Bioconcentration factor; ^{‡‡}: Negative logarithm of the acid dissociation constant (K_a).

2.2. Selenium

Se is a natural trace element that belongs to Group VIA of the Periodic Table of Elements. It is a semi-metal (or metalloid) element that exhibits an intermediate characteristic between metal and non-metals. Most Se compounds show similar trends to sulfur (S) compounds regarding bond energies, ionization potentials, and oxidation states (Mehdi et al., 2013; Naga-Jyothi et al., 2020). However, unlike the S, Se has a lower oxygen capacity, so not too many oxides are known. The mass numbers of six isotopes of Se found in nature are 74, 76, 77, 78, 80, and 82, and their relative abundances are 0.87%, 9.02%, 7.58%, 23.52%, 49.82%, and 9.19%, respectively (Cappelletti et al., 2021). In nature,

Se exists in organic or inorganic forms and the four oxidation states of it are -2 , 0 , $+4$, and $+6$. The inorganic forms (SeO_3^{-2}), and (SeO_4^{-2}) are commonly present in water bodies, whereas the organic forms, selenomethionine, and selenocysteine exist in plant-based foods (Barceloux, 1999). The physical properties of selenium are given in Table 2.3.

Table 2.3. The physical properties of elemental Se (Naga-Jyothi et al., 2020).

Electron configuration	(Ar) $3d^{10} 4s^2$ $4p^4$
Atomic number	34
Atomic weight	78.97
Atomic radius, Å	1.90
Covalent radius, Å	1.18
Ionic radius, Å	1.98
Melting point	220.8°C
Boiling point	685°C
Electron affinity, eV	-4.21
Electronegativity	2.55
State at 25°C	Solid
Specific heat, $\text{J kg}^{-1} \text{K}^{-1}$	321

Se is an essential micronutrient for various organisms, including humans, animals, and microorganisms (Vu et al., 2019; Zou et al., 2020). Humans generally take Se from plant-based food with varying concentrations depending on the type and composition of soil and geographical location (Kieliszek & Lipinski, 2020). Interestingly, depending on the concentration, Se can be beneficial or harmful to the organisms (Gan et al., 2019; Zou et al., 2020). Some properties of Se such as being antioxidative, supporting thyroid hormone synthesis, preventing DNA damage by neutralizing free radicals, being protective against stomach and colon cancer, and slowing aging make it a significant micronutrient (Sun et al., 2014; Özakman et al., 2018). A Se-deficient diet can increase the spread of malignant tumors and viral infections (Kieliszek & Lipinski, 2020). In so much, Se deficiency leads to a lethal disease known as Keshan disease (Dinh et al., 2018; Kieliszek & Lipinski, 2020). A recent study published during the COVID-19 pandemic argues that Se supplementation, especially in the form of sodium selenite (Na_2SeO_3), inhibits penetration of SARS-CoV-2 viruses to cell membrane, and makes them ineffective by oxidizing thiol groups in the virus protein (Kieliszek & Lipinski, 2020). In addition, Moghaddam et al. (2020) indicated that Se-deficient people infected with SARS-CoV-2 exhibited more severe symptoms compared to those with a better Se status. Thus, Se

supplementation can be an adjuvant treatment option for the recovery of COVID-19 patients (Moghaddam et al., 2020). A daily intake of 50-100 μg of Se increases cellular immune response and modulates viral respiratory infections (Jayawardena et al., 2020). On the other hand, excessive concentrations, even slightly above the required amounts, cause the formation of reactive oxygen species (ROS), DNA damage, selenosis, and cancer in humans (Sun et al., 2014; Vu et al., 2019). In aquatic environments, dissolved Se induces reproductive failure and mortality, the formation of ROS in fish, DNA damage in fish liver, and equilibrium breakdown in the aquatic system (Gobi et al., 2018; Gan et al., 2019). The toxic concentrations of Se compounds depend on the organisms and their form (Saçan & Özkovalak, 2007). Additionally, Se causes significant bioaccumulation in fish tissue (Wright & Welbourn, 2002; Gobi et al., 2018), algae (Saçan & Balcıoğlu, 2001), and the food web (Hamilton, 2004; Lemly, 2004).

Se exists naturally on earth, but its mobilization has been fastened in the environment by coal and mining processes, coal combustion, oil refining, agricultural drainage, and many other reasons mentioned before. The estimated annual production of Se is around 150-750, 930, 730, 150, and 500 tons in the USA, China, Japan, Russia, and Germany, respectively, and these countries are the leading producers of Se in the world (Cappelletti et al., 2021). The reported Se concentration in saltwater is higher than the overall Se levels in natural water, which vary from 0.1 to 400 $\mu\text{g L}^{-1}$ (Akl et al., 2006). Mehdi et al. (2013) reported higher concentrations of Se in saltwater ranging from 0.04 to 0.12 g L^{-1} . Se concentration in groundwater varies from 0.50 to 1 $\mu\text{g L}^{-1}$ across the world. The concentrations of 11.6, and 0.01 $\mu\text{g L}^{-1}$ were detected in the surface water of Canada, and Norway, respectively (Hu et al., 2009; Reimann et al., 2018). Levels of Se in natural waters are usually lower than 1 $\mu\text{g L}^{-1}$, in soils, range from 0.005 to 3.5 mg kg^{-1} (average value is 0.33 mg kg^{-1} worldwide), and in most urban air, vary from 0.1 to 10 ng m^{-3} (El-Ramady et al., 2015). However, in Se-extravagant regions, the Se content in soil can extend to 79.08 mg kg^{-1} with a mean of 27.81 (Dinh et al., 2018). Se is widely distributed in nature, and concentrations of Se compounds above the homeostatic levels required for organisms threaten them and lead to environmental problems. That's why, the US EPA (Environmental Protection Agency) limits the maximum concentration of Se in surface water to 5.0 $\mu\text{g L}^{-1}$ under which the aquatic life can survive without any harm (US EPA, 2014, as cited in Ullah et al., 2021). However, in most cases, higher concentrations of chemicals have been found in the tissue of organisms than in the nearby environment (Adeleye et al., 2022). Thus, without considering other parameters such as the bioaccumulation factor, setting absolute levels of environmental concentrations may result in the underestimation of the threats that they pose in aquatic ecosystems.

2.2.1. Selenite and Selenate

As aforementioned, Se can be present in nature in five oxidation states and each form of it exhibits different characteristics in terms of bioavailability, mobility, and toxicity (Li et al., 2020). For instance, in oxygen-deficient acidic soils, Se mainly exists in the elemental or other insoluble forms (selenium sulfide, heavy metal selenides), which are less mobile so less bioavailable for the organisms (Gad & Pham, 2014). In most of the water bodies with a pH of 6 to 8 and in redox conditions, Se is widely present in the forms of (SeO_3^{2-}) and (SeO_4^{2-}) (Lemly, 2004; Winkel et al., 2012). Thus, selenium oxyanions, SeO_3^{2-} and SeO_4^{2-} are two predominant inorganic forms of Se in water bodies, and highly soluble compared to elemental Se making them more bioavailable for aquatic organisms (Gad & Pham, 2014). As a result, besides elemental and organic forms, the toxic effect of Se in aquatic environments on several species originates from the presence of its water-soluble forms (Guimarães et al., 2021). In most cases, much attention is given to the toxicity of organic selenium compounds owing to their high potential to bioaccumulate in tissues (Okonji et al., 2021). However, SeO_3^{2-} and SeO_4^{2-} uptake by the aquatic organism can be metabolized to organic Se and bioaccumulate in the tissues, and subsequently, cause selenium toxicity (Mechora et al., 2015). In addition to being metabolized to the organic forms, inorganic forms of Se are toxic to organisms at specific concentrations above the homeostatic requirements (Okonji et al., 2021). For instance, Li et al. (2020) reported that *Lemna minuta* (a species of duckweed) gave a rapid response to 5.0 mg L^{-1} of SeO_4^{2-} and stopped the growth of the root, whereas it happens after 3 d incubation when exposed to the same concentrations of SeO_3^{2-} . At low concentrations, inorganic forms of Se taken up by algae increase algal growth (stimulation) and photosynthesis and protect them against oxidative damage; on the other side, high amounts of Se may be toxic and cause growth inhibition, malformed proteins, and chloroplast alterations (Geoffroy et al., 2007; Sun et al., 2014; Vallentine et al., 2014). Sun et al. (2014) reported that selenium concentrations below 75 mg L^{-1} increase the growth of *C. vulgaris* and inhibit ROS, whereas concentration above 100 mg L^{-1} creates opposite effects on *C. vulgaris*.

In this study, single and mixture toxicity of SeO_3^{2-} and SeO_4^{2-} with RAM, and SMX on the green freshwater alga *C. vulgaris*, were investigated. The toxic effect of these two inorganic forms of Se on *C. vulgaris* is calculated regarding the Se content of SeO_3^{2-} and SeO_4^{2-} salts. 96 h toxicity assessment of SeO_3^{2-} and SeO_4^{2-} on *C. vulgaris* was investigated in this thesis to use data attained from one laboratory for mixture toxicity evaluations.

2.3. Algae As a Test Organism for Ecotoxicology Studies

It is now a very well-known fact that aquatic ecosystems have been exposed to intentional or accidental discharges of numerous chemicals. Inevitably, this unpleasant chemical pollution is affecting water quality and the stability of aquatic ecosystems. Physical and chemical assessments, conventional methods such as chemical oxygen demand and suspended solids can supply quantitative data on the pollution but may not be enough or reflect environmental stress and adverse effects on the aquatic organisms (Saçan & Balcioğlu, 2006; Omar, 2010). To sufficiently monitor the aquatic environments and for risk assessment purposes, ecotoxicological studies address bioindicators that are sensitive to environmental changes, able to exhibit indirect biotic effects of toxic compounds, and respond quickly to environmental disruptions (Omar, 2010; Rotondo et al., 2023).

As primary producers, microalgae are ecologically essential species in aquatic ecosystems. Algae are significant bioindicators for the biological monitoring of contaminated water bodies since a change in the algal biomass directly affects higher trophic organisms. A change in algal biomass or toxic effects of microalgae can disrupt the stability of aquatic ecosystems. However, despite their importance, the toxicity assessments are mostly done with higher trophic organisms (Xin et al., 2021). In fact, for ecotoxicological studies, microalgae are ideal due to many reasons such as their rapid growth rates, short life cycles, immediate response to toxic compounds in samples, strong sensitivity, ease of obtaining and maintenance in cultures (Lee et al., 2023; Rotondo et al., 2023).

Microalgae toxicity testing aims to observe the effects of tested chemical substances on algal growth. Along the test duration, the response of the algae containing great numbers of cells is continuous and distributed due to being not affected by each single organism response (Christensen et al., 2009). The main idea of the algal growth inhibition test is to expose exponentially growing microalgae to increasing concentrations of test chemicals over a predetermined period. The response of algae to toxicants is evaluated with a reduction in algal growth; therefore, growth inhibition is the endpoint of the assay. Two response variables, average specific growth rate, and yield calculations, are proposed by OECD (OECD, 2011) to assess the effects of the test substances. The average specific growth rate is defined as calculating the logarithmic increase of the biomass per day over a specific time period, while yield is expressed as the biomass at the end of the test period minus the biomass at beginning the test. For calculating algal biomass, parameters such as optical density, fluorescence, cell counts, cell volume, etc., are preferably used. The guideline notes that these two response variables are different from each other and not comparable. In this study, we used the specific growth rate of algae to assess growth inhibition as suggested by the guideline.

Bioassays for growth inhibition can be done on many species of microalgae and also cyanobacteria, as indicated by OECD (2011) guideline. The guideline (OECD, 2011) recommends some strains of green algae for the toxicity assessments but does not restrain the test organism to them. In this thesis, *C. vulgaris* is selected as the test organism owing to being extensively and naturally present in freshwater ecosystems, rapid growth within a short duration, and easy to obtain and maintain in cultures (Safi et al., 2014; Geiger, 2014; Tugcu et al., 2017; Ru et al., 2020).

2.3.1. *Chlorella vulgaris*

Chlorella vulgaris is a green freshwater alga that was discovered in 1890 by Martinus Willem Beijerinck and cultured for the first time as a pure eukaryotic microalga (Ru et al., 2020). It is a spherical microscopic cell with 2-10 μm which can be in the form of single cells or is capable of constructing colonies up to a maximum of 64 cells (Safi et al., 2014; Ru et al., 2020) (Figure 2.1). This freshwater alga can grow under autotrophic, mixotrophic, and heterotrophic conditions (Tomaselli, 2003). In optimal conditions, a single non-motile *C. vulgaris* cell can multiply asexually and rapidly within 24 hours by autosporeulation (Safi et al., 2014).

Considering the algal taxa, the majority of species of green algae in marine, freshwater, and terrestrial environments belong to the Chlorophyta (Leliaert et al., 2012). Those widely distributed species are one of the main oxygen sources on earth and have a significant role in biogeochemical cycles (Lobus, 2022). As a primary producer, they are the key organisms of the food chains. In addition, algae have a high capacity to catch CO_2 ; for instance, one kilogram of dry algae biomass can capture nearly 1.8 kg of CO_2 (Lobus, 2022). That's why the effects of toxicants on algae need to be evaluated to sustain a balance of aquatic ecosystems and to protect aquatic organisms.

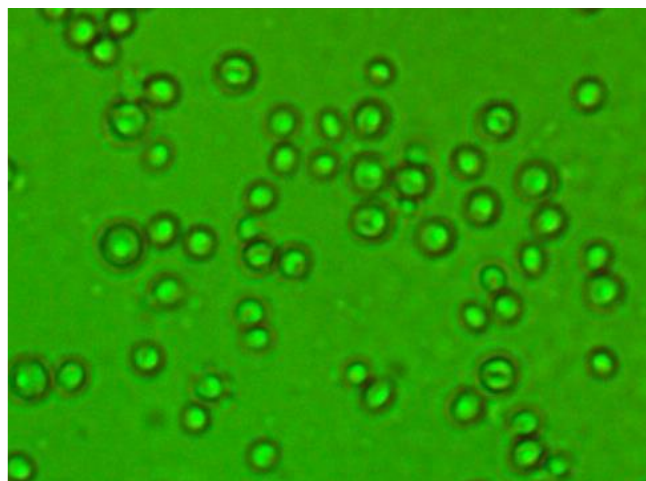


Figure 2.1. The microscopic view of *C. vulgaris* (Gökçe, 2019)

2.4. Toxicity Assays for The Single Chemicals

Chemicals exist in the environment in different amounts, and from the late 19th century onwards, it was accepted that the environmental hazards they pose highly depend on the concentrations (Calabrese, 2014a). In that regard, the dose-response or concentration-response relationship took its place at the center of toxicological studies, and it is now essential in terms of the environmental risk assessments of chemicals. A dose-response relationship shows the change in the effect (quantitatively measurable biological response) of an increasing range of concentrations of chemicals on a test organism in a determined period. The response of the test organism to certain amounts of the chemicals is measured in distinct periods, and the relationships are represented by dose-response curves. The most commonly used concentration-response/dose-response curves are EC₅₀ and LD₅₀ curves. EC₅₀ is the half-maximum effective concentration stands for the concentration of a substance at which 50% of its maximal effect is observed, whereas LD₅₀ is the median lethal dose required to reach 50% mortality from toxicity, compared to the control group (organisms or cultures not exposed to the toxicants).

In algal toxicity testing, one of the responses of algae exposed to toxicants is growth inhibition (Ig). The relationship between the increased amount of chemicals and the growth inhibition is shown in a concentration-response curve. An increasing concentration of a toxicant can lead to successive increments in effect, whereas no response can be observed until a certain concentration gives a significant response, which is the threshold dose. Moreover, low concentrations of a toxicant can stimulate for instance growth, while high concentrations inhibit; this phenomenon is known as hormesis, and details are given under the headline 'Hormetic Effect.' To sum up, all these properties result in a non-linear or threshold concentration-response relationship, which mostly appears as U-shaped, inverted U-shaped, or J-shaped curves (Moffett, 2007).

The statistical analysis of inhibitory concentrations (IC) reducing the response variable (specific growth rate of algal cultures) can be done with regression models such as probit, logit, and linear interpolation. To obtain a concentration-response curve, the percent inhibitions obtained at the end of specific periods are fitted against corresponding concentrations.

2.5. Toxicity Assays for The Chemical Mixtures

It is indisputable that several toxicants from different classes are present in aquatic ecosystems. Determining the joint effects of the contaminants on aquatic organisms merely by looking at the single

toxicity testing is challenging because together, these contaminants may perform different and unpredictable mode of action (MoA), which refers to the behavior of a chemical upon interaction with the exposed organism. The possible interactions between chemicals may affect each other's individual MoA, or those chemicals can work in a non-interactive manner and do not affect each other's MoA (Bopp et al., 2019). The influence of one chemical on the MoA of another chemical can result in a more significant or lower toxic effect than expected (additive). Thus, the impact of the combined chemicals can be less than additive, called antagonistic interaction, or greater than expected, which is a synergistic interaction (Bopp et al., 2019).

In order to assess the characteristics of the chemical interactions, some approaches and models have been proposed from the past to now. Loewe and Muischnek (1926, as cited in Sigurnjak-Bureš et al., 2021) introduced a frequently used and rather old graphical method called isobologram. An isobole is a curve usually appearing in a line with rectangular coordinates, where the axes show the concentration of each mixture component. Thus, isobole is a line of additivity wherein components contribute to toxicity proportionally to their concentration and are used to differentiate between synergistic and antagonistic interactions (Tallarida, 2006). If the data points fall below the line of additivity, the interaction between the chemicals is synergistic; if they lie above the line, the interaction is antagonistic. Figure 2.2 shows the isobologram representation of the interactions.

Since the isobolograms are applicable exclusively to binary mixtures, Chou and Talalay (1984) developed a F_a -CI or CI plot, a plot of combination index (CI) on the y-axis as a function of effect levels (f_a) on the x-axis. The algorithm behind the F_a -CI plot is akin to those for constructing isobole, but the former is effect-dependent, whereas the latter is dose-dependent.

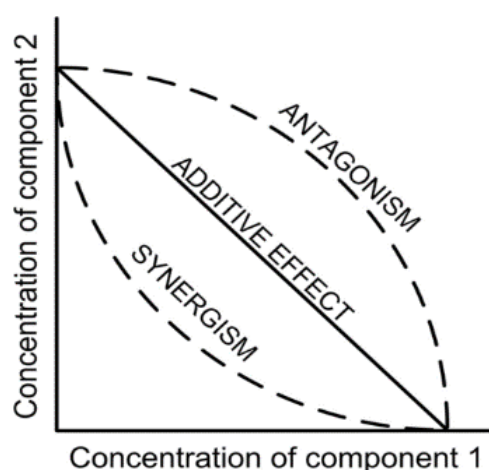


Figure 2.2. The isobologram representation of additive, synergistic, and antagonistic interactions (Sigurnjak-Bureš et al., 2021).

CA, and IA are the two most known and conventional models applied as a default approach to decide whether a mixture of chemicals produces antagonistic, synergistic, or additive effects (Syberg et al., 2009; Geiger et al., 2016; Sigurnjak-Bureš et al., 2021). Both concepts allow the prediction of non-interactive joint effects of the chemicals based on the known impacts of the each single chemical in the mixture. The CA model assumes that mixture components have similar MoA, while IA depends on the assumption of dissimilar MoA. The predictive power of both models has been evaluated in many *in vitro* assessments (González-Pleiter et al., 2013; Geiger et al., 2016; Thrupp et al., 2018; Xiong et al., 2019a; Silva et al., 2021; Domínguez-García et al., 2023).

2.5.1. Concentration Addition (CA)

CA assumes that the extent of the response to a chemical combination can be forecasted by summing the doses or concentrations of components with similar effects after adjusting individual variations in efficiencies (Hernández et al., 2017; Sigurnjak-Bureš et al., 2021). The CA or additive model assumes that components of a mixture with a similar mode of action do not interfere with each other; they present together as diluted forms. When two substances with similar MoA are simultaneously combined, an additive effect occurs, meaning the toxic effect of the mixture equals the sum of the impact of the single toxicants. Thus, the overall impact of a mixture on an organism does not change if the sum of toxic units does not. The CA model assumes that the toxicity of a substance has a constant characteristic; the only difference between individual and combined cases is being exposed to one compound and the mixture, respectively (Backhaus et al., 2004). Any deviation from the additive effect can result in synergism, more than additive, or antagonism, less than additive.

The model fundamentally employs the concept of TU, formulated as in equation 2.1 (Backhaus et al., 2004). TU is the ratio between the concentration of the substance i in the mixture, (c_i), and the equivalent effect concentration of the same substance that independently produces a preselected inhibition level in the exposed test organism.

$$TU_i = \sum_i \frac{c_i}{EC_{x,i}} \quad (2.1)$$

Here, x refers to the concentration causing a distinct response level. The sum of the individual TUs (equation 2.2) indicates the toxic unit of a mixture (Backhaus et al., 2004).

$$TU_{MIX} = \sum_{i=1}^n TU_i = \frac{c_{mix}}{EC_{x,mix}} \quad (2.2)$$

where c_{mix} is the total concentration of the chemicals in the mixture and $EC_{x,mix}$ is the CA-predicted effective concentration containing equal proportions of the same chemicals causing x% effect on the test organism. For the predictive purpose of the CA model, equation 2.2. has been revised to equation 2.3. to calculate the predicted EC_x values of the mixture ($EC_{x,mix}$) (Backhaus et al., 2004):

$$\frac{c_{mix}}{\sum_{i=1}^n \frac{c_i}{EC_{x,i}}} = \frac{\sum_{i=1}^n c_i}{\sum_{i=1}^n \frac{c_i}{EC_{x,i}}} = EC_{x,mix} \quad (2.3)$$

In the situation of equal concentrations of c_{mix} and $EC_{x,mix}$, TU_{MIX} equals one, where an additive effect occurs (Eq 2.4). This is also the famous formulation of CA defined by Berenbaum (1985)

$$\sum_{i=1}^n \frac{c_i}{EC_{x,i}} = 1 \quad (2.4)$$

2.5.2. Independent Action (IA)

Independent action, contrary to the CA model, assumes that components of a mixture target different sides of an organism, in other words, have different MoAs upon interaction with the organism (Backhaus et al., 2004). According to the model, since the compounds in a mixture target different sides of an organism, the toxic action of each is independent of the other. Cedergreen et al. (2008) argue that the model has a questionable theoretical background when thinking of the highly organized and integrated body system of living organisms. Assuming the action of a compound on a target receptor does not affect to some degree the response of the other receptors interacting with the concurrently present second compound has raised questions regarding the applicability of the IA model. Moreover, the IA model was first employed for quantal response assessments (i.e., mortality, survival), but the graded response analysis, the response graded regarding the intensity or the dose of the applied toxicant, was not feasible to the theoretical assumption of IA (Backhaus et al., 2004; Cedergreen et al., 2008). However, the model successfully predicted toxic effect of chemicals with different MoAs that coexist simultaneously in a test medium on the algae and bacteria (Cedergreen et al., 2008). Thus, while uncertainties in the theoretical background of the model remain, besides the quantal responses, the model has also been widely used in many toxicology and ecotoxicology studies

to investigate the sublethal effects of chemicals on organisms, given its good predictions. The algorithm of the model is formulated as in equation 2.5 below (Backhaus et al., 2004):

$$EC_{mix} = 1 - \prod_{i=1}^n [1 - E_{c_i}] \quad (2.5)$$

in which, E_{c_i} stands for the effect that a certain concentration of compound i in the mixture provokes when applied to the test organism individually. EC_{mix} is the expected effect of the total concentrations of the components of the mixture on the test organism.

2.5.3. Combination Index (CI)-Isobologram

Combination index (CI)-isobologram, known as the Fa -CI plot of Chou-Talalay, or CI plot stems from a unified theory of to assess the effect of a drug combination and provide comparison with the individual components of it (Chou, 2010). The method was first used in pharmacological studies to investigate drug interactions, but later, its application extended to toxicology and ecotoxicology studies (Rodea-Palomares et al., 2010). The model developers argue that the dose-dependent effect of the chemicals highly depends on and is governed by mass-action law, instead of MoA. Therefore, unlike the predictive models based on the MoA of chemicals, the CI method considers the mass-action characteristics of the components of a mixture. In that regard, Chou (1976) developed the median effect equation (Eq 2.6), derived from a unified theory encompassing many equations from a wide range of disciplines:

$$\frac{f_a}{f_u} = \left(\frac{D}{D_m} \right)^m \quad (2.6)$$

here, D stands for the dose or concentration of a compound, while f_a indicates the fraction or the percentage influenced by the applied D (% of inhibition), and f_u is the fraction unaffected (so, $f_u = 1 - f_a$). D_m denotes the median-effect dose analogous to IC_{50} , EC_{50} , or LD_{50} , and m serves as the coefficient shaping the dose-effect curve; in the cases of $m = 1$, $m > 1$, and $m < 1$ hyperbolic, sigmoidal, and negative sigmoidal curves occur, respectively.

Equation 2.6 was rearranged as in equation 2.7 below (Chou, 1976) allowing to make dose and effect as interchangeable parameters, where each can be easily calculated by D_m and m values. From

the logarithm of equation 2.6, the median-effect plot can be constructed ($x = \log(D)$, $y = \log(f_a/f_u)$), where m is the slope and $\log(D_m)$ is the x -intercept (Rodea-Palomares et al., 2010).

$$D = D_m[f_a/(1 - f_a)]^{1/m} \quad (2.7)$$

Subsequently, the median effect equation for individual compounds was enlarged to multiple compounds and their mixture. When two chemicals present together and undergo consecutive dilutions, the mixture of them acts as a third drug for a concentration-effect/dose-effect relationship (Chou, 2006). The result is a new logarithmic equation; $x = \log[(D)_1 + (D)_2]$, $y = \log[(f_a)_{1,2}/(f_u)_{1,2}]$, giving $m_{1,2}$, $(D_m)_{1,2}$. Based on all successive median effect equations, Chou and Talalay (1984) developed a CI calculating each effect level and their combinations for quantifying synergism and antagonism between the combined chemicals, where CI takes the values CI=1, <1, and >1 stand for additive, synergistic, and antagonistic effect, respectively. Equation 2.8 shows the first version of the index set for binary mixture of the compounds (Chou, 2006):

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} = \frac{(D)_1}{(D_m)_1[f_a/(1 - f_a)]^{1/m_1}} + \frac{(D)_2}{(D_m)_2[f_a/(1 - f_a)]^{1/m_2}} \quad (2.8)$$

For more than two drug combinations, a general equation (equation 2.9) was developed for the CI model, which is the ultimate equation of the CI method (Chou, 2006):

$${}_x^n(CI) = \sum_{j=1}^n \frac{(D)_j}{(D_x)_j} = \sum_{j=1}^n \frac{(D_x)_{(1-n)} \{[D]_j / \sum_1^n [D]\}}{(D_m)_{(j)} \left\{ (f_{ax})_j / [1 - (f_{ax})_j] \right\}^{1/m_j}} \quad (2.9)$$

here, ${}_x^n(CI)$ indicates the CI for n drugs at $x\%$ inhibition. $(D_x)_{(1-n)}$ is the total dose of the drugs in a mixture causing $x\%$ of inhibition, whereas $[D]_j / \sum_1^n [D]$ is the proportion of the dose of each component exerting $x\%$ inhibition in the mixture. $(D_m)_{(j)} \left\{ (f_{ax})_j / [1 - (f_{ax})_j] \right\}^{1/m_j}$ is the dose of each drug causing $x\%$ inhibition as to be exposed individually, where D_m is the median-effect dose as IC_{50} , EC_{50} , f_{ax} fractional inhibition at $x\%$ inhibition, m is the slope of the plot (Chou, 2006).

The algorithms behind the F_a -CI plot of Chou-Talalay, a plot of CI on the y-axis as a function of effect level (f_a) on the x-axis, or CI-isobologram similar to those behind the isobologram method, but the former is effect-dependent, the latter is dose-dependent (Chou, 2006). One limitation of the isobologram, which makes the F_a -CI plot preferable, is that it can assess only the binary mixtures of

the compounds. In addition, classical isobolograms are unsuitable for showing all effect levels of the drug due to the overlapping data points (Chou, 2006). However, a F_a -CI plot can be depicted at all f_a levels concurrently with more than two combined chemicals (Chou, 2006). The distinctive property of the model is that it is independent of the MoA of the compounds in a mixture, as in the conventional methods, e.g., CA and IA. It is argued that in most cases, the synergistic and antagonistic interactions between the chemical substances are independent of MoA (Rodea-Palomares et al., 2010). Furthermore, knowing the pharmacological MoA of a chemical may not provide sufficient data in terms of the toxic MoA of it (Chou, 2006; Rodea-Palomares et al., 2010).

2.6. Hormesis

Hormesis is a term describing two opposing situations in which a chemical has a stimulatory impact at low concentration/doses and an inhibitory impact at high concentration/doses. A dose or concentration-response relationship can be categorized as hormetic in the case of a stimulatory response to at least one exposure level, i.e., higher growth than the control, with a subsequent decrease in the response (Agathokleous et al., 2019). The result is a biphasic concentration-response relationship. Stimulatory responses have been encountered frequently in growth parameters such as individual growth, increased production of eggs in insects and crustaceans, and a prolonged life span; however, hormesis can be seen in physiological and biochemical situations, to name a few, gene expression, enzyme activity, and tumor occurrence (Cedergreen et al., 2005). Hormesis is independent of organisms, in other words, can be depicted in all organisms from animals and plants to microorganisms (Cedergreen et al., 2005).

The hormetic response is a common phenomenon encountered in most of the algal growth inhibition tests (Fu et al., 2019; Mao et al., 2021; Gomaa et al., 2023). A reasonable increase in algal growth benefits the aquatic ecosystems, considering the role of algae mentioned before. However, the health of all the ecosystems is built on balance. Understanding the impacts of chemicals and their mixtures on algal populations requires comprehensive analyses. Both the excessive decrease and increase in algal growth can be equally detrimental to aquatic life. The overgrowth of algae can end up with harmful algal bloom (HAB) (Hallegraeff, 2003). The toxins released after HAB can lead to severe impacts on other organisms (including humans) such as “altering cellular process, mortalities, respiratory or digestive tract problems, memory loss, seizures, lesions and skin irritation, as well as losses of coastal resources” (Sellner et al., 2003). For instance, *Ceratium* blooms can result in odor (Ewerts et al., 2018), lead to oxygen depletion, and decrease water transparency (Meichtry de Zaburlin et al., 2016). Deterioration in the overall quality of water and physical habitats (such as

disrupted visual predation and hindered movement), in available food, and changes in the composition of the species are the other adverse impacts of algal bloom (Coffey et al., 2019). Therefore, a dichotomous identification can be done for the quantity of algal populations owing to the delicate balance between being detrimental and beneficial.

2.7. Risk Assessment

From the manufacture through the point of disposal, a chemical poses a risk to the environment. Calculations should be made to account for these effects on each part of the environment. A newly produced chemical's environmental concentration cannot be tested, hence, modeling, extrapolating, or predicting its abundance in each environmental segment is necessary. For risk assessment objectives, low-toxic-effect metrics including NOEC and LOEC, and PNEC, and MEC or predicted environmental concentration (PEC) are evaluated. The term "LOEC value" refers to the lowest determined concentration that considerably deviates from the control. The tested concentration that is below the LOEC is referred to as the NOEC. Lower concentrations are thought to have no harmful effects on aquatic species at the PNEC level. Tugcu & Saçan (2018) provided a summary on the use of low-toxic-effect metrics for risk analysis. ACR is computed using the low-toxic-effect concentrations (NOEC, LOEC) and the acute median effective concentration (EC_{50}). ACR is determined either using the EC_{50} and maximum acceptable toxicant concentration (MATC) values or the EC_{50} and NOEC values. The geometric mean of the chemicals' NOEC and LOEC values, also referred to as MATC, is referred to as chronic value (ChV) in the ECOSAR model (ECOSAR, 2012). Additionally, according to the US Environmental Protection Agency (EPA), ACR is the ratio of acute LC_{50}/EC_{50} to chronic MATC value or regression derived EC_{20} value (Hoff et al., 2010). The ratio of the EC_{50} value to the NOEC value is another indication of ACR in the EU Technical Guidance Document (TGD) (EU TGD, 2003).

The PEC or MEC to PNEC ratio, also known as the risk factor (RF), can be used to depict the possible adverse impacts of a compound. A possible risk exists when the ratio is greater than 1. This ratio can be used to understand the risk scale roughly. The concern levels can be divided into three categories: low concern (1–10), new data needed (10–100), and major concern (above 100). If the ratio is greater than 100, action must be taken to lower the environmental risk.

3. MATERIALS AND METHODS

3.1. Test Organism

Algal growth inhibition tests were performed in batch cultures applying OECD test protocol No. 201 with *C. vulgaris* (OECD, 2011). The algae's parental cultures, *C. vulgaris* strain (CCAP 211/11B), were taken from the Ecotoxicology and Chemometrics Lab of the Institute of Environmental Sciences at Boğaziçi University in Istanbul, Turkey. The strain was acquired from the Culture Collection of Algae and Protozoa (CCAP, The Scottish Association for Marine Science, Scottish Marine Institute, Dunbeg, Argyll, UK) and has been cultivated in appropriate laboratory conditions over an extended period.

3.2. Test Chemicals

The pharmaceuticals SMX and RAM, and Se compounds (anhydrous sodium selenite (Na_2SeO_3), and sodium selenate (Na_2SeO_4)) were purchased from Sigma Aldrich. The chemicals had a purity of $\geq 98\%$; thus, no further purification was done, and all of them were stored at room temperature in the dark.

The stock solutions of Na_2SeO_3 and Na_2SeO_4 were prepared using de-ionized water. The reported aqueous solubility of SMX and RAM are $< 1 \text{ mg mL}^{-1}$, so these chemicals are poorly soluble in water (Shafiq et al, 2007; Garner et al, 2012; de Moura Oliveira et al., 2019). The estimated solubilities of RAM and SMX from the *n*-octanol-water partition coefficient (K_{ow}), which were given in Table 2.1 and Table 2.2, respectively, are much higher compared to the experimental. Since both RAM and SMX are sparingly soluble in water, stock solutions of them were prepared in the organic solvent dimethyl sulfoxide (DMSO) ($> 99.9\%$ purity) purchased from Merck and Co. Inc. Therefore, for the growth inhibition analyses of RAM and SMX an additional control group with a maximum of 0.1% (v/v) DMSO was prepared regarding OECD test protocol No. 201 to observe the growth of the algae with and without DMSO (OECD, 2011). To see the difference between them, a t-test was performed ($p\text{-value} \leq 0.05$). All the stock solutions were sterile-filtered with $0.2 \mu\text{m}$ filters to eliminate prospective impurities and microorganisms.

3.3. Algal Growth Inhibition Assay

Algal growth inhibition assays were conducted in batch cultures with the test organism *C. vulgaris* following the standard procedures (OECD, 2011). The detailed descriptions of conditions for the algal bioassay are provided by Ertürk & Saçan (2013). The growth medium to harvest the algae was a Bold Basal Medium with 3-fold Nitrogen and Vitamins (3N BBM + V) (Bilanovic et al., 2009). The chemicals and their concentrations in 3N BBM + V are given in Table 3.1. The inoculation of the medium with the algae was done four days before the start of the test to ensure the algae were in their exponential growth phase. The initial biomass of the inoculum contained approximately 1.5×10^5 cells mL⁻¹, which has to be sufficiently low to eliminate the possibility of algal growth inhibition due to nutrient depletion.

Table 3.1. Bold Basal Medium with 3-fold Nitrogen and Vitamins for *C. vulgaris* (Bilanovic et al., 2009).

Constituents	Concentration (mg L ⁻¹)
NaNO ₃	750
CaCl ₂ .2H ₂ O	25
MgSO ₄ .7H ₂ O	75
K ₂ HPO ₄ .3H ₂ O	75
KH ₂ PO ₄	175
NaCl	25
Na ₂ EDTA	4.5
FeCl ₃ .6H ₂ O	0.584
MnCl ₂ .4H ₂ O	0.246
ZnCl ₂	0.03
CoCl ₂ .6H ₂ O	0.012
Na ₂ MoO ₄ .2H ₂ O	0.024
Vitamin B1	1.2
Vitamin B2	0.01

Following the end of the lag phase, 100 mL of the harvested algal culture was poured into 500 mL borosilicate Erlenmeyer flasks for the test. Before conducting the final inhibition test, preliminary range finding experiments were performed to identify the concentration range where growth inhibition potentially occurs. After defining the concentration range, the ultimate growth inhibition tests were employed by exposing the algae cultures in the log phase to five different concentrations of the test chemicals. For each concentration, three replications were set up. From the stock solutions of the chemicals, 50 mL of test solutions containing the test chemical and the growth medium of the

algae were prepared in 100 mL borosilicate Erlenmeyer flasks for each concentration to control the solvent. Algae harvesting and all the growth inhibition tests were done in a laminar air-flow cabinet which was sterilized before. All the materials used in algae harvesting and experiments are also exposed to UV light before the tests are performed. Figure 3.1 shows the test conditions before the experiments.

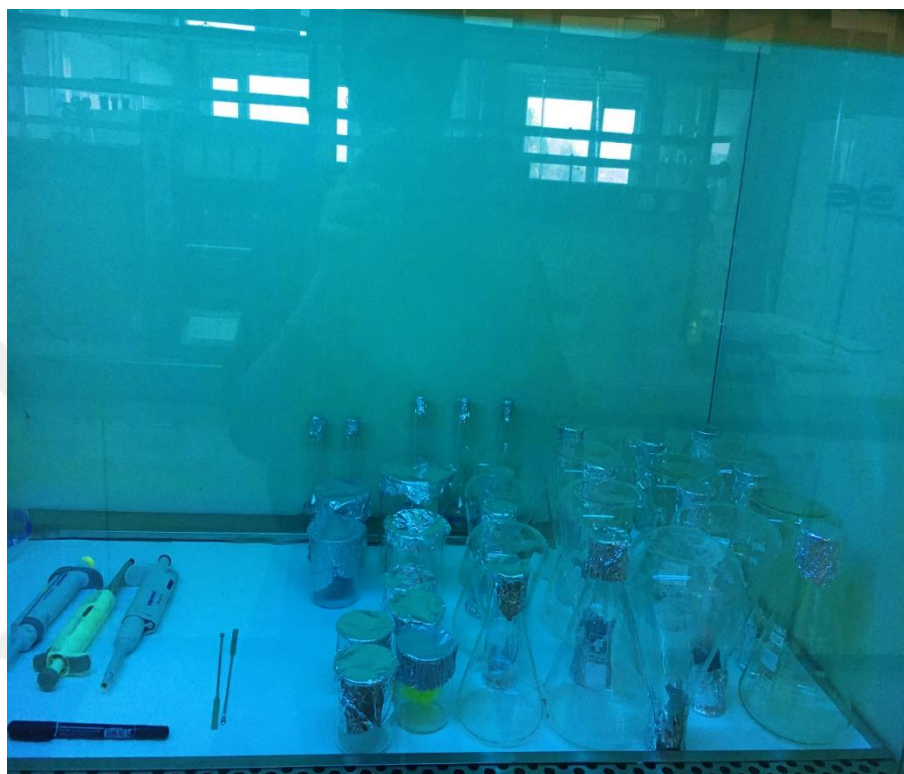


Figure 3.1. The laminar air flow cabinet containing test materials exposed the UV light before the experiment.

Sterile equipment was used for every experiment to avoid contamination. All the glassware was washed with phosphate-free detergent (Sigma Aldrich), then rinsed 2-3 times with tap water. After this process, they were cleaned with hexane to remove any organic residue, then, washed with tap water 2-3 times. To remove any inorganic residue, they were subsequently rinsed with diluted nitric acid (10% v/v), then washed with tap water 2-3 times, and finally rinsed with distilled water. All the cleaned glassware was stored in the oven (WiseVen, Daihan Scientific; Korea) for three hours at 180°C before the experiments. Other materials that were used in the experiment, such as plastic pipette tips, algal media and filter papers, were autoclaved for 15 minutes at 120 °C under 2 atm. pressure. The vitamins were sterile-filtered using 0.2 µm filters before being added to the growth medium for algae.

Both the incubation of the inoculum in the test medium and the test cultures were kept in a temperature-controlled growth chamber at 24 ± 0.5 °C with continuous lighting with 60 µmol.

photons $\text{m}^{-2}\text{s}^{-1}$ light intensity in the test duration. The growth chamber containing test cultures and the inoculum in the test medium are shown in Figure 3.2 and Figure 3.3, respectively. All the glassware containing test cultures were shaken by hand daily.



Figure 3.2. Test cultures in the light and temperature-controlled growth chamber under continuous illumination



Figure 3.3. Incubation of the inoculum in the test medium done in the temperature-controlled growth chamber under continuous illumination

3.3.1. Control Test for Algal Culture

Since the pH in the control vessel shouldn't vary by more than 0.5 between the first and last day of the experiment (OECD, 2011), the pH control of bioassays was done at the beginning and the end of the test duration (96 h) with a pH-meter (WTW pH 330i). According to OECD test protocol No. 201, 16-factor rise in algal growth for controls after 72 h of incubation is advised to ensure the test's validity (OECD, 2011). 3,5-dichlorophenol (3,5-DCP) was employed as a reference toxicant in addition to the above-mentioned test acceptability criteria to see if algae had the same response. Chemical concentrations of the test solutions were controlled by UV-Vis spectrophotometer on the

first and last day of each experiment to check whether there was a decrease in the concentration of chemicals due to volatilization, photolysis in the test conditions, or adsorption to the vessels.

3.3.2. Monitoring Algal Growth

The algal growth was observed daily during the 96 h test period by measuring the optical density at 680 nm (OD₆₈₀) using a UV-Vis spectrophotometer (Lasany, LI-2804). The selected wavelength is where the maximum chlorophyll absorption can be observed for *C. vulgaris*. The growth inhibition caused by the test chemicals was determined regarding the growth of the algae not exposed to the chemical (control group). The graph showing a linear relationship between algal cell numbers and corresponding OD₆₈₀ for *C. vulgaris* has been set by Tugcu et al. (2017) using the same laboratory conditions and materials as in this study. The plot was redrawn by Gökçe (2019) with the addition of new experimental results for *C. vulgaris* (Figure 3.4). In this study, instead of counting algal cells in a hemocytometer, the plot optimized by Gökçe (2019) was used to determine the cell numbers by corresponding absorbance value.

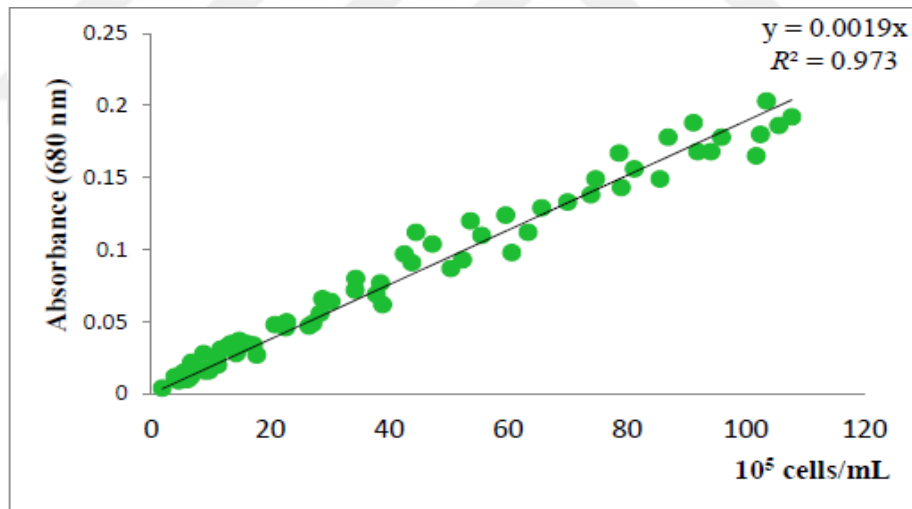


Figure 3.4. The plot of absorbance (680 nm) versus algal biomass (cells/mL) for *C. vulgaris* (Gökçe, 2019)

3.3.3. Calculation of Algal Growth

The average specific growth rate for a predetermined period was computed for each vessel of the controls and treatments on (day⁻¹) of the chemical logarithmic increase in the biomass as in the following equation (3.1):

$$\mu_{i-j} = \frac{\ln X_j - \ln X_i}{t_j - t_i} \quad (3.1)$$

where:

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

X_i : concentration of biomass at time i (cells mL^{-1});

X_j : concentration of biomass at time j (cells mL^{-1}).

The percent growth inhibition calculation based on the average specific growth rate of the test solutions and solvent controls is given in equation (3.2):

$$\%I_r = \frac{\mu_c - \mu_T}{\mu_c} \times 100 \quad (3.2)$$

where:

$\%I_r$: percent inhibition in average specific growth rate;

μ_c : mean value for average specific growth rate (μ) in the control group;

μ_T : average specific growth rate for the treatment replicate.

3.3.4. Statistical Analysis of Single and Mixture Toxicity

The toxicity of each chemical and their mixture to the algae were assessed by statistical analysis of the specific growth rate (SGR) suggested by OECD test protocol No. 201 (OECD, 2011). The inhibitory concentrations that decrease SGR by 50% (IC_{50}) and 20% (IC_{20}) were calculated with 95% confidence intervals using the statistical methods in ToxCalc™ Software (ver. 5.0.32, Tidepool Scientific, CA, USA) at the end of the 48 h, 72 h, 96 h. To determine whether the toxicity calculation model has a notable impact on the data, different statistical methods such as linear interpolation and maximum likelihood including probit, logit, Weibull, and angular were used to compute IC values. Normality and homogeneity of data were analyzed, and LOEC and NOEC values were calculated based on the statistical analysis suggested by parametric tests USEPA (2002) and applied in ToxCalc™ software (version 5.0.32).

A flow diagram of the applied statistical methodology is depicted in Figure 3.5. For the calculation of NOEC and LOEC values of each chemical hypothesis testing is applied. A hypothesis test approach is performed to define a statistically notable difference between the SGR of the treated cultures and the control. When the input data meets the assumptions of normality and homogeneity of variance, a parametric multiple comparison test, i.e., Dunnett's test is applied. Thus, Dunnett's test requires normality and homogeneity of variances, which are tested by the Shapiro-Wilks test and

Bartlett's test, respectively. If the data fails to meet the requirements of the normality and homogeneity test, NOEC and LOEC are calculated by non-parametric tests, such as Steel's Many One Rank test or Wilcoxon Rank Sum Test.

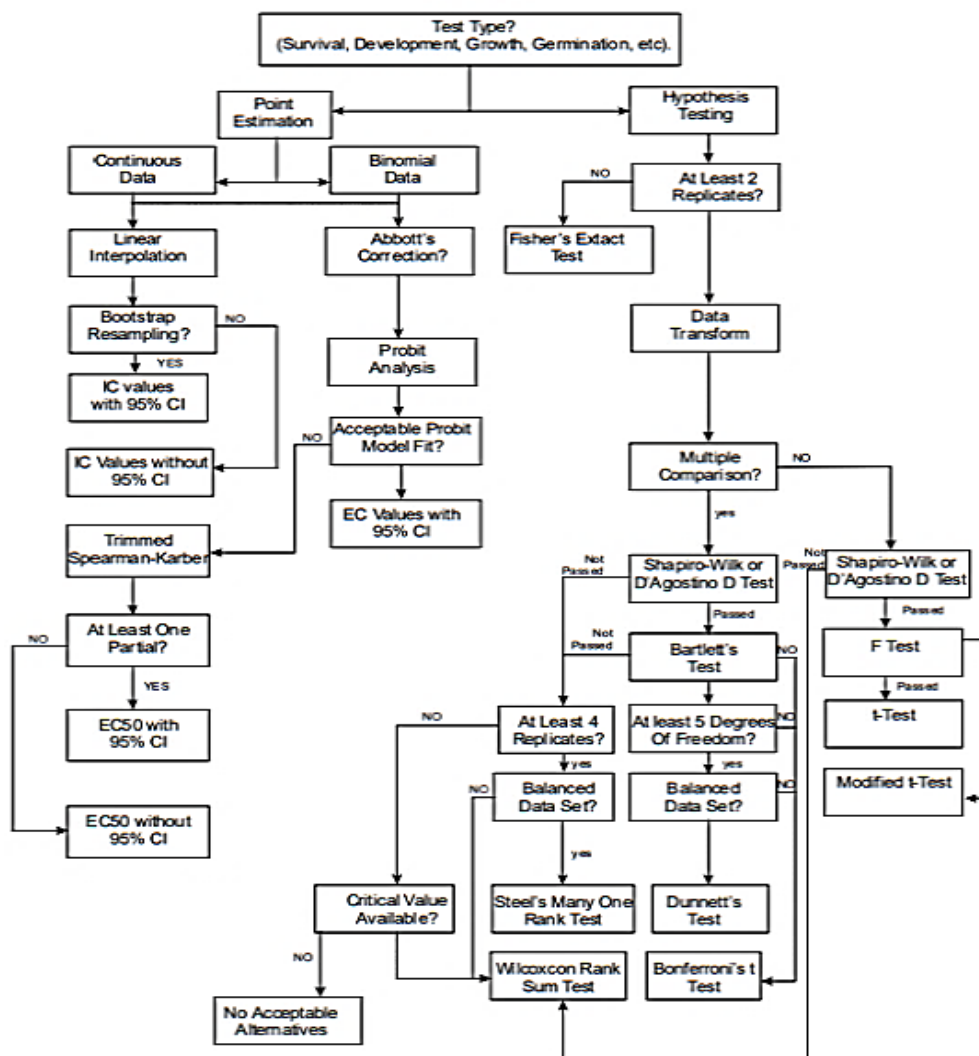


Figure 3.5. Flow diagram of statistical methodologies accepted by the USEPA (2002)

TU method (Marking, 1985) which is based on the concentration additivity was employed to evaluate the combined effects of the used chemicals, where binary mixture tests were performed using proportions of the respective IC_{50} s ($=1$ toxic unit (TU)). In this study, binary mixture experiments were done with the following concentrations: $\Sigma 0.25$ TU, $\Sigma 0.5$ TU, $\Sigma 1$ TU, $\Sigma 2$ TU, and $\Sigma 4$ TU. A 50% growth inhibition in a mixture is assumed to take place at 1 TU, which is simply an additive effect where each single component of the mixture presents at a concentration of half of their single IC_{50} values. If 50% growth inhibition takes place at less than 1 TU, the mixture shows synergism; if it is observed at more than 1 TU, the mixture depicts antagonism. On the other hand, according to Lin et al. (2004), the mixture is considered additive if a 50% effect occurs at 1 TU (± 0.2). If the 50%

effect corresponds to a TU lower than 0.8 TU, there is a synergistic interaction, and if it is higher than 1.2 TU, the interaction is supposed to be antagonistic (Lin et al., 2004).

The mixture toxicity values predicted by CA, IA, and CI-isobologram methods were compared to the experimental data to assess their predictability power. The results obtained from CA, IA, and CI-isobologram methods were analyzed by calculating the model deviation ratios (MDRs). If the computed MDR was within a factor of two or if the concentration response curve for the experimental data's 95% confidence interval overlapped with the abovementioned models, additive effects were thought to be possible ($0.5 \leq \text{MDR} \leq 2$). The MDR, which is the ratio of the experimental concentration divided by the anticipated effect concentration, was utilized to evaluate the deviance throughout the whole dataset. MDR values of 1 implies that the models used have the best possible fit.

MDR values < 1 show that the predictive model underestimated mixture toxicity, meaning the experimentally tested joint effect is more toxic than predicted. MDR scores of > 1 show that the mixture's toxicity has been overestimated.

3.4. Risk Assessment

PNEC, and PECs were evaluated as part of the regulatory assessment of chemicals. The lowest NOEC value or short-term (acute) LC_{50} value is divided by a pertinent assessment factor to determine the PNEC. In the case of fresh water, the PNEC is determined by taking the lowest EC_{50} , LC_{50} , or NOEC value and dividing it by the proper assessment factor for each aquatic organism. In other words, the data's PNEC value, which represents the organism with the greatest sensitivity, is selected. For PNEC calculation utilizing freshwater data, common assessment factors (AF) are provided in Table 3.2.

According to the availability of the toxicity data, either equation (3.3) or (3.4) given below can be used to determine the PNEC value:

$$\text{PNEC} = \text{EC}_{50} \text{ or } \text{LC}_{50} / \text{AF} \quad (3.3)$$

$$\text{PNEC} = \text{"NOEC"} / \text{"AF"} \quad (3.4)$$

The RF was calculated by dividing the PEC, or if available MEC, by the PNEC value, as shown in the following equation (3.5), one can compute the risk factor.

$$RF = PEC / PNEC \quad (3.5)$$

ACR can be determined in two ways using the following equations:

1) The U.S. Environmental Protection Agency (EPA) set a formulation for ACR as shown in the following equations (3.6, and 3.7) (Hoff et al., 2010):

$$MATC = \sqrt{NOEC/LOEC} \quad (3.6)$$

$$ACR_{MATC} = \frac{LC_{50}}{MATC} \quad (3.7)$$

2) The EU Technical Guidance Document (EU TGD, 2003) uses two formulations for ACR depending on NOEC (Eq. 3.8) and IC₂₀ (Eq. 3.9), respectively.

$$ACR_{NOEC} = \frac{EC_{50}}{NOEC} \quad (3.8)$$

$$ACR_{IC_{20}} = \frac{EC_{50}}{IC_{20}} \quad (3.9)$$

Table 3.2. Assessment factors used in the calculation of PNEC*s (Pickup et al., 2017)

Available Data	Assessment Factor
At least 1 short-term L(EC) ₅₀ from each of 3 trophic levels of the base set (fish, <i>Daphnia</i> , or algae)	1000
One long-term NOEC (either fish or <i>Daphnia</i>)	100
Two long-term NOECs from species representing 2 trophic levels (fish and/or <i>Daphnia</i> and/or algae)	50
Long-term NOECs from at least 3 species (normally fish, <i>Daphnia</i> , and algae representing 3 trophic levels)	10
SSD** method	5-1
Field data or model ecosystems	Reviewed on a case-by-case basis

*: PNEC= predicted no effect concentration; **: SSD: species sensitivity distribution.

4. RESULTS AND DISCUSSION

4.1. Algal Growth Assay

At least a 16-fold increment in algal biomass (cells mL⁻¹) corresponding to a specific growth rate of 0.92 day⁻¹ was observed at the end of 96 h. The average of the data obtained from the control cultures throughout the experiments was taken for each day of test duration. Based on these data, the exponentially growing algal biomass throughout the test period is graphed as in Figure 4.1. The trend line of the graph is $y = 0.6797e^{0.7652x}$ with $R^2 = 0.9485$.

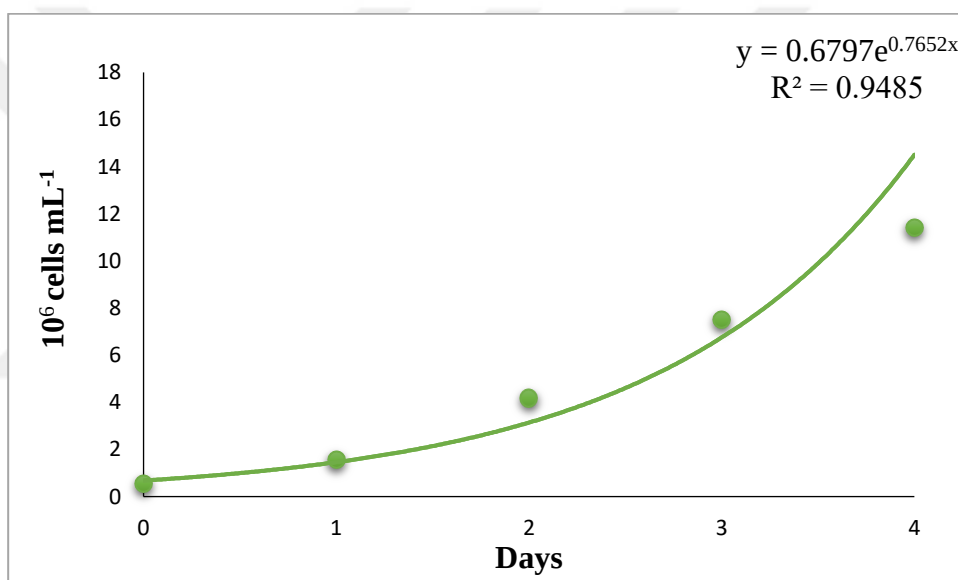


Figure 4.1. The exponential growth curve for algal biomass is based on the average number of cells mL⁻¹ per day obtained from all control cultures of the tests.

The ratio of standard deviation to mean, in other words, the coefficient of variation of the average specific growth rate in the controls, was <10% for all the tests throughout the assays. The recorded pH values of the controls at the beginning and the end of the test duration were 6.00 (±0.5) and 6.10 (±0.5), respectively. The pH changes for each test run are given in Appendix A (Table A1). Since the change in the pH of the controls was less than 1.5-fold at the end of the experiments (OECD, 2011), the tests were valid regarding the pH criterion set by the OECD. For the growth inhibition analyses of RAM and SMX, a control group with a maximum of 0.1% (v/v) DMSO was also prepared to observe if there is a difference due to usage of DMSO. In that regard, a *t*-test was run (*p*-value > 0.05), and no noticeable difference was observed. 3,5-dichlorophenol (3,5-DCP) was employed as a

reference toxicant in addition to the above-mentioned test acceptability criteria to see if algae had the same response. The IC_{50} of 3,5-DCP to *C. vulgaris* was calculated as 2.91 mg L^{-1} , which corresponds with earlier results obtained in our lab and ISO guidelines (ISO 8692, 2012). The UV-Vis spectrum of each test chemical and their binary mixture were taken between 200 and 800 nm at the beginning and end of the experiments. The used concentrations for taking spectrums were 4, 0.8, 40, and 12.5 mg L^{-1} for SeO_3^{2-} , SeO_4^{2-} , RAM, and SMX, respectively. For the binary mixtures, 1 TU of each was analyzed. The UV-Vis spectrum of chemicals is provided in Appendix B (Figure B1-B5). The results show that neither a single chemical nor a binary mixture of chemicals showed significant chemical loss. As a result, all the experiments carried out with *C. vulgaris* were in accordance with the validation and acceptability criteria set by the OECD Test Protocol No. 201 (OECD, 2011).

4.2. Single Toxicity Tests

Each experiment to evaluate the effect of the chemicals on *C. vulgaris* included a control culture and test cultures exposed to equally spaced concentrations of the chemicals. The used concentrations are determined regarding the range finding assays. Chemicals with IC_{50} values greater than 100 mg L^{-1} in their range finding assay were not further tested. OECD (2011) indicates that if no response occurs at concentrations up to 100 mg L^{-1} the chemical is non-toxic to the test organism. Among the tested chemicals, only RAM did not inhibit the growth up to its maximum solubility in DMSO (80 mg L^{-1}). Apart from RAM, all the experiments were carried out with five equally spaced concentrations of the tested chemicals following the range-finding assays. CAS registry number of the test chemicals and their concentrations used in experiments are given in Table 4.1.

Table 4.1. The equally spaced concentrations of the chemicals for the assessment of algal toxicity.

Chemical Name	Cas No.	Concentrations (mg L^{-1})
Na_2SeO_3	10102-18-8	1, 2, 4, 8, 16
Na_2SeO_4	13410-01-0	0.2, 0.4, 0.8, 1.6, 3.2
RAM	87333-19-5	20, 40, 80
SMX	723-46-6	3.125, 6.25, 12.5, 25, 50

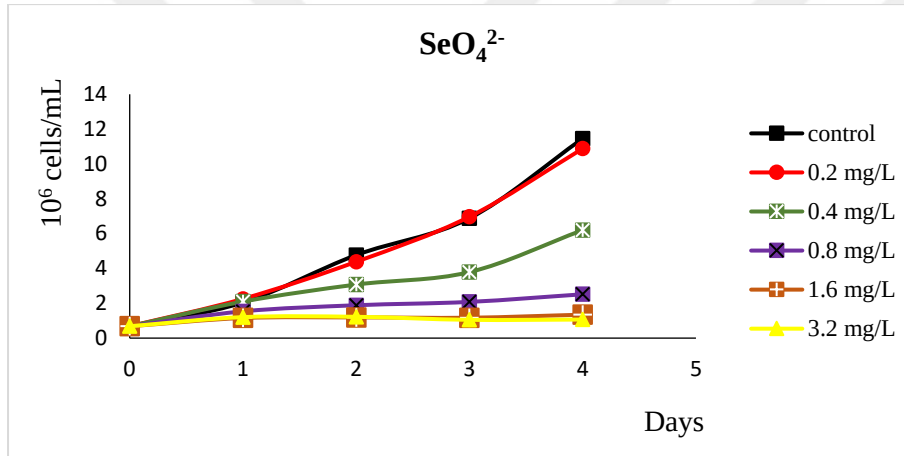
The response of *C. vulgaris* to each chemical in terms of algal biomass is shown in Figure 4.2. The trend lines of control solvents with DMSO and the control coincide with each other, showing there is no significant difference regarding algal growth between them. The percent growth inhibitions (Ig (%)) of the algae caused by the test chemicals were calculated every 24 h over the 96 h test period

based on the specific growth rates and were shown in Figure 4.3. Among all chemicals, SeO_4^{2-} showed the highest toxicity at a concentration of 3.2 mg L^{-1} , where 90.76% growth inhibition occurred. From the $\text{Ig (\%)} \text{ vs. concentration}$ graph of the SeO_3^{2-} treatment, an apparent stimulatory effect was observed at a concentration of 1 mg L^{-1} during the whole test period. The stimulation effect of SeO_3^{2-} at low concentrations might be related to its Se content enhancing the neutralization of free radicals, which decreases the rate of algal cell degradation (Özkovalak, 2005). In addition, Se may typically exhibit a pattern of sulfur metabolism when interact with proteins and amino acids (Özkovalak, 2005). Moreover, Sun et al. (2014) observed that when *C. vulgaris* exposed to low concentrations of Se, it reduces intracellular reactive oxygen species (ROS) and lipid peroxidation (LPO); hence, it acts as an antioxidant, and increases growth of algae by increasing the number of photosynthetic pigments.

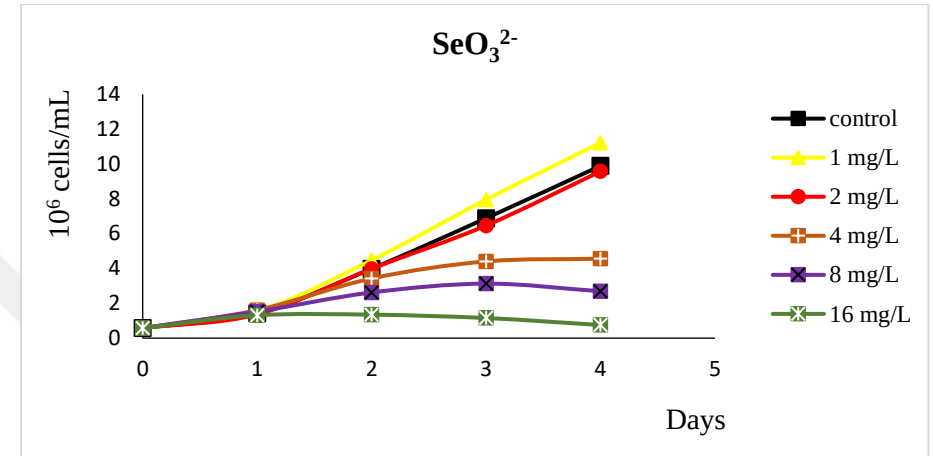
The increased concentration of SeO_3^{2-} , and SeO_4^{2-} showed a corresponding increase in growth inhibition. SMX revealed a different concentration-response relationship; the highest toxicity occurred at a concentration of 12.5 mg L^{-1} at 72 h where the highest test concentration was 50 mg L^{-1} . The concentration of 12.5 mg L^{-1} at 72 h caused growth inhibition of 88%, while it was 70.84% at the end of the 96 h test period. The reason for the weakened inhibitory effect of SMX on algal growth after 72 h might be due to the adaptation of *C. vulgaris* to the toxicity of SMX. Our results were consistent with the results of studies assessing the toxicity of SMX on *S. obliquus* (Xiong et al., 2019b) and *R. subcapitata* (Zhang et al., 2021). These studies also revealed similar responses from the abovementioned algae to SMX. This implies that algae gradually reduce growth inhibition as a result of their adaptation to SMX as the duration is extended.

Inhibitory concentrations (IC) with 95% confidence intervals were computed by statistical methods including linear interpolation (ICp), Probit, Logit, and Weibull using ToxCalc software (Table 4.2). The results showed that SeO_4^{2-} with the IC_{50} of 0.75 mg L^{-1} was the most toxic compound to *C. vulgaris* among the test chemicals at the end of the 96 h. SeO_4^{2-} was followed by SeO_3^{2-} , and SMX with IC_{50} s of 6.68 and 10.12 mg L^{-1} , respectively, in terms of their toxic effects on algae. LOEC and NOEC of the test chemical was calculated with ToxCalc software and toxicity assessment of each chemical was done based on the IC_{50} values. The toxicity of the chemicals is classified according to GHS as follows:

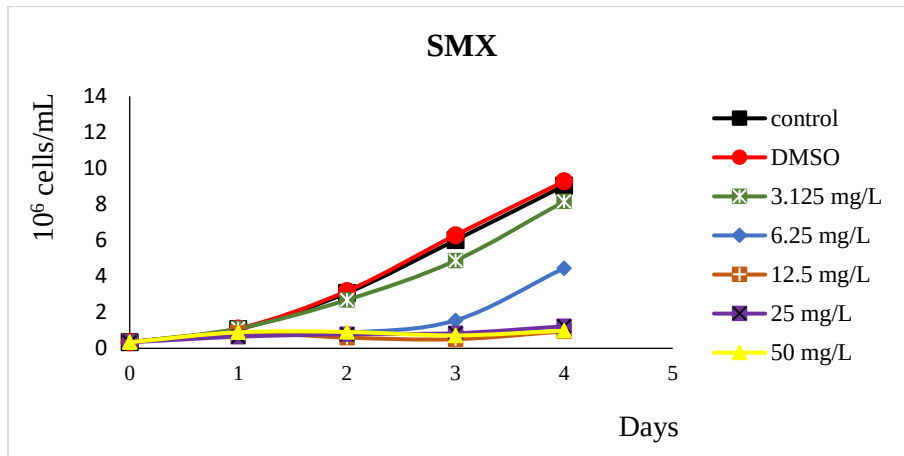
- Highly toxic: $\text{EC}_{50} \leq 1 \text{ mg L}^{-1}$
- Toxic: $1 \text{ mg L}^{-1} < \text{EC}_{50} \leq 10 \text{ mg L}^{-1}$
- Harmful to aquatic organisms: $10 \text{ mg L}^{-1} < \text{EC}_{50} \leq 100 \text{ mg L}^{-1}$
- Non-toxic: $\text{EC}_{50} > 100 \text{ mg L}^{-1}$



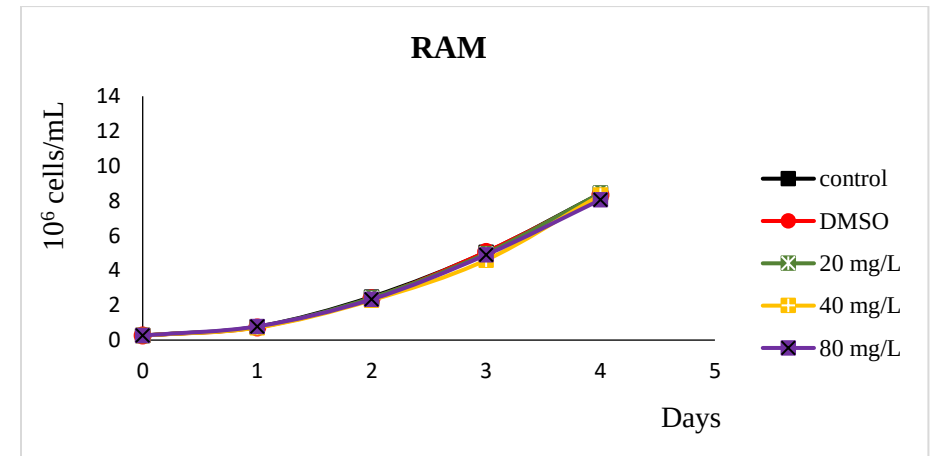
(a)



(b)

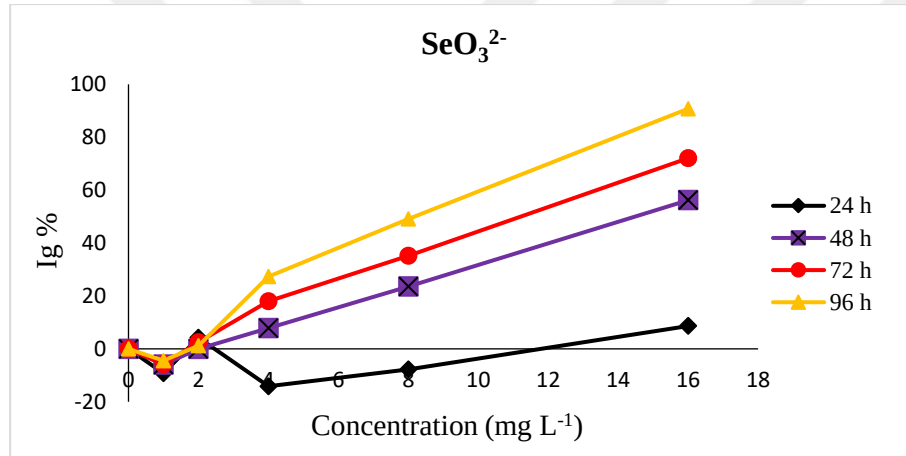


(c)

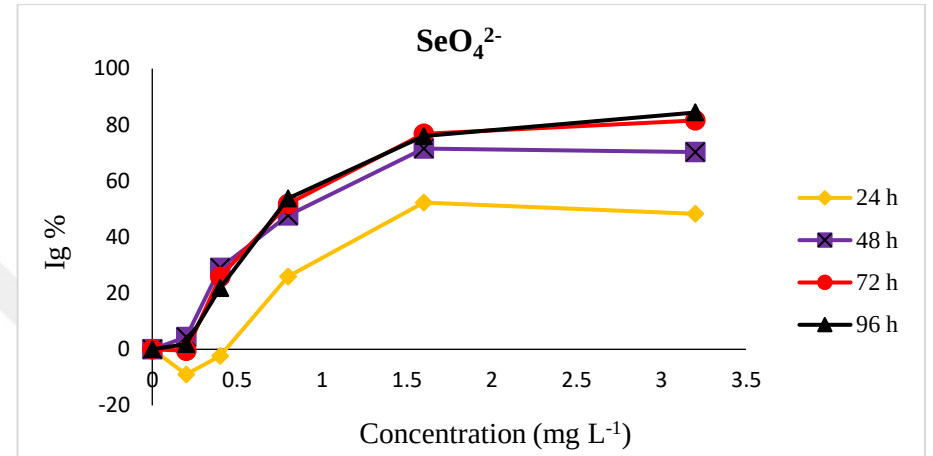


(d)

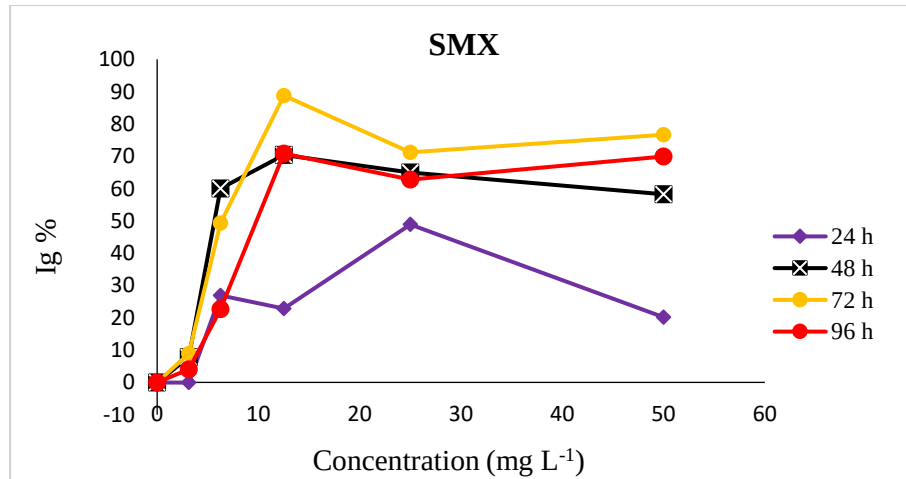
Figure 4.2. Change in biomass of algae when exposed to (a) SeO₄²⁻, (b) SeO₃²⁻, (c) RAM, and (d) SMX throughout the test period.



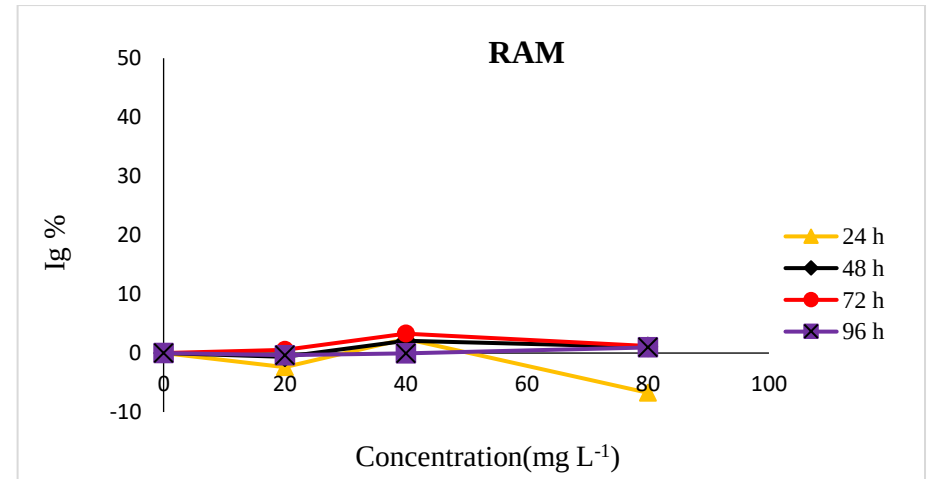
(a)



(b)



(c)



(d)

Figure 4.3. Percent growth inhibition of *C. vulgaris* exposed to (a) SeO₃²⁻, (b) SeO₄²⁻, (c) RAM, and (d) SMX during 96 h test period.

Table 4.2. IC₅₀ and IC₂₀, NOEC, and LOEC of SeO₄²⁻, SeO₃²⁻, RAM, and SMX computed using the specific growth rates (SGR) as input by statistical methods linear interpolation (ICp), Probit, Logit, and Weibull at the end of 48, 72 and 96 h.

Compound	Response Variable	Method	48 h		72 h		96 h		NOEC/ LOEC (mg/L) ^b	Toxic Class ^c
			IC ₂₀ (mg L ⁻¹)	IC ₅₀ (mg L ⁻¹)	IC ₂₀ (mg L ⁻¹)	IC ₅₀ (mg L ⁻¹)	IC ₂₀ (mg L ⁻¹)	IC ₅₀ (mg L ⁻¹)		
SeO ₃ ²⁻	SGR	ICp	6.50 (3.74-13.2) ^a	14.12 (10.06-16.02)	3.95 (3.43-12.94)	10.90 (4.14-14.5)	3.30 (2.99-3.67)	6.68 (5.81-7.62)	2/4	Toxic
		Probit	7.08 (0.09-10.45)	14.16 (9.25-125.01)	4.92622 (0.51-7.53)	10.16 (6.14-22.94)	3.87 (0.95-5.64)	7.22 (4.58-11.6)		
		Logit	7.28 (3.70-9.41)	14.14 (11.3-20.46)	5.08 (2.68-6.74)	10.22 (8.00-13.72)	3.97 (2.37-5.10)	7.29 (5.84-9.14)		
		Weibull	7.48 (1.53-10.28)	14.34 (10.50-23.8)	5.14 (1.34-7.52)	10.71 (7.18-15.4)	3.96 (1.42-5.62)	7.78 (5.42-10.6)		
SeO ₄ ²⁻	SGR	ICp	0.37 (0.31-0.44)	0.88 (0.72-1.01)	0.35 (0.30-0.40)	0.77 (0.68-0.88)	0.38 (0.34-0.42)	0.75 (0.70-0.80)	0.2/0.4	Very toxic
		Probit	0.3 (0.007-0.65)	1.00 (0.42-2.9)	0.35 (0.043-0.59)	0.83 (0.43-1.60)	0.36 (0.05-0.60)	0.82 (0.44-1.53)		
		Logit	0.33 (0.1-0.52)	1.00 (0.6-1.5)	0.35 (0.16-0.50)	0.82 (0.60-1.13)	0.36 (0.18-0.51)	0.81 (0.60-1.10)		
		Weibull	0.55 (—) ^d	9.22 (—)	0.32 (0.04-0.56)	0.90 (0.48-1.45)	0.33 (0.05-0.56)	0.89 (0.49-1.40)		
SMX	SGR	ICp	3.86 (3.60-4.12)	5.65 (5.28-6.05)	3.98 (3.63-4.48)	6.39 (5.58-8.15)	5.80 (5.08-6.68)	10.12 (9.64-10.6)	3.125/ 6.125	Harmful
		Probit	1.21111 (—)	8.95 (—)	2.70 (0.06-5.20)	7.35 (2.58-14.0)	4.08 (0.02-8.63)	13.69 (4.69-44.9)		
		Logit	1.18108 (0.00-3.49)	8.79 (2.3-19.3)	2.88 (1.10-4.32)	7.11 (4.92-9.82)	4.01 (1.01-6.79)	13.43 (8.46-21.6)		
		Weibull	0.55 (—)	9.22 (—)	1.65 (0.0-4.36)	7.76 (1.11-15.2)	3.24 (0.01-7.79)	15.11 (4.58-33.8)		
RAM	SGR	(—) ^d	—	—	—	—	—	—	—	Not toxic

a: 95% confidence interval; b, c: Calculated using 96-h experimental values; d: Not calculated.

The IC_{50} values obtained for inorganic Se compounds are lower than the results of the studies conducted on the toxicity assessment of inorganic forms of Se to *C. vulgaris* in the literature, apart from the lowest recorded IC_{50} value of $5.48 \mu\text{g L}^{-1}$ for *C. vulgaris* exposed to SeO_3^{2-} (US EPA, 1987). In a bioassay using *C. vulgaris* as a test organism, $30.0 \pm 2.04 \text{ mg L}^{-1}$ IC_{50} value was determined at the end of 168 h for SeO_3^{2-} (Zhang, Z. et al., 2023). Pastierová et al. (2009, as cited in Guimarães et al., 2021) reported 73.2 and 322.7 mg L^{-1} IC_{50} values of SeO_3^{2-} , and SeO_4^{2-} for *C. vulgaris*, respectively.

The 96 h IC_{50} of 0.75 mg L^{-1} calculated for *C. vulgaris* exposed to SeO_4^{2-} in this study is lower than those reported for other freshwater green microalgae such as *Scenedesmus quadricauda* ($IC_{50}= 33 \text{ mg L}^{-1}$, 96 h exposure) (Vítová et al., 2011), *Chlorella sorokiniana* ($IC_{50}= 45 \text{ mg L}^{-1}$, 168 h exposure) (Gojkovic et al., 2014). Our result was close to the results of one of the studies that reported the toxicity of selenate to *Chlorella pyrenoidosa* (a freshwater green microalgae with $IC_{50}= 0.79 \text{ mg L}^{-1}$) (Bennett, 1988), and higher than the results for *Chlorella reinhardtii* (a freshwater green microalgae with an IC_{50} ranging from 0.032 to 0.355 mg L^{-1}) (Geoffroy et al., 2007; Fournier et al., 2010). All the above-mentioned studies show that *C. vulgaris* is less tolerant to selenate compared to *Scenedesmus quadricauda*, *Selenastrum capricornutum*, and *Chlorella sorokiniana*, whereas it has a higher tolerance to selenate than *Chlorella reinhardtii*. Besides the freshwater microalgae, the studies conducted on the toxicity assessment of the Se salts to marine microalgae are also investigated. Brix et al. (2004) reported an EC_{50} of 45 mg L^{-1} at the end of 96 h test duration for a hypersaline alga *Dunaliella viridis* exposed to selenate, while Guimarães et al. (2021) reported an EC_{50} of 6.22 mg L^{-1} for marine microalga *Nannochloropsis oceanica*. These results show that *C. vulgaris* is more sensitive to selenate than those marine microalgae.

The studies analyzing the effect of Se on different freshwater and marine microalgae are mostly conducted with SeO_3^{2-} rather than SeO_4^{2-} . Thus, comparatively more data can be obtained for the toxic effect of Se in the form of selenite regarding IC_{50} values in the related literature. Morlon et al. (2005) reported EC_{50} of 6.3 mg L^{-1} for *Chlorella reinhardtii* exposed to SeO_3^{2-} , which is the closest value to our result (6.68 mg L^{-1}). *Chlorella pyrenoidosa* revealed EC_{50} at a concentration of 15.37 mg L^{-1} (Zhao et al., 2019), while the same ratio of inhibition was seen in the growth of *Haematococcus pluvialis* (a freshwater green microalga) at a concentration of 24.02 mg L^{-1} (Zheng et al., 2017) when exposed to SeO_3^{2-} . Moreover, Al-Hasawi et al. (2020) reported a 3.0 mg L^{-1} EC_{50} value for *Pseudokirchneriella subcapitata* exposed to SeO_3^{2-} . Kramárová et al. (2012) obtained an EC_{50} of 12.24 mg L^{-1} at the end of the 96 h for *Scenedesmus quadricauda* while Vítová et al.'s (2011) study ended up with a lower value of 4 mg L^{-1} for *Scenedesmus quadricauda*. When we compare *C. vulgaris*

to other freshwater green microalgae, it is more sensitive to SeO_3^{2-} than the species *C. pyrenoidosa*, and *Haematococcus pluvialis*. In the case of the species *S. capricornutum*, and *S. quadricauda*, different results obtained for the toxicity of selenite make a comparison with *C. vulgaris* hard. Singh et al. (2023) reported an EC_{50} of 195 mg L^{-1} for marine microalgae *Dunaliella salina* exposed to SeO_3^{2-} , whereas it was 12.94 mg L^{-1} for another marine microalga *N. oceanica* (Guimarães et al., 2021).

To date, a considerable number of studies have been done on the ecotoxicological impacts of SMX on various aquatic organisms by both individual and mixture toxicity assessments as aforementioned in the literature review, part 2.1.1. We also assessed the single toxicity of SMX and obtained an IC_{50} of 10.12 mg L^{-1} for *C. vulgaris* at the end of 96 h. Baran et al. (2006) reported a 48 h EC_{50} of $1.57 \mu\text{g L}^{-1}$ for *C. vulgaris* exposed to SMX. Borecka et al. (2016) obtained 0.98 and 1.51 mg L^{-1} EC_{50} values for *C. vulgaris* exposed to SMX at the end of the 48 h and 72 h test periods, respectively. Dong et al. (2020) observed nearly 40% growth inhibition (IC_{40}) at a concentration of 200 mg L^{-1} at the end of 72 h for SMX using *C. vulgaris* as a test organism. Aderemi et al. (2021) reported an EC_{50} of 47.9 mg L^{-1} at the end of 96 h for *C. vulgaris* exposed to SMX, while Zhang, T. et al. (2023) detected a very close EC_{50} value of 48.36 mg L^{-1} for it.

Apart from *C. vulgaris*, Zhang et al. (2021) and Chang et al. (2022) reported 96 h EC_{50} values of 0.49 and 0.612 mg L^{-1} for *P. subcapitata* exposed to SMX, respectively. On the other hand, Aderemi et al. (2021) and Eguchi et al. (2004) reported EC_{50} values of $> 2 \text{ mg L}^{-1}$ and 1.53 mg L^{-1} for *Raphidocelis subcapitata* (other names for it are *P. subcapitata*, and *S. capricornutum*) at the end of 96 h test period, respectively. Isidori et al. (2005) reported a 72 h EC_{50} value of 0.52 mg L^{-1} for SMX using *R. subcapitata* as a test organism. Xiong et al. (2019a) calculated a 96 h EC_{50} value of 0.12 mg L^{-1} for *Scenedesmus obliquus* (a green microalga) exposed to SMX. The data on the ecotoxicological impact of SMX on marine microalgae is very limited. We found only one study reporting 96 h EC_{50} values of 0.93 and 4.65 mg L^{-1} for marine diatoms *Skeletonema costatum* and *Phaeodactylum tricornutum* which are exposed to SMX, respectively (Feng et al., 2024). Therefore, there is a data gap on the toxicity of SMX towards marine microalgae. The reported studies depict that a wide range growth response was obtained for *C. vulgaris* exposed to SMX, extending from $1.57 \mu\text{g L}^{-1}$ to 200 mg L^{-1} (test durations are different ranging from 48 h to 96 h). Regarding the reported algal toxicity data of SMX, *C. vulgaris* overall seems to be more tolerant to SMX compared to other microalga mentioned in this study.

Overall, the differences in the IC_{50} values test chemicals show that the toxicity of chemical compounds changes with different microalgal species, different strains of the same species, and variations among the laboratories. The variation in the degree of inorganic Se compounds and SMX toxicity may be ascribed to differences in the kinetics of their absorption, utilization, and removal among the algal species (Karthikeyan et al., 2019). The same species exposed to the same chemical compounds in different laboratories may give different toxicity results because temperature, pH, nutrients, light density, test protocols, and even personal treatments may differ (Geiger, 2014). Moreover, the existence of other compounds in the medium composition may prevent chemical uptake by test organisms. Brix et al. (2004) indicates that species of freshwater microalgae are less tolerant to inorganic selenium compounds than marine microalgae because sulfate in marine water reduces especially the bioavailability of selenate. In the case of SMX, the growth response differences between the different ecotoxicological studies on the freshwater green algae *C. vulgaris* may stem from the genotype and phenotype differences among the species in the *Chlorella* clades, resulting in variations in the SMX tolerance (Zhang, Z. et al., 2023). Differences in the initial density of the algae may also lead to variations in the response because with a lower initial density the algal population will not be able to recover from the toxic shock and more severe stress may occur on the algae (Zhang, Z. et al., 2023).

The different consequences obtained from growth inhibition tests of microalgae may stem from the chosen mathematical calculation of the response. Geiger (2014) calculated both the specific growth rate and yield (or biomass) as response variables for the growth inhibition analysis of *C. vulgaris* exposed to pharmaceuticals and phenols. The results reveal that IC_{50} values calculated based on yield response are numerically lower than those using the specific growth rate. Fettweis et al. (2021) study on the metal sensitivities of *S. quadricauda* resulted in different IC values when different test endpoints (i.e., yield and specific growth rates) are used, and they indicate that approximately two-fold high IC values calculated using specific growth rate (SGR) showing SGR are a less sensitive test endpoint compared to yield. On the other hand, Simpson et al. (2003) calculated lower IC values using the growth rate as the test endpoint than those calculated using the biomass at the end of the 72 h test period for *Phaeodactylum tricornutum* exposed to copper. Those variations in IC value are a result of the change in mathematical calculation, rather than a sign of a higher or lower sensitivity for cell yield compared with growth rate as endpoints (Simpson et al., 2003). In addition, the OECD guideline (2011) for the testing of chemicals notes that these two response variables are different from each other and not comparable. EC_x (the effective concentration leading to x% inhibition of growth) values based on the average specific growth rate (E_rC_x) are generally higher than the values based on

yield ($E_y C_x$) due to the mathematically different results that obtained at the end, not from the sensitivity differences between the two variables.

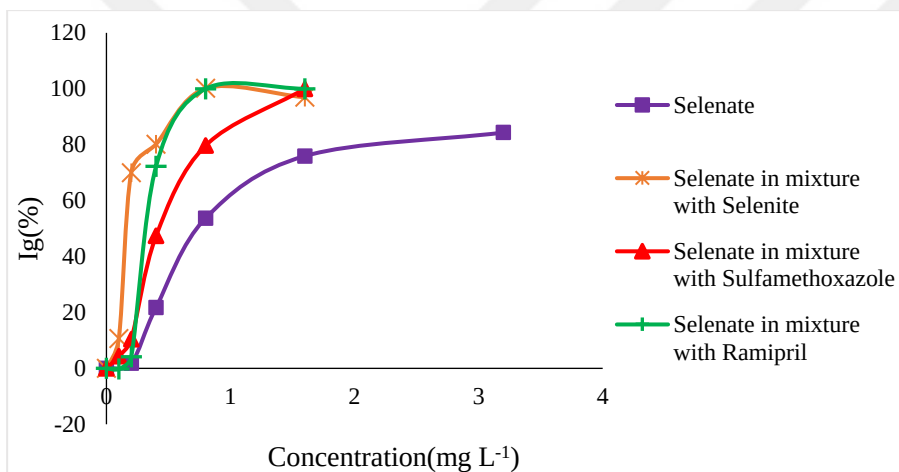
4.3. Mixture Toxicity Tests

The mixture toxicity of the chemicals was evaluated by exposing the algae to the binary mixtures of the chemicals using the following concentrations: Σ 0.25 TU, Σ 0.5 TU, Σ 1 TU, Σ 2 TU, and Σ 4 TU. Here, Σ 1 TU equals to sum of half of the IC_{50} values of each component of the mixtures obtained from single toxicity assessments. ICs from the binary mixture experiments of SeO_3^{2-} , SeO_4^{2-} , SMX, and RAM significantly decreased compared to the single exposure experiments. This means that individual test chemicals showed higher toxicity in the binary mixtures. The responses ($Ig(\%)$) of microalgae to the single toxicants were compared with those obtained from the mixture toxicity experiments in Figure 4.4.

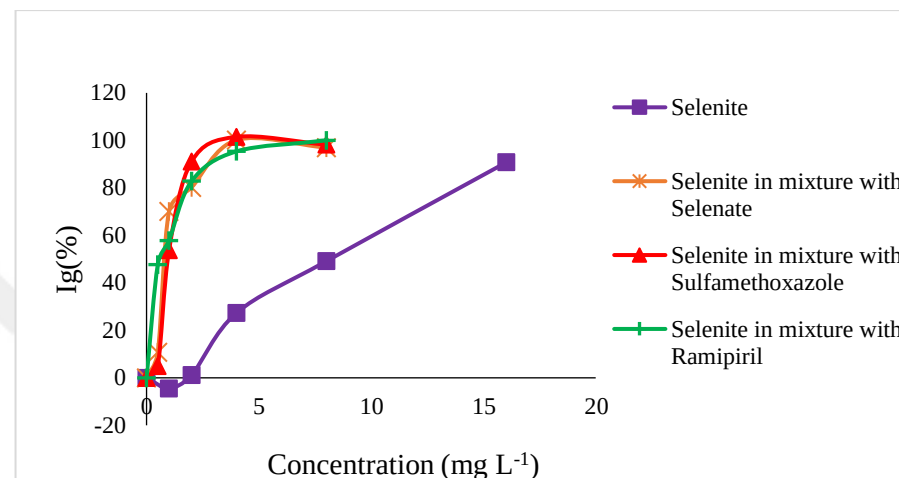
The most significant decrease in IC_{50} values between single and mixture exposures was found for the binary mixture of SeO_3^{2-} , and SeO_4^{2-} , where the IC_{50} value decreased from 6.68 to 1.39 $mg\ L^{-1}$ and 0.75 to 0.156 $mg\ L^{-1}$, respectively. This indicates that approximately an 80% increase occurs in toxicity. In addition, the hormetic effect of SeO_3^{2-} disappears when present in the mixtures. Among the tested chemicals, only the single form of RAM did not inhibit the growth of *C. vulgaris*. However, its binary mixture with the studied chemicals showed a toxic effect. The IC_{50} values of RAM were 28.54 $mg\ L^{-1}$, 16.72 $mg\ L^{-1}$, and 6.15 $mg\ L^{-1}$ when combined with SMX, SeO_4^{2-} , and SeO_3^{2-} respectively. Table 4.3 shows the IC_{50} values of the single chemicals and how the IC_{50} values decrease in binary mixtures.

Table 4.3. IC_{50} of SeO_4^{2-} , SeO_3^{2-} , SMX, and RAM compared to IC_{50} of each in the mixtures.

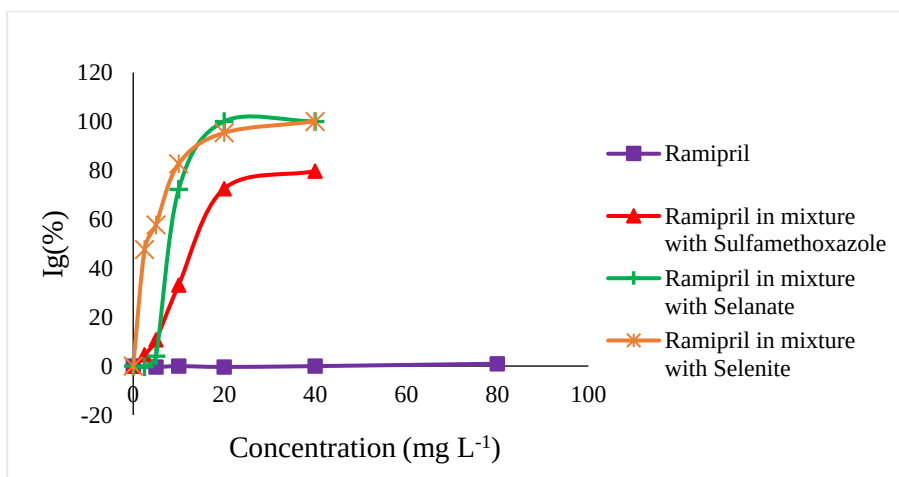
Chemicals	IC_{50} of single chemicals ($mg\ L^{-1}$)	IC_{50} of the chemicals in the binary mixtures ($mg\ L^{-1}$)			
		Selenite	Selenate	SMX	RAM
Selenite	6.68	-	1.39	1.60	1.02
Selenate	0.75	0.156	-	0.40	0.31
SMX	10.12	2.43	5.47	-	7.2
RAM	>100	6.15	16.72	28.54	-



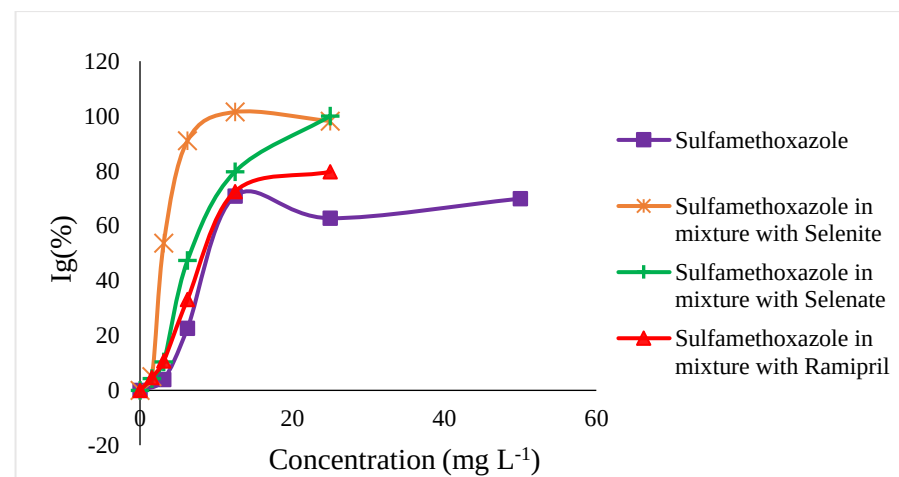
(a)



(b)



(c)



(d)

Figure 4.4. Concentration-response curve of (a) SeO_4^{2-} , (b) SeO_3^{2-} , (c) RAM, and (d) SMX individually compared to binary mixtures that each formed

4.4. Toxicity Prediction of CA, IA, and CI Models versus Experimental Results

The predicted concentrations for assessing the growth inhibition of the mixed chemicals were calculated using CA, IA, and CI-isobologram methods, and they were compared with the results of the binary mixture experiments. The models' calculation of the mixture toxicity is based on the IC values of the tested chemicals obtained from the single toxicity experiments. The mixture toxicity experiments were performed using the IC₅₀ values of the single chemicals derived from the linear interpolation method (ICp). However, to estimate the mixture toxicity by CA and IA, IC values of SMX and SeO₄²⁻ obtained from the logit method were used, while the IC values of SeO₃²⁻ and SeO₄²⁻ obtained from probit and Weibull methods were used, respectively. We used the statistical method that calculated the lowest IC₅₀ values to be more conservative for the environment. We did not use IC₅₀ values of the single chemicals derived from the ICp method for estimating chemical toxicity by CA and IA models for two reasons. First, ICp could not compute IC values greater than 75% inhibition for SeO₄²⁻, 90% for SeO₃²⁻, and 60% for SMX. Second, we aimed to predict IC values for combined chemicals up to 95% growth inhibition by CA and IA models, so we used statistical methods that can calculate IC values up to 95% growth inhibition for single chemicals with 95% confidence intervals. The CI model does not rely on IC₅₀ and other growth inhibition percentages of the single chemicals to forecast different growth inhibition levels of the mixed chemicals.

It should be noted that ramipril did not inhibit the growth of *C. vulgaris* up to its maximum solubility in DMSO, which is 80 mg L⁻¹. For this reason, we used five different concentrations of RAM, starting from the maximum soluble concentration (80-40-20-10-5 mg L⁻¹) in binary mixture toxicity experiments. In order to predict the toxicity of a mixture using CA and IA, we need to know the IC₅₀ value of each chemical as mentioned above. For the CI-isobologram method, at least two IC values are needed to predict mixture toxicity. However, we didn't have an exact experimental IC value for RAM, as the measured value should be above 80 mg L⁻¹. Therefore, we estimated the IC values (from 1 to 95% Ig) of RAM using ToxCalc software prediction, which gave us an estimated IC₅₀ value of 239.5 mg L⁻¹. In addition to the IC₅₀ value obtained from the ToxCalc software for RAM, its IC₅₀ value was also predicted from Quantitative Structure-Toxicity Relationship (QSTR) models published for algae in the literature. The model equation of the selected QSTR model (Önlü & Saçan, 2017) is given below.

$$\begin{aligned} \text{pEC}_{50}(72 \text{ h}) = & 5.14 (\pm 0.93) + 3.48 (\pm 1.40) \text{ SPAM} - 1.92 (\pm 0.49) \text{ Mor31p} + 0.24 (\pm 0.12) \text{ NdsCH} - \\ & 0.44 (\pm 0.18) \text{ CATS2D_02_AP} + 0.95 (\pm 0.30) \text{ B05[C-S]} + 0.15 (\pm 0.03) \text{ F03[C-N]} + 0.10 (\pm 0.01) \\ & \text{MLOGP2} - 0.76 (\pm 0.14) \text{ Hardness} \end{aligned} \quad (4.1)$$

Table 4.4. Type and chemical meanings of descriptors appearing in the model Equation 4.1 (Önlü & Saçan, 2017)

Descriptor	Type Chemical meaning Model	Descriptor Value
SPAM	Geometrical descriptors Average span R	0.3427
Mor31p	3D-MoRSE descriptors Signal 31/weighted by polarizability	0.4763
NdsCH	Atom-type E-state indices Number of atoms of type dsCH	0
CATS2D_02_AP	CATS 2D CATS2D acceptor-positive at lag 02	0
B05[C-S] 2D	Atom Pairs Presence/absence of C-S at topological distance 5	0
F03[C-N] 2D	Atom Pairs Frequency of C-N at topological distance 3	4
MLOGP2	Molecular properties Squared Moriguchi octanol-water partition coefficient	5.2532
Hardness	Quantum chemical (energy-related descriptor) Half of the energy difference between the lowest unoccupied and highest occupied molecular orbitals.	4.73

Based on the QSTR model, the predicted IC_{50} value of RAM for algae is 393.56 mg L^{-1} . The same QSTR model has been used previously to predict IC_{50} values of pesticides and to compare the model's predictive performance with experimental IC_{50} values (Gökçe & Saçan, 2019). The model's predicted order of algal toxicity agreed with the order of experimental algal toxicity. To be more conservative for the aquatic environment, the IC_{50} value of RAM predicted by the ToxCalc program which used some of the experimental data was utilized to calculate the toxicity of RAM in binary mixtures. However, there is a significant difference between the experimental concentrations and estimated inhibitory concentrations, which means that CA and IA models may overestimate IC values when calculating them in mg L^{-1} . Consequently, to calculate toxic units (TU), we divided the experimental results by the estimated IC_{50} value of RAM to find the TU for each concentration used. Since one toxic unit equals the IC_{50} of a given endpoint, we calculated the number of TU for each of the experimental concentrations using the predicted IC_{50} . The concentration-response curves of binary mixtures of RAM have produced incompatible results when the concentrations were used as mg L^{-1} and TU. However, the concentration-response curves of CA and IA obtained from the predicted TU along with their corresponding I_g (%) values, seem to be more reliable than the concentration unit mg L^{-1} .

4.4.1. Selenite and Selenate in Mixture

The observed IC_{50} value of SeO_3^{2-} and SeO_4^{2-} in the mixture was 1.55 mg L^{-1} ($1.48 - 1.58 \text{ mg L}^{-1}$ confidence interval 95%). The corresponding TU at 50% growth inhibition was 0.384 TU, showing a synergistic effect of the mixed chemicals. The CA, IA, and CI models predicted IC_{50} values of 4.03, 5.48, and 1.80 mg L^{-1} , respectively. All IC_{50} values estimated by the models do not overlap with the confidence interval of the experimental result. Although all three models underestimated the toxicity, the CI model predicted IC_{50} to be closer to the experimental result than the CA and IA models. Figure 4.5 (a) compares the concentration-response curves of CA, IA, and CI models with experimental results regarding mg L^{-1} and TU units, respectively.

4.4.2. Selenite and Sulfamethoxazole in Mixture

The observed IC_{50} value of SeO_3^{2-} and SMX in the mixture was 4.04 mg L^{-1} ($3.64 - 4.72 \text{ mg L}^{-1}$ confidence interval 95%). The corresponding TU at 50% growth inhibition was 0.404 TU, showing a synergistic effect of the joint chemicals. The CA, IA, and CI models predicted IC_{50} values of 10.0, 11.64, and 4.86 mg L^{-1} , respectively. Thus, all the models underestimate toxicity, and the closest prediction by CI is not in the confidence interval of the experimental IC_{50} . The concentration-response curves of CA, IA, and CI models and the experimental results are compared in Figure 4.5 (b).

4.4.3. Selenate and Sulfamethoxazole in Mixture

The experimental IC_{50} value of SeO_4^{2-} and SMX in the mixture was 5.88 mg L^{-1} ($4.70 - 7.04 \text{ mg L}^{-1}$ confidence interval 95%). The corresponding TU at 50% growth inhibition was 0.905 TU, showing an additive effect of the joint chemicals. The CA, IA, and CI models predicted IC_{50} values of 6.49, 6.98, and 5.67 mg L^{-1} , respectively. All three models' IC_{50} values were within the confidence interval of the experimental result. Although the results are acceptable, CA and IA slightly underestimate the joint effect of combined chemicals, while CI overestimates it. The CI model does the best and closer estimation. In addition, since all the models estimate toxicity within the confidence interval of the experimental result, it is hard to discuss the MoA of the combined chemicals.

The concentration-response curves of CA, IA, and CI and the experimental results are compared in Figure 4.6. (a).

4.4.4. Selenite and Ramipril in Mixture

The experimental IC_{50} value of the SeO_3^{2-} and RAM in the mixture was 7.18 mg L^{-1} ($3.97 - 12.8 \text{ mg L}^{-1}$ confidence interval 95%). The corresponding TU at 50% growth inhibition was 0.168 TU, showing a synergistic effect of the joint chemicals. The CA, IA, and CI models predicted IC_{50} values of 42.7, 205, and 6.76 mg L^{-1} , respectively. The calculated IC_{50} values regarding TU by CA, IA, and CI models were 1.00, 1.53, and 0.158 TU, respectively. As aforementioned, since the experimental concentration of RAM is smaller than the estimated IC values, and the CA and IA models are highly dependent on the single toxicity of the mixed chemicals, these models most probably cannot estimate the toxicity using the inhibitory concentrations with mg L^{-1} unit. Since $1 \text{ TU} = IC_{50}$ of the single chemicals, one can calculate the corresponding TU of any given concentration. Therefore, comparing experimental IC values with the estimated ones using a toxic unit (TU) gave more reliable results for all binary mixtures of RAM with the tested chemicals. The CI model estimated the toxicity of mixed SeO_3^{2-} and RAM closely to the experimental value, and the result overlaps the confidence interval of experimental IC_{50} . Considering the estimated TUs by CA and IA, both models highly underestimate the toxicity. The concentration-response curves of CA, IA, and CI models and the experimental results are compared in Figure 4.6 (b).

4.4.5. Selenate and Ramipril in Mixture

The experimental IC_{50} value of the SeO_4^{2-} and RAM in the mixture was 17.04 mg L^{-1} ($15.68 - 18.45 \text{ mg L}^{-1}$ confidence interval 95%). The corresponding TU at 50% growth inhibition was 0.455 TU, showing a synergistic effect of the joint chemicals. The CA, IA, and CI models predicted IC_{50} values of 37.5, 200, and 17.11 mg L^{-1} , respectively. The calculated IC_{50} values regarding TU by CA, IA, and CI models were 1.00, 1.52, and 0.456 TU, respectively. CA and IA's toxicity prediction of the combined chemicals is far from the observed value, and these two models highly underestimate the toxicity. On the other hand, the CI model very well estimated the toxicity. Figure 4.7. (a) compares the concentration-response curves of CA, IA, and CI models with experimental results regarding mg L^{-1} and TU units, respectively.

4.4.6. Sulfamethoxazole and Ramipril in Mixture

The observed IC_{50} value of the SMX and RAM mixture was 35.77 mg L^{-1} ($31.8 - 39.2 \text{ mg L}^{-1}$ confidence interval 95%). The corresponding TU at 50% growth inhibition was 0.66 TU, showing the synergistic effect of the joint chemicals. The CA, IA, and CI models predicted IC_{50} values of 54.5,

206, and 32.21 mg L⁻¹, respectively. The calculated IC₅₀ values regarding TU by CA, IA, and CI models were 1.00, 1.35, and 0.59 TU, respectively. CA and IA models again highly underestimated toxicity, Figure 4.7 (b) compares the concentration-response curves of CA, IA, and CI models with experimental results regarding mg L⁻¹ and TU units, respectively.

The result of all binary mixtures of test chemicals regarding being additive, synergistic, or antagonistic when present in the test medium simultaneously is summarized in Table 4.5 below. The results show that except for the SeO₄²⁻ – SMX mixture, the omnipresence of tested chemicals has a synergistic effect, in other words more toxic to algae.

Table 4.5. The characteristics of the interaction between the binary mixture of SeO₄²⁻, SeO₃²⁻, RAM, and SMX determined by calculated TUs.

Mixture	TU	Effect
SeO ₃ ²⁻ – SeO ₄ ²⁻	0.384	Synergistic
SeO ₃ ²⁻ – SMX	0.404	Synergistic
SeO ₄ ²⁻ – SMX	0.905	Additive
SeO ₃ ²⁻ – RAM	0.168	Synergistic
SeO ₄ ²⁻ – RAM	0.455	Synergistic
SMX – RAM	0.660	Synergistic

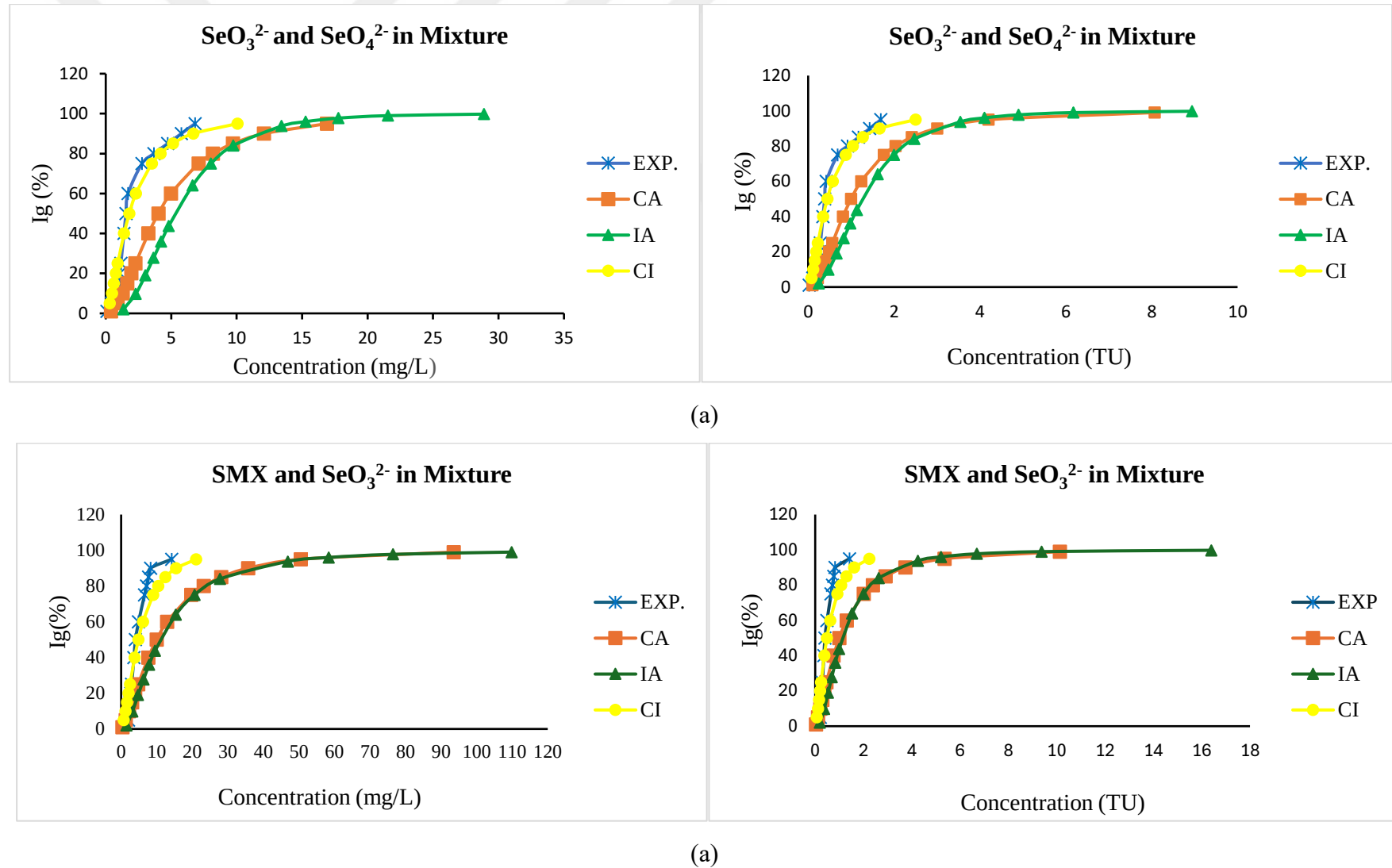


Figure 4.5. Comparison of concentration-growth inhibition (Ig(%)) curves of CA, IA, and CI with experimental results (Exp) from the mixture toxicity test of (a) SeO₃²⁻ and SeO₄²⁻, (b) SMX and SeO₃²⁻ regarding both mg L⁻¹ and TU

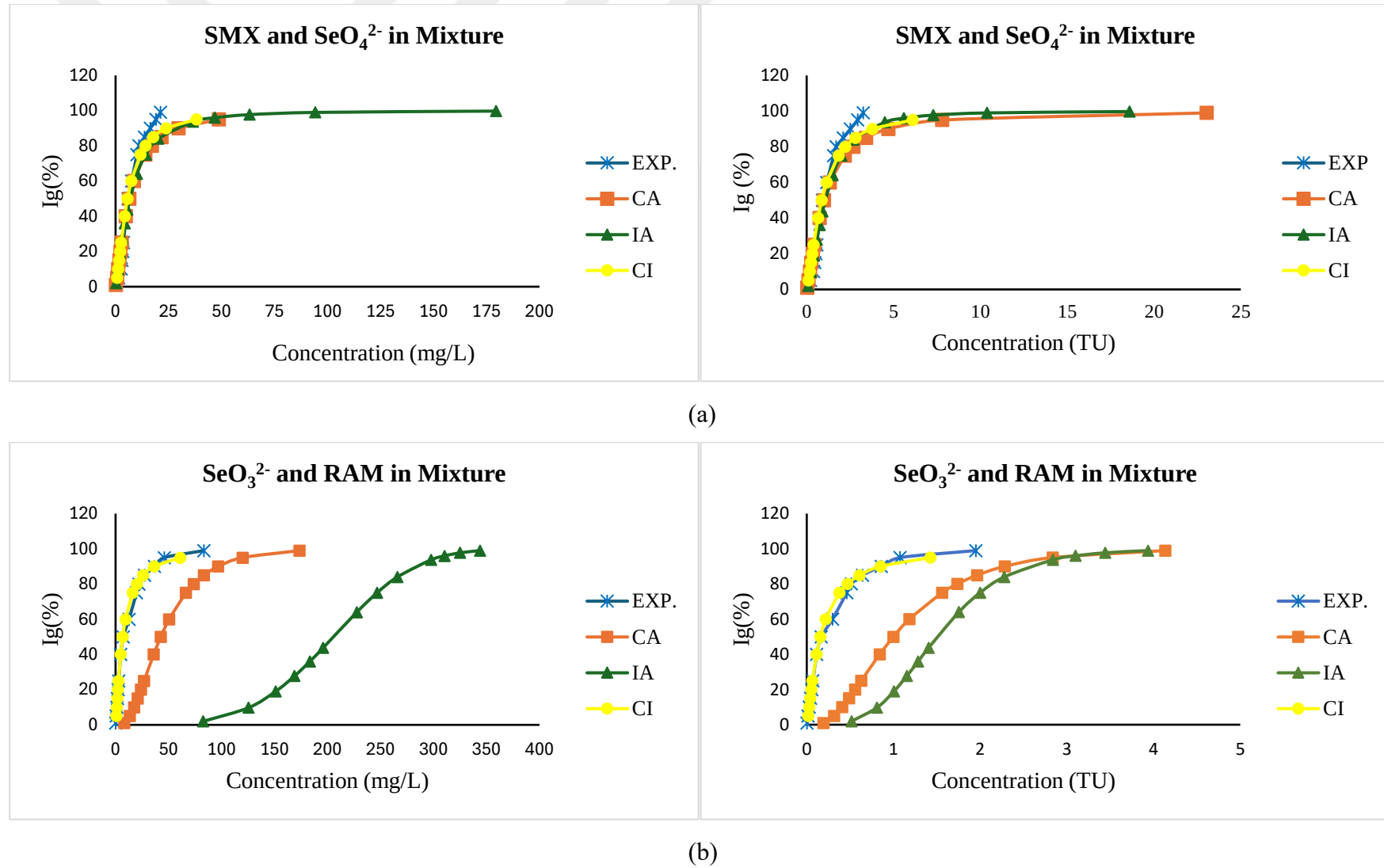


Figure 4.6. Comparison of concentration-growth inhibition (Ig(%)) curves of CA, IA, and CI with experimental results (Exp) from the mixture toxicity test of (a) SMX and SeO_4^{2-} , (b) SeO_3^{2-} and RAM regarding mg L^{-1} and TU.

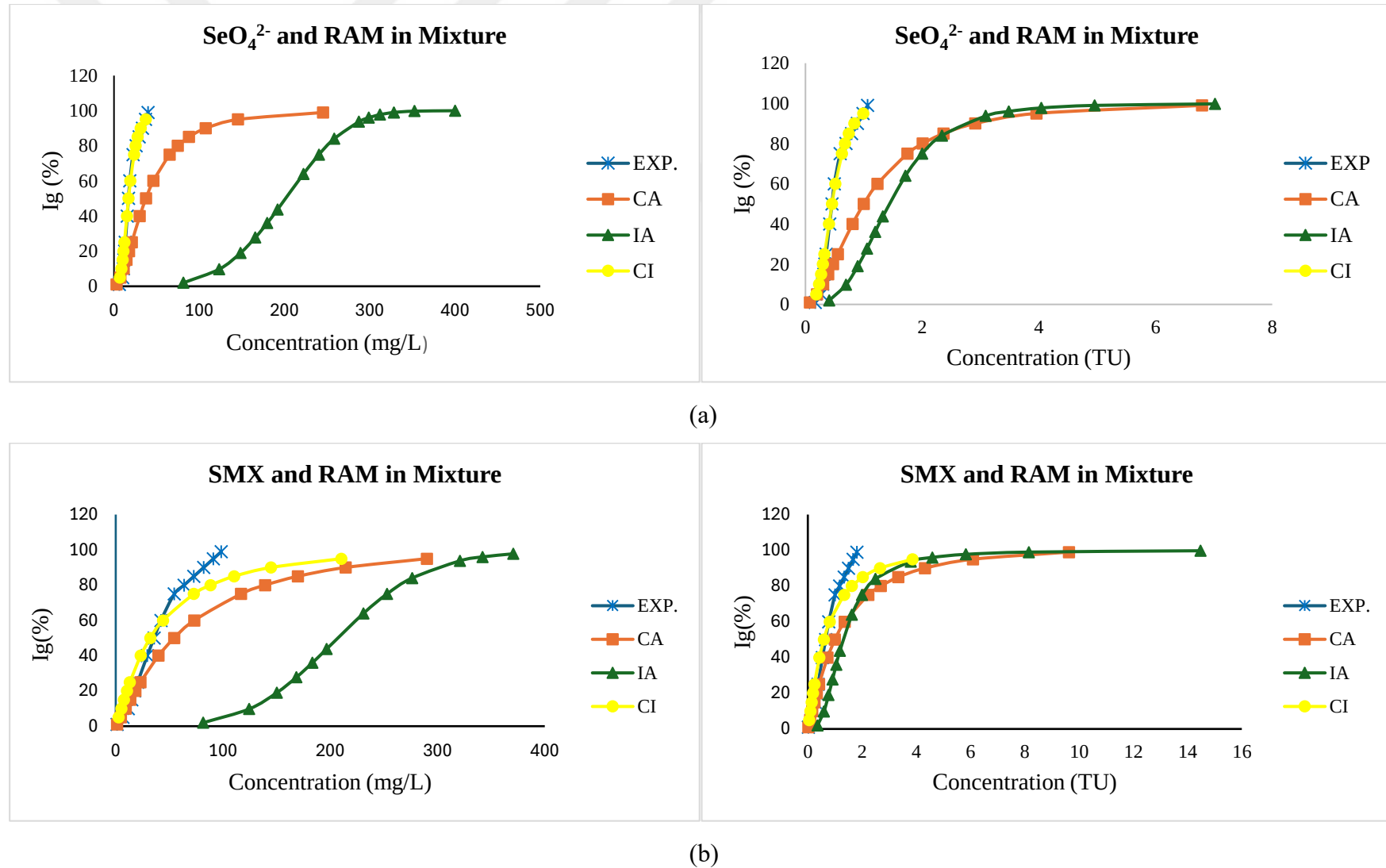


Figure 4.7. Comparison of concentration-growth inhibition (Ig(%)) curves of CA, IA, and CI with experimental results (Exp) from the mixture toxicity test of (a) SeO₄²⁻ and RAM, (b) SMX and RAM regarding mg L⁻¹ and TU

4.5. Model Deviation Ratios (MDR) of CA, IA, and CI-Isobologram

The MDRs of the models' predictions at different concentrations causing certain algal growth inhibition are calculated, and the results are shown in Table 4.6. Bold numbers in the table indicate the MDRs within the range of 0.5 to 2. As aforementioned, MDR values of 1 depict that the models used have the best possible fit. MDR values <1 show that mixture toxicity was underestimated, and MDR scores of >1 show that it has been overestimated.

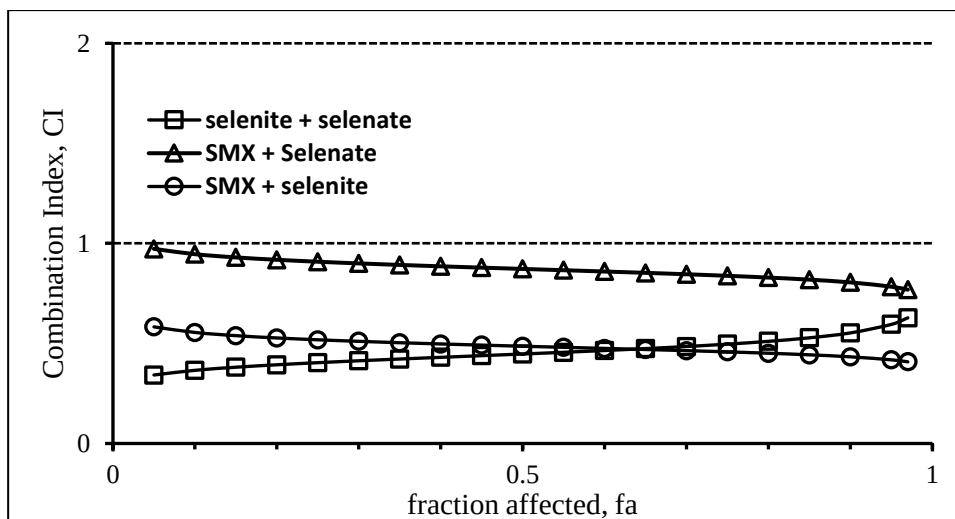
The combined effect of the tested substances was mostly well estimated by the CI-isobologram method within the range of $0.5 \leq \text{MDR} \leq 2$. Except for the SeO_3^{2-} and RAM mixture, the CI model overestimates toxicity at lower concentrations (mostly causing $\text{IC}_5 - \text{IC}_{25}$) and underestimates the toxic effect at higher concentrations. Geiger et al. (2016) also detected a similar trend for the CI model, where an overestimation of toxicity exists at a lower range Ig. The CI method overall well-predicted the effect of SeO_3^{2-} and RAM in the mixture at most Ig levels. The CA and IA models could not precisely predict the toxicity of the binary mixtures at most growth inhibition levels even in the case of SMX and SeO_4^{2-} , where most MDR values are in the set range. None of the predicted IC values of the SeO_3^{2-} and RAM mixture by CA and IA models were within the range of MDR. CA and IA models underestimate toxicity for the IC_{50} values of all binary mixtures. As a result, if we compare the predictive power of the three models, CI is the most robust model for predicting the toxic effect of the joint chemicals. The graphical representation of the interaction between all tested binarily mixed chemicals was also achieved using the CI-isobologram method proposed by Chou (2006). The fa-CI plot in Figure 4.8. was obtained from the program CompuSyn. This effect-oriented plot shows the interaction at all growth inhibition levels, in other words, as fraction affected (fa). In the fa-CI plot, $\text{CI} < 1$, $= 1$, and > 1 , represents synergistic, additive, and antagonistic interaction, respectively.

Considering the fa-CI plot in Figure 4.8, the interaction seems to be synergistic at all levels for all binary mixtures. However, we accepted the interaction between the SeO_4^{2-} and SMX as additive regarding the range that Broderius et al. (1995) set for, where strict additivity occurs at $\text{TU} \pm 0.2$ as used in other studies (Lin et al., 2004; Hodges et al., 2006; Geiger et al., 2016)

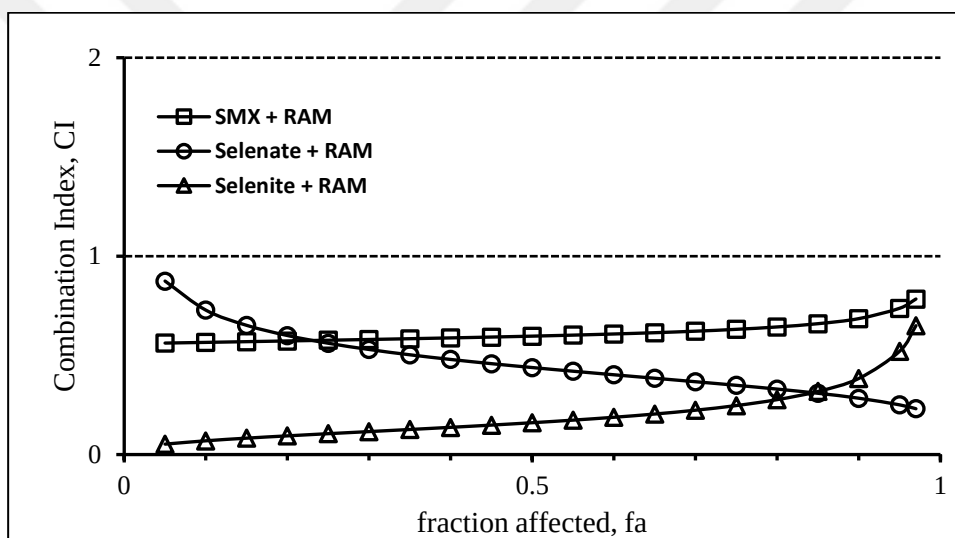
Table 4.6. Model deviation ratios (MDRs) for different growth inhibition level (Ig%) for CA, IA, and CI models.

	$\text{SeO}_3^{2-} - \text{SeO}_4^{2-}$			$\text{SeO}_4^{2-} - \text{SMX}$			$\text{SeO}_3^{2-} - \text{SMX}$			SMX – RAM			$\text{SeO}_4^{2-} - \text{RAM}$			$\text{SeO}_3^{2-} - \text{RAM}$		
Ig (%)	CA	IA	CI	CA	IA	CI	CA	IA	CI	CA	IA	CI	CA	IA	CI	CA	IA	CI
1	0.22	0.20		1.67	1.54		1.28	0.71		1.18	0.16		2.17	0.73		0.01	0.01	
5	0.48	0.38	1.41	2.15	1.89	2.21	1.72	1.12	2.96	1.39	0.35	2.39	1.39	0.58	1.48	0.05	0.02	0.86
10	0.67*	0.50	1.83	2.10	1.80	2.22	1.08	0.74	1.96	1.31	0.42	2.25	0.98	0.45	1.26	0.07	0.04	1.01
15	0.61	0.45	1.59	1.71	1.45	1.84	0.85	0.60	1.57	1.12	0.43	1.92	0.81	0.40	1.17	0.09	0.05	1.07
20	0.55	0.40	1.39	1.47	1.25	1.61	0.72	0.52	1.36	1.00	0.43	1.70	0.71	0.37	1.12	0.10	0.06	1.09
25	0.51	0.37	1.26	1.31	1.11	1.45	0.63	0.47	1.22	0.91	0.44	1.55	0.64	0.35	1.08	0.11	0.06	1.09
40	0.43	0.32	0.99	1.01	0.89	1.14	0.47	0.38	0.95	0.74	0.47	1.26	0.51	0.31	1.03	0.14	0.08	1.00
50	0.38	0.29	0.86	0.91	0.82	1.04	0.40	0.34	0.83	0.66	0.48	1.11	0.45	0.30	1.00	0.17	0.11	1.06
60	0.34	0.27	0.74	0.87	0.83	1.01	0.38	0.34	0.79	0.57	0.49	0.96	0.40	0.29	0.96	0.25	0.17	1.36
75	0.39	0.35	0.79	0.71	0.77	0.84	0.33	0.33	0.71	0.45	0.51	0.75	0.34	0.29	0.95	0.30	0.23	1.22
80	0.45	0.42	0.88	0.63	0.74	0.76	0.30	0.32	0.66	0.43	0.55	0.72	0.35	0.31	1.02	0.30	0.24	1.10
85	0.49	0.48	0.92	0.62	0.80	0.75	0.26	0.31	0.60	0.40	0.57	0.66	0.33	0.33	1.06	0.33	0.28	1.05
90	0.48	0.51	0.86	0.54	0.79	0.67	0.22	0.28	0.51	0.35	0.56	0.57	0.31	0.33	1.06	0.38	0.35	1.01
95	0.40	0.46	0.68	0.38	0.69	0.48	0.27	0.36	0.63	0.27	0.49	0.43	0.25	0.32	1.00	0.38	0.39	0.75

*:Bold numbers indicate the MDRs within the range of 0.5 and 2.



(a)



(b)

Figure 4.8. The combination index plot (fa-CI plot) obtained from the program CompuSyn shows the growth inhibition levels from $fa = 0.05$ (IC_{05}) to $fa = 0.95$ (IC_{95}) for the binary mixtures of (a) selenite (SeO_3^{2-}) – selenate (SeO_4^{2-}), SMX - selenate (SeO_4^{2-}), and SMX - selenite (SeO_3^{2-}), and (b) SMX – RAM, selenate (SeO_4^{2-}) – RAM, and selenite (SeO_3^{2-}) – RAM

As far as we know, there have been no studies assessing the toxicity of the binary mixtures of the selected chemicals on the algae. Therefore, we are unable to compare our experimental results and predictions with existing literature. Thus, our study, in that regard, seems to be the first study evaluating experimental and predicted toxicity of the binary mixtures of SeO_3^{2-} , SeO_4^{2-} , SMX, and RAM.

4.6. Risk Assessment

PNEC and MECs were evaluated as part of the regulatory assessment of test chemicals and their binary mixtures. PNEC values are calculated to evaluate the concentration of test chemicals in freshwater that won't cause any damage to aquatic organisms. To determine the PNEC, the NOEC or short-term (acute) LC_{50}/IC_{50} value is divided by a pertinent AF. Following the guidelines mentioned in REACH Chapter 10, AF values change between 10 to 1000 depending on the availability of the data (ECHA, 2008). In the case of fresh water, the PNEC is determined by taking the lowest value of the IC_{50} , LC_{50} , or NOEC and dividing it by the proper AF for each aquatic organism. In other words, the data's PNEC value, representing the organism with the greatest sensitivity, is selected.

We reviewed the literature for the toxicity of test chemicals to the species from three trophic levels in freshwater ecosystems. The reported LC/IC_{50} and NOEC values for different species of algae, fish, and *Daphnia magna* from the literature are depicted in Table 4.7. For SMX, since there is chronic toxicity data (e.g., NOEC) for three trophic levels, an AF of 10 is used for PNEC calculation. For SeO_4^{2-} , SeO_3^{2-} , and RAM a full base set (fish, invertebrate, algae) short-term toxicity study is available, so the AF of 1000 is used for PNEC calculation of these chemicals. Among all data in Table 4.7, the maximum and minimum IC_{50} s and NOECs, the calculated PNEC values from the minimum LC/IC_{50} and NOEC values, and proper AF for each single chemical and their binary mixtures are given in Table 4.9.

We utilized the PNEC values to assess the risk associated with the test chemicals. In this respect, as a case study, the measured environmental concentrations (MECs) of the single chemicals from different local areas were obtained from previously published sources to decide whether the environmental concentrations of the test chemicals can pose a risk to aquatic organisms. For the freshwater bodies in which different concentrations of the chemicals were detected on different sides, we considered the mean values of them. Apart from such situations, we took just the reported concentrations of the chemical compounds in a certain freshwater body.

All the MECs that we found in the literature are given in Table 4.8. As a result, among all the data, we took the highest MECs of the single chemicals for the calculation of risk factors. The maximum and minimum MECs are taken from Table 4.8 and are given in Table 4.9.

As far as we know, there has not been any study in the literature showing the omnipresence of the tested chemicals in aquatic environments. Hence, for the risk status of the binary mixture of the chemicals, we summed the measured concentrations of the single chemicals, thinking of the scenario

if the detected amounts of the single chemicals in a natural aquatic environment were found to be together, what will be the risks? Considering this scenario, the risk factor (RF) calculation for chemical mixtures is based on the sum of the MECs of the single chemicals and the calculated PNEC values. The highest MEC data from the literature is divided by the PNEC values obtained for single and binary mixtures of the test chemicals to calculate RF showing the risk status of them in aquatic environments (Table 4.9). Lastly, the toxic effect of selenite and selenate salts on *C. vulgaris* is calculated based on their Se content in this study. Therefore, the selenite and selenate risk status are calculated using the detected local selenium concentration.

In our study, except for RAM, all single substances are categorized as risky ($RF > 100$) The RF increased when we assumed that these chemicals were found in a freshwater body with the detected amounts, and so, the mixtures also have a risky status. The risk status of SMX calculated from both acute and chronic data results in very high RFs. Therefore, special concern should be given to this antibiotic. On the other hand, Straub (2016), based on MECs and chronic NOECs/EC₁₀s (with an AF of 10 for PNEC calculation) indicates that except for some specific locations such as WWTP effluents, there is a low risk stem from SMX existence to surface water. Isidori et al. (2005) reported no risk for aquatic organisms arising from SMX using the acute toxicity values (EC₅₀s) for PNEC calculation (with an AF of 1000), and MECs. Li et al. (2019) and Liu et al. (2020) reported long-term risk status (chronic toxicity) of SMX in surface water as an RF of between 158.09 -6989, and 5964, respectively. In that regard, our study is consistent with more recent studies. What we see from the literature is more studies done on the toxicity of SMX, and the concentration of it in aquatic bodies increases over time because the recently reported MECs are higher than those in the past (Duan et al., 2022). Therefore, as the concentration increases the risk status of SMX increases as well.

Table 4.7. E/LC₅₀s, and NOEC of SMX, SeO₄²⁻, SeO₃²⁻, and RAM reported in the literature for algae, daphnia magna, and fish.

Chemicals	Species Group	Species	Test end-point	Test duration	E/LC ₅₀ (mg L ⁻¹)	NOEC (mg L ⁻¹)	References
SMX	Algae	<i>Chlorella vulgaris</i>	Growth inhibition	96 h	10.12	3.125	This study
		<i>Chlorella vulgaris</i>	Growth inhibition	48 h	0.98		Borecka et al. (2016)
		<i>Chlorella vulgaris</i>	Growth inhibition	72 h	1.51		Borecka et al. (2016)
		<i>Chlorella vulgaris</i>	Growth inhibition	96 h	47.9	12.5	Aderemi et al. (2021)
		<i>Chlorella vulgaris</i>	Growth inhibition	96 h	47.36		Zhang, T. et al. (2023)
					(10.12 < 48.36) ^a		
		<i>Raphidocelis subcapitata</i>	Growth inhibition	96 h	1.53	0.614	Eguchi et al. (2004)
		<i>Raphidocelis subcapitata</i>	Growth inhibition	72 h	0.52		Isidori et al. (2005)
		<i>Raphidocelis subcapitata</i>	Growth inhibition	96 h	0.49		Zhang et al. (2021)
		<i>Raphidocelis subcapitata</i>	Growth inhibition	96 h	0.612		Chang et al. (2022)
		<i>Scenedesmus obliquus</i>	Growth inhibition	96 h	0.12		Xiong et al. (2019a)
		<i>Skeletonema costatum</i>	Growth inhibition	96 h	0.93		Feng et al. (2024).
		<i>Phaeodactylum tricornutum</i>	Growth inhibition	96 h	4.65		Feng et al. (2024).
		<i>Pseudokirchneriella subcapitata</i>	Growth inhibition	96 h	0.146	0.09	Ferrari et al. (2004)
	Crustacean	<i>Daphnia magna</i>	Mortality	48 h	> 100		Ferrari et al. (2004)
		<i>Daphnia magna</i>	Genotoxicity	24 h	25.20		Isidori et al. (2005)
		<i>Daphnia magna</i>	Immobility	48 h	189.2		Kim et al. (2007)
		<i>Daphnia magna</i>	Immobility	96 h	177.3		Kim et al. (2007)
		<i>Daphnia magna</i>	Immobility	48 h	123.1		Park & Choi (2008)

Table 4.7. continued

Chemicals	Species Group	Species	Test end-point	Test duration	E/LC ₅₀ (mg L ⁻¹)	NOEC (mg L ⁻¹)	References
SMX	Crustacean	<i>Daphnia magna</i>	Mortality	48 h	234.18		Lu et al. (2013)
		<i>Daphnia magna</i>	Reproduction	21 days	3.30		Lu et al. (2013)
		<i>Daphnia magna</i>	Ingestion rate	21 days	1.29		Lu et al. (2013)
		<i>Daphnia magna</i>	Immobility	48 h	43.97		Drzymala & Kalka (2020)
	Fish	<i>Danio rerio</i>	Mortality	96 h	> 100		Isidori et al. (2005)
		<i>Danio rerio</i>	Mortality	10 d		> 8	Ferrari et. al (2004)
		<i>Oryzias latipes</i>	Mortality	96 h	562.5		Kim et al. (2007)
SeO₄²⁻	Algae	<i>Chlorella vulgaris</i>	Growth inhibition	96 h	0.75		This study
		<i>Chlorella vulgaris</i>	Growth inhibition	14 days	322.7		Pastierová et al. (2009)
		<i>Chlorella pyrenoidosa</i>	Growth inhibition	96 h	0.79		Bennett (1988)
		<i>Chlorella reinhardtii</i>	Growth inhibition	96 h	0.032		Geoffroy et al. (2007)
		<i>Scenedesmus quadricauda</i>	Growth inhibition	96 h	33		Vítová et al. (2011)
		<i>Chlorella reinhardtii</i>	Growth inhibition	96 h	0.355		Fournier et al. (2010)
		<i>Chlorella sorokiniana</i>	Growth inhibition	168 h	45		Gojkovic et al. (2014)
	Crustacean	<i>Daphnia magna</i>	Body weight	48 h	5.3		Dunbar et al. (1983)
		<i>Daphnia magna</i>	Mortality	48 h	4.07		Martins et al. (2007)
	Fish	<i>Pimephales promelas</i>	Mortality	96 h	11.8		Adams & Johnson (1981)

Table 4.7. continued

Chemicals	Species Group	Species	Test end-point	Test duration	E/LC ₅₀ (mg L ⁻¹)	NOEC (mg L ⁻¹)	References
SeO ₃ ²⁻	Algae	<i>Chlorella vulgaris</i>	Growth inhibition	96 h	6.68		This study
		<i>Chlorella vulgaris</i>	Growth inhibition	96 h	5.48		U.S. EPA (1987).
		<i>Chlorella vulgaris</i>	Growth inhibition	14 days	73.2		Pastierová et al. (2009)
		<i>Chlorella vulgaris</i>	Growth inhibition	168 h	30		Zhang, Z. et al. (2023)
				(6.68 > 5.48) ^a			
		<i>Chlorella reinhardtii</i>	Growth inhibition	96 h	6.3		Morlon et al. (2005)
		<i>Scenedesmus quadricauda</i>	Growth inhibition	96 h	4		Vítová et al.'s (2011)
		<i>Scenedesmus quadricauda</i>	Growth inhibition	96 h	12.24		Kramárová et al. (2012)
		<i>Haematococcus pluvialis</i>	Growth inhibition	96 h	24.02		Zheng et al. (2017)
		<i>Chlorella pyrenoidosa</i>	Growth inhibition	96 h	15.37		Zhao et al. (2019)
		<i>Pseudokirchneriella subcapitata</i>	Growth inhibition	96 h	3.0		Al-Hasawi et al. (2020)
	Crustacean	<i>Daphnia magna</i>	Immobility	48 h	0.47		Adams & Johnson (1981)
		<i>Daphnia magna</i>	Body weight	48 h	1.1		Dunbar et al. (1983)
		<i>Daphnia magna</i>	Mortality	48 h	3.02		Martins et al. (2007)
	Fish	<i>Danio rerio</i>	Mortality	96 h	23		Adams & Johnson (1981)
		<i>Pimephales promelas</i>	Mortality	96 h	10.9		Adams & Johnson (1981)
		<i>Oncorhynchus mykiss</i>	Mortality	96 h	4.4		Adams & Johnson (1981)
		<i>Sander vitreus</i>	Mortality	96 h	11.7		Mauk & Brown (1999)
		<i>Danio rerio</i>	Mortality	120 h	0.55		Mal et al. (2017)

Table 4.7. continued

Chemicals	Species Group	Species	Test end-point	Test duration	E/LC ₅₀ (mg L ⁻¹)	NOEC (mg L ⁻¹)	References
Ramipril	Algae	<i>Chlorella vulgaris</i>	Growth inhibition	96 h	239 ^b		This study
		<i>Pseudokirchneriella subcapitata</i>	Growth inhibition	96 h	>100		Merck (2023)
	Crustacean	<i>Daphnia magna</i>	Immobility	48 h	>100		Merck (2023)
	Fish	<i>Danio rerio</i>	Mortality	96 h	>100		Merck (2023)

a: 96 h EC₅₀ value of the tested chemicals for *C. vulgaris* obtained from this study is compared with the same studies in the literature

b: Predicted by ToxCalc. Experimentally up to 100 mg L⁻¹ RAM did not show toxicity to *C. vulgaris*, so it has a EC₅₀ of > 100 mg L

Table 4.8. The measured environmental concentrations (MECs) of Se, RAM, and SMX in the aquatic environments of different regions.

Compound	Detected area	Country	MEC ($\mu\text{g L}^{-1}$)	References
Se	Southern Manitoba, surface waters	Canada	11.6	Hu et al. (2009)
	Ziyang of Shaanxi province, drinking water	China	7.85	Cui et al. (2017)
	Rivers of Shaanxi province	China	5.43*	Du et al. (2018)
	Olifants River	South Africa	207.8	Genthe et al. (2018)
	Sirmaur district, groundwater	India	2.00 – 4475.0	Kashyap et al. (2018)
	Oppdal district, stream water	Norway	0.15	Reimann et al. (2018)
	Vulture Lake, East Basin	Canada	2.74	Peixoto Mendes et al. (2022)
	Danube River	Croatian part	0.1683	Špoljarić Maronić et al. (2024)
RAM	City of Gdansk, WWTP effluents	Poland	0.06*	Kot-Wasik et al. (2016)
	City of Dresden, WWTP effluents	Germany	0.092*	Gurke et. al (2015)
	City of Dresden, WWTP influents	Germany	0.114.3*	Gurke et al. (2015)
	Dartmouth district of Nova Scotia, sewage treatment plant (STP)	Canada	0.102	Ghoshdastidar et al. (2015)
	Several STPs	Canada	< 6.05*	Greenham et al. (2019)
SMX**	Belém and Barigui Rivers	Brazil	771	Böger et al. (2021)
	João Mendes River	Brazil	743	Sabino et al. (2021)
	Hai river system	China	157	Chen et al. (2018)
	Weihe River	China	70.8	Wang et al. (2019)
	Dhaka city	Bangladesh	190.5	Angeles et al. (2020)
	Erlong Lake	China	771.4	Li et al. (2019)
	Xiaoqing River	China	78.3	Li et al. (2020)
	Yitong River	China	551	Yu et al. (2019)
	Shandong Province, drinking water	China	2.09	Hanna et al. (2018)
	Charmoise River, France	France	576	Dinh et al. (2017)

Table 4.8. continued

Compound	Detected area	Country	MEC ($\mu\text{g L}^{-1}$)	References
SMX**	Kumasi	Ghana	2861	Azanu et al. (2018)
	Kshipra River, India	India	4660	Hanna et al. (2020)
	Lake Victoria, freshwater systems within the lake	Kenya	86500	Kairigo et al. (2020)
	Rivers in Machakos, Nyeri, and Meru	Kenya	11686	Kairigo et al. (2020)
	Nairobi River Basin	Kenya	1800(median)	Kairigo et al. (2020)
	Yeongsan River, Korea	Korea	113.2	Na et al. (2019)
	Navigable streams	USA	28.2	Batt et al. (2016)
	Hanoi, urban lakes	Vietnam	255 (median)	Tran et al. (2019)

* Mean value of the measured concentrations at detected areas.

** All data on the measured SMX concentration in freshwaters are cited in Duan et al. (2022). These are the mean concentrations detected in the relevant areas, and for some median values are taken which are indicated in parenthesis. We directly took MECs of SMX from Duan et al. (2022) study due to being the most recent MECs of it.

Table 4.9. Experimental IC₅₀ of the single chemicals and the binary mixtures with the corresponding PNEC values and the MECs of test chemicals in freshwaters are given. Risk factor (RF) is calculated by dividing MEC by PNEC.

Chemical compound	IC ₅₀ (mg L ⁻¹)		PNEC	MEC (mg L ⁻¹)		Detected area		Country		RF	Risk Status	References
	Min	Max		Min	Max	Min	Max	Min	Max			
Se	N.A ^a	N.A	N.A	0.00017	0.208	Danube River	Olifants River	Croatian part	South africa	N.A	N.A	(Genthe et al., 2018; Maronić et al., 2024)
SeO ₃ ²⁻ - Se	0.47	24.02	0.00047	— ^b	—	—	—	—	—	442	Risky	(Adams & Johnson, 1981; Zheng et al., 2017)
SeO ₄ ²⁻ - Se	0.75 ^c	11.8	0.00075	—	—	—	—	—	—	277	Risky	(Adams & Johnson, 1981; This study)
SMX	0.09^d	>1000	0.009	0.028	86.5	Weihe River	Lake Victoria	China	Kenya	9,611	Risky	(Isidori et al., 2005; Wang et al., 2019; Ferrari et al., 2004; Duan et al., 2022)
RAM	>100	>100	0.2395 ^f	0.00006	0.000092	WWTP effluents	WWTP effluents	Gdanks, Poland	Dresden, Germany	0.0004	N.R ^e	(Gurke et al., 2015; Wasik et al., 2016; Merck, 2023)
SeO ₃ ²⁻ and SeO ₄ ²⁻	1.5	—	0.00015	0.00034	0.416	—	—	—	—	1477	Risky	This study
SeO ₃ ²⁻ and SMX	4.0	—	0.0004	0.0282	86.71	—	—	—	—	35668	Risky	This study
SeO ₄ ²⁻ and SMX	5.9	—	0.00059	0.0284	86.71	—	—	—	—	16315	Risky	This study
SeO ₃ ²⁻ and RAM	7.2	—	0.00072	0.00023	0.02089	—	—	—	—	202	Risky	This study
SeO ₄ ²⁻ and RAM	17.0	—	0.0017	0.00023	0.02089	—	—	—	—	659	Risky	This study
SMX and RAM	35.7	—	0.00357	0.0283	86.50009	—	—	—	—	11976	Risky	This study

^a: Not applicable; ^b: Not available; ^c: The lowest IC₅₀ for selenate toxicity obtained from this study; ^d: NOEC value; ^e: Not risky; ^f: The estimated IC₅₀ value of RAM calculated in this study used for the PNEC calculation

4.6.1. Acute-to-Chronic Ratios (ACRs)

ACRs are utilized to forecast chronic toxicity in aquatic organisms in cases where chronic data are absent, limited, or indefinite, but acute toxicity is known (Hoff et al., 2010). ACRs are usually calculated for specific species for which both acute and chronic data are available. These ratios are then employed to estimate chronic toxicity to a different species exposed to the same compound. Since a matched acute and chronic value from the same laboratory is not commonly accessible, there is a significant variation among the reported ACRs in the literature (Hoff et al., 2010). Therefore, as an alternative, fixed values can be used instead of ACRs that are calculated based on chemical and species-specific data. Certain regulatory programs, such as the Methodology Document for Ecological Structure-Activity Relationship Model (ECOSAR) by the U.S Environmental Protection Agency set an ACR value of 4 for green algae using the average of their data set based on the 72/96h IC_{50} s and Chronic values ($ChV = MATC = \sqrt{(NOEC)(LOEC)}$) (ECOSAR, 2012). The average of ACRs based on the test results, in accordance with the European Union Technical Guidance Document (EU TGD) (2003) is reported to be closer to 5.4 for green algae (Ahlers et al. 2006).

In this study, three types of ACR were used to compare the results with the literature: $ACR_{MATC} = IC_{50}/MATC$, $ACR_{20} = IC_{50}/IC_{20}$ and $ACR_{NOEC} = IC_{50}/NOEC$ (Hoff et al., 2010; EU TGD, 2003) (Table 4.8). If the ACR for algae is less than an application factor of 10, closer to 4, indicating that regulations are conservative for this taxon (Brill et al., 2021).

Table 4.10. The NOEC, LOEC, MATC (ChV) of test chemicals and the relevant ACR values

Chemical	IC_{50} (mg L ⁻¹)	NOEC	LOEC	MATC	IC_{20}	ACR_{NOEC}	ACR_{MATC}	ACR_{IC20}
SeO ₃ ²⁻	6.68	2	4	2.82	3.30	3.34	2.37	2.02
SeO ₄ ²⁻	0.75	0.2	0.4	0.28	0.38	3.75	2.67	1.97
SMX	10.12	3.12	6.25	4.42	5.8	3.24	2.28	1.74
RAM	239.5	80	150	109.5	179.4	2.99	2.18	1.33

5. CONCLUSION

In the presented study, using the green freshwater algae *C. vulgaris* as a test organism, the 96 h toxicity values of four chemicals from different categories (RAM, SMX, SeO_4^{2-} , and SeO_3^{2-}) were investigated by the OECD No. 201 standardized algal growth inhibition test (OECD, 2011). In addition to the particular effects of each chemical, experiments are conducted to understand the characteristics of the interactions (antagonistic, synergistic, and additive) if the test chemicals are simultaneously present in an aquatic medium. Among the four chemicals, SeO_4^{2-} , showed the highest toxicity with an IC_{50} of 0.75 mg L^{-1} . RAM did not inhibit the growth of the algae up to its solubility limit and had an IC_{50} of $> 100 \text{ mg L}^{-1}$, therefore, it is non-toxic to *C. vulgaris*. When binarily mixed, the toxicity of all tested substances, even of RAM, increased. RAM which is not toxic to *C. vulgaris* in single form, gave IC_{50} values of 28.54 mg L^{-1} , 16.72 mg L^{-1} , and 6.15 mg L^{-1} when combined with, SMX, SeO_4^{2-} , and SeO_3^{2-} . Apart from this study, as aforementioned (section 2.1.2), RAM is potentially hazardous to aquatic species from different trophic levels in the presence of other chemicals due to possible synergistic interactions. The simultaneous presence of chemicals with RAM increases the severity of the effect, which will even result in mortality in fish. Therefore, more focus should be placed on the toxicity of chemical mixtures, even if they are not toxic when applied individually. However, there is still a considerable knowledge gap regarding the ecotoxicity of RAM in the literature, so, further studies need to be done on the toxicity of RAM to aquatic organisms, especially for the mixture toxicity of it.

The interaction between the binarily mixed chemicals was synergistic, except for SeO_4^{2-} and SMX combination (additive) considering the TU approach. In other words, the effect of all the mixed chemicals on the growth of algae is greater than the sum of the effect of single compounds, except SeO_4^{2-} - SMX mixture where the effect equals the sum of the effect of single compounds (additive).

The mixture toxicity of the chemicals is also assessed by CA, IA, and CI-isobologram methods. The prediction of the actual toxicity of the mixtures was found to be more robust using the CI method compared to those offered by the CA and IA methods. In addition, the high dependence of CA and IA models on the IC values of each chemical in a mixture makes it difficult to predict toxicity of the mixtures containing a chemical not toxic to test organisms in a single form. In that regard, the CI model may be more applicable for predicting the toxicity of chemicals in mixtures.

The risk factors were calculated for freshwater bodies as a case study using PNEC and data

available in the literature regarding MECs of all chemicals. Except for a single form of RAM, all the single and binarily mixed chemicals have a risky status in freshwater and are in the status of major concern. However, it should be noted that there was no available data in the literature for the concentration of RAM detected in freshwater bodies. Therefore, we used the concentration measured in a certain WWTP effluent for the risk assessment. The reason for being detected in low amounts or not detected in freshwater can be attributed to RAM pharmacokinetics. When enters the body, RAM quickly metabolizes to its active metabolites, such as ramiprilat. Only a few amounts of it (2%) excreted unchanged from the body. Thus, even RAM is among the most prescribed cardiovascular drugs, its MECs in surface waters are lower compared to other cardiovascular drugs.

Acute-to-chronic ratio (ACR) was calculated using the low-toxic-effect concentrations (LOEC, NOEC, and IC20) to check whether the ACR values are within the safe level of chemicals set for algae. The average of ACR_{MATC} , ACR_{NOEC} , ACR_{IC20} are 2.38, 3.33, and 1.76, respectively. The calculated ACR values comply with the proposed safety level of chemicals for algae as specified by ECOSAR (2012).

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APPENDIX A

Table A.1 The initial and the final pH values of the single and mixture toxicity bioassays in 96 h test duration

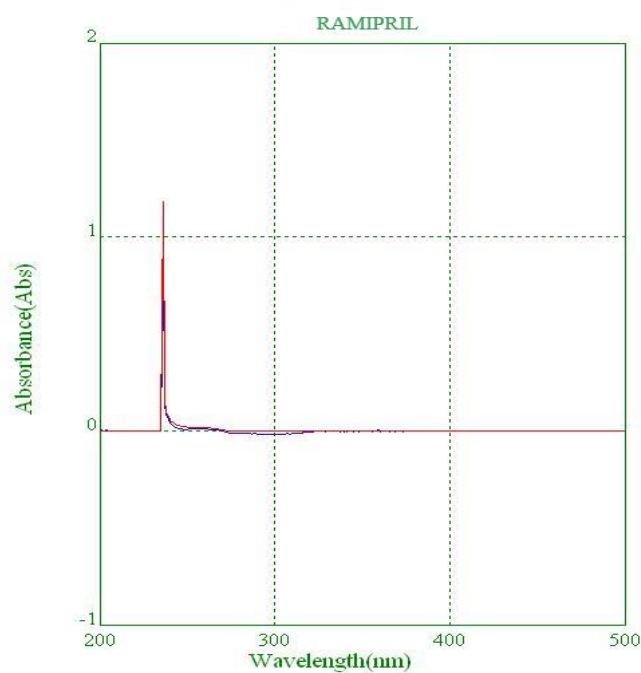
Chemicals	pH (1st day)	pH (4th day)
SeO ₃ ²⁻	6.03	6.20
SeO ₄ ²⁻	5.98	6.08
SMX	6.04	6.32
RAM	6.04	6.30
SeO ₃ ²⁻ – SeO ₄ ²⁻	6.07	6.32
SeO ₃ ²⁻ – SMX	6.01	6.37
SeO ₄ ²⁻ – SMX	6.05	6.40
SeO ₃ ²⁻ – RAM	6.00	6.35
SeO ₄ ²⁻ – RAM	6.05	6.32
SMX – RAM	6.02	6.29

APPENDIX B

Chemical

Spectrum

Ramipril



Sulfamethoxazole

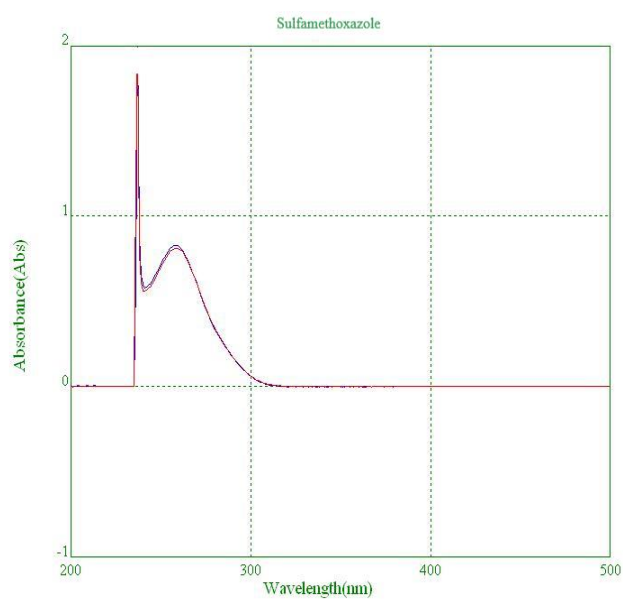
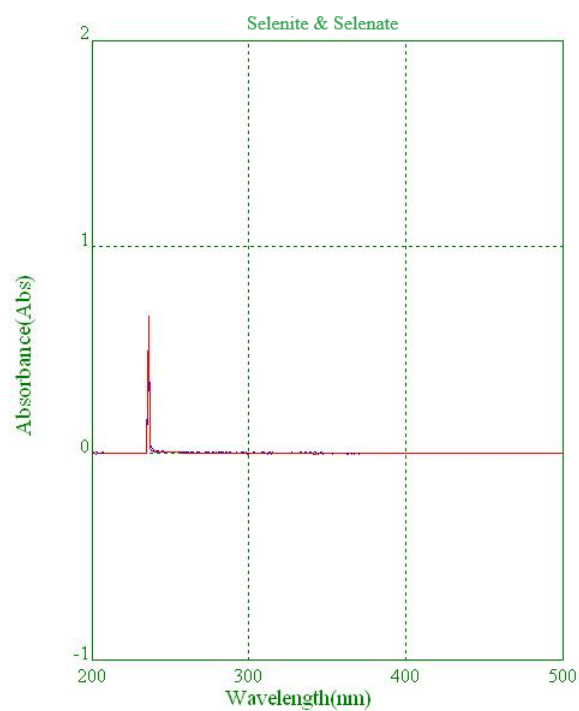


Figure B.1. UV-Vis spectroscopy of ramipril and sulfamethoxazole at concentrations of 40 and 12.5 mg L⁻¹, respectively. Red and blue lines indicate the tested chemicals' first and last day spectrum, respectively.

Chemical**Spectrum**

Selenite and Selenate in mixture



Selenite and Ramipril in mixture

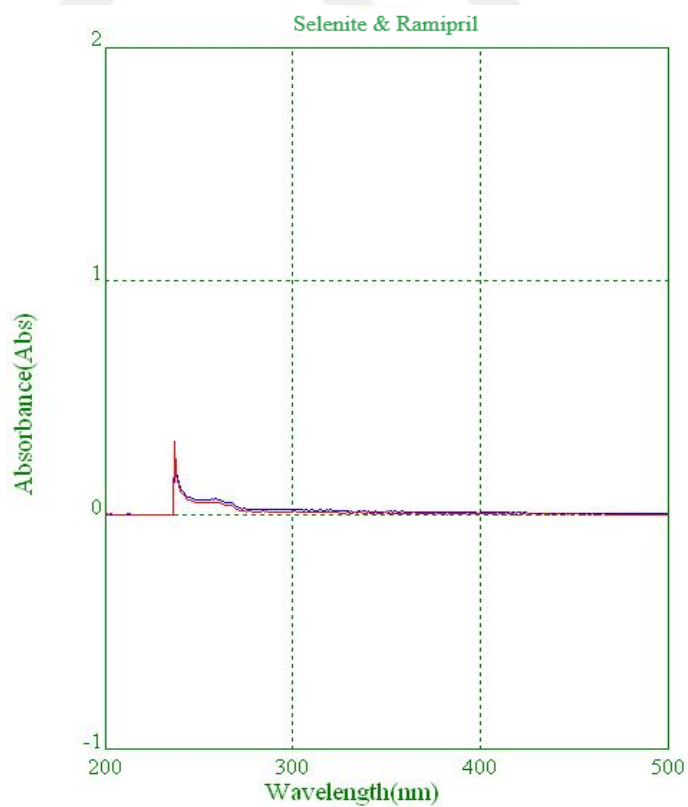
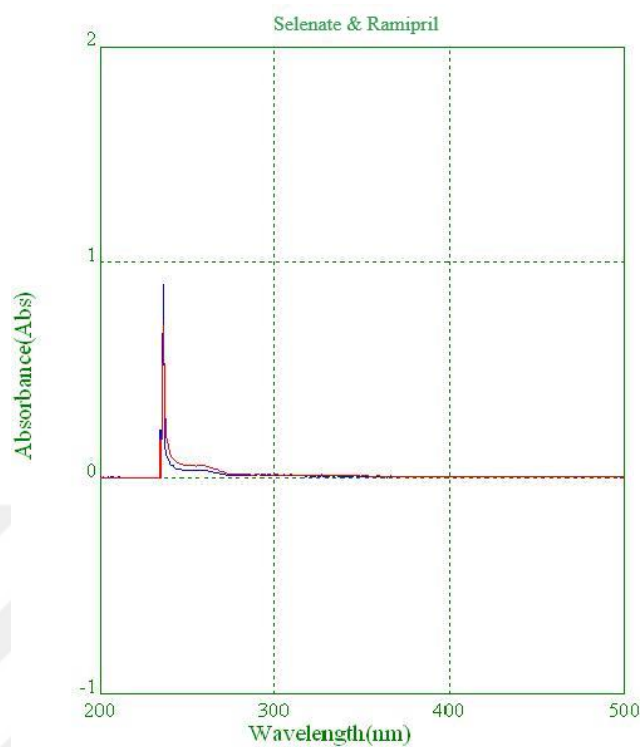


Figure B.2. UV-Vis spectroscopy of binary mixture of selenite and selenate, selenite and ramipril at a concentration of 1 TU. Red and blue lines indicate the chemicals' first and last day spectrum, respectively.

Chemical

Spectrum

Selenate and Ramipril in mixture



Sulfamethoxazole and Selenate in mixture

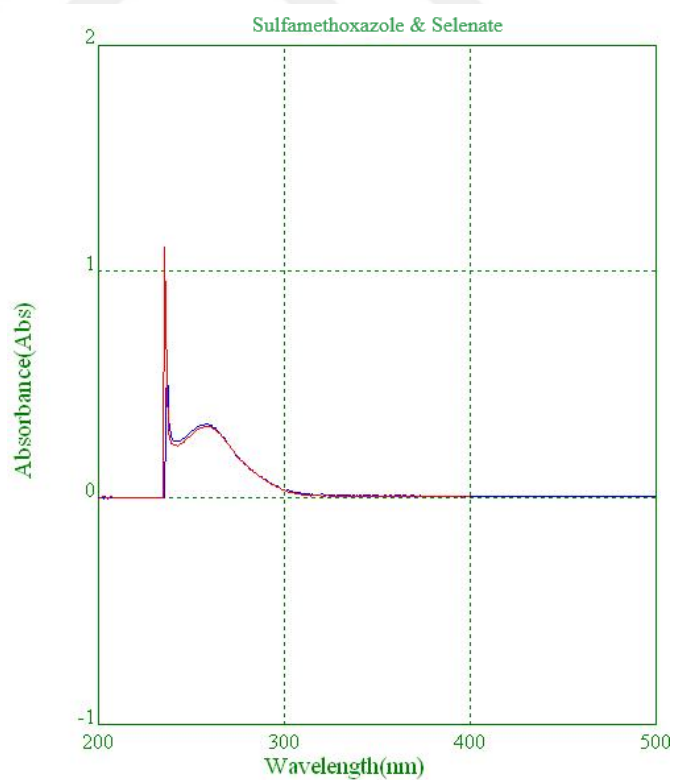
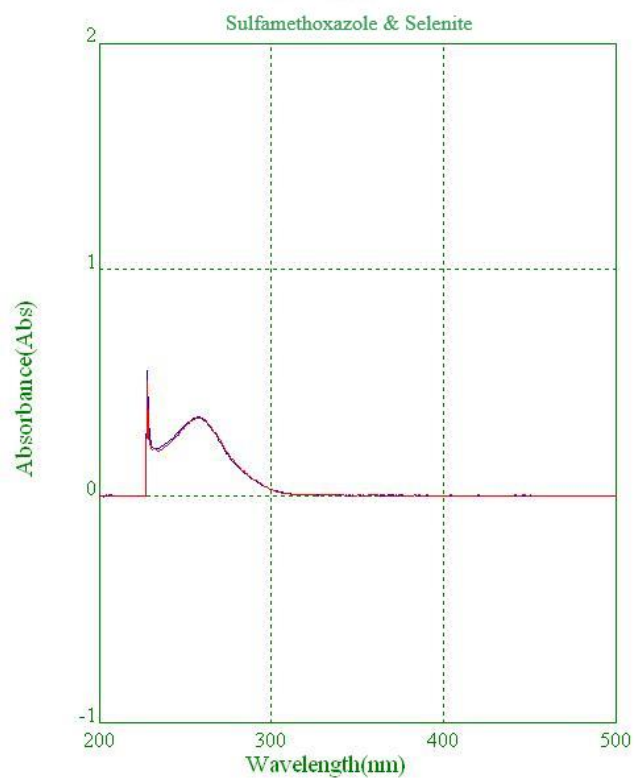


Figure B.3. UV-Vis spectroscopy of binary mixture of selenate and ramipril, sulfamethoxazole and selenate at a concentration of 1 TU. Red and blue lines indicate the chemicals' first and last day spectrum, respectively.

Chemical**Spectrum**

Sulfamethoxazole and Selenite in mixture



Sulfamethoxazole and Ramipril in mixture

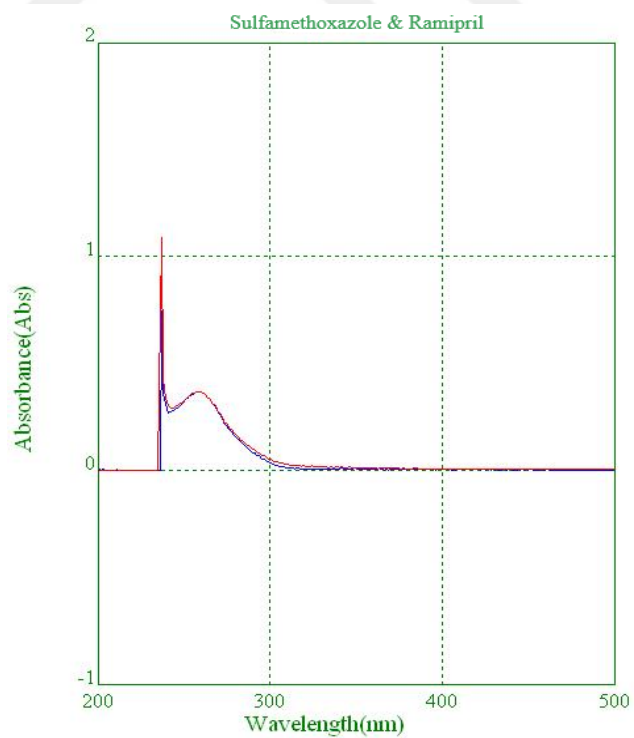
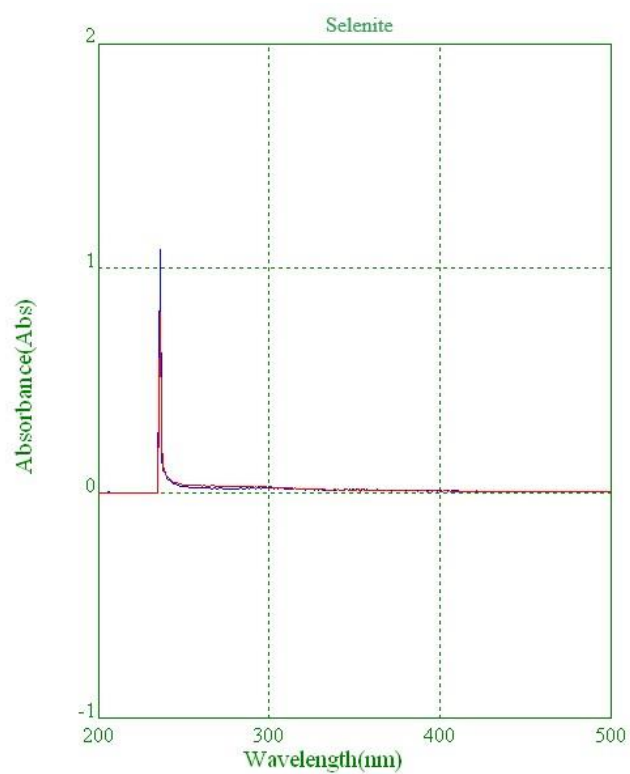


Figure B.4. UV-Vis spectroscopy of binary mixture of sulfamethoxazole and selenite, sulfamethoxazole and ramipril at a concentration of 1 TU. Red and blue lines indicate the chemicals' first and last day spectrum, respectively.

Chemical

Spectrum

Selenite



Selenate

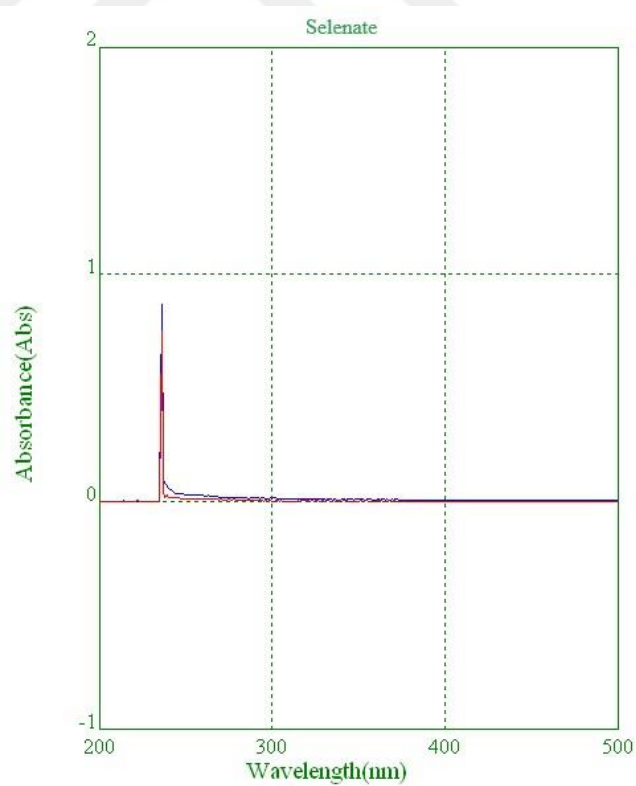


Figure B.5. UV-Vis spectroscopy of selenite and selenate at concentrations of 4 and 0.8 mg L⁻¹, respectively. Red and blue lines indicate the tested chemicals' first and last day spectrum, respectively.