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Polytechnic and Basic Sciences School

Ph.D. School in Chemical Sciences



**Study of urban and industrial wastewater
for the removal of organic pollutants, in
order to reuse the treated waters**

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Abstract

The increasing presence of emerging contaminants and the complexity of industrial effluents represent one of the most pressing challenges in the field of sustainable water management and environmental protection.

Different oxidation/disinfection processes were studied, such as chlorination (currently one of the most widely used) but also innovative advanced oxidation processes (AOP) from a chemical and ecotoxicological point of view. The research mainly focused on the study of by-products generated by the oxidation of various model emerging pollutants generally found in wastewater. Two different organic molecules used in sunscreens to absorb harmful UV rays, namely Ethylhexyl triazone and Octocrylene, were studied, which, following classical treatment with hypochlorite, showed the formation of even more toxic by-products, demonstrating how the process with hypochlorite, although necessary to reduce the bacterial load in the effluent, can lead to the generation of by-products that are extremely more toxic than the starting molecules. Subsequently, other model molecules such as sartans, Losartan, Irbesartan, Valsartan, were studied, investigating different processes, both classical and advanced oxidation through Fenton processes, with oxidants such as peroxymonosulphate and percarbonate catalytically activated by iron.

It was shown how these advanced processes lead to the formation of by-products that are in any case toxic to the species tested, except for the Fenton-like process with Fe(II)-activated peroxymonosulphate, which led in the case of Valsartan to potentially less toxic products. In addition, an innovative AOP process was investigated for wastewater treatment based on the use of hypochlorite and sunlight/UVA on another emerging pollutant, Nimesulide. It has been shown that treatment in the absence of light at near-neutral pH can also degrade the pollutant in wastewater in a short time only in the presence of UVA or sunlight, resulting in sub-pipelines at an advanced oxidation stage, characterized by high-resolution mass analysis, and a clear reduction of organic carbon in solution compared to the process in absence of light.

Regarding industrial wastewater, processes to reduce the toxicity of vegetation water, generated by the oil industry, were studied by means of classical chemical, adsorption and precipitation processes that showed, through ecotoxicological tests, the attractiveness of flocculation processes to decrease its toxicity with respect to a model organism. Finally, a non-destructive multivariate chemometric (partial least square regression) method via Near Infrared spectroscopy (NIRs) was developed for the on-line control of certain tanning substances used by the tanning industry such as synthetic and natural tannins, glutaraldehyde, Cr(III) and even innovative tanning agents such as zeolites. The method enables the evaluation of the concentration of substances used during the tanning process through diffuse reflectance NIRS analysis of liquid solutions. This allows for direct monitoring without the need for sample pretreatment, thereby optimizing the process by assessing concentrations in real time. Consequently, it could improve the efficiency of chemical usage, reduce waste generation, and minimize the environmental impact of the pollutants involved.

This PhD thesis aims to identify and optimise more sustainable treatment approaches that are effective in abating industrial wastewater pollution. The goal is to provide a solid scientific basis and tools to support a more conscious management of water resources, to reduce the environmental impact of production processes and to ensure better protection of biodiversity and human health.

Chapter 1: Water Resources, Wastewater, and Pollution: An Overview

1.1 The Global Water Crisis

Water, so much associated with life and the survival of the humans, has always been threatened by population growth, industrialisation processes and climate change. The Global Commission on the Economics of Water identifies water crises as a major global risk, since water is the basis of economic stability, environmental sustainability and human health. There are two major challenges facing the world with regarding water: sufficient supply and maintaining its quality.

Over the last century, water use has steadily increased, mainly due to technological and industrial development, which in turn leads to increased pollution of water bodies, thus worsening the supply of clean water. For this reason, freshwater, approximately 2.5% of the total water availability on Earth, is unequally distributed and more and more limited by human activities.

According to the Global Commission on the Economics of Water (2023), by 2050 we may face a global crisis due to the scarcity of available freshwater. While 50 to 100 litres per person per day are essential for health and hygiene, at least 4,000 litres per person are necessary for adequate nutrition and consumption. This water crisis could result in a global average Gross domestic product (GDP) loss of 8% by 2050, with lower-income countries potentially suffering losses of up to 15%, alongside even greater economic consequences beyond that. Water is not only the medium for many biological activities, but also an essential resource for agriculture, industry and domestic consumption. Although the hydrosphere is vast, it contains only a small portion that is accessible and suitable for human use, so efficient and sustainable management of water resources is an urgent priority.

1.2 The Global Water Cycle and Availability of Freshwater

The hydrological cycle or global water cycle is the relentless movement of water across, above and below the Earth's surface. It includes various processes of evaporation, condensation, precipitation, infiltration and runoff that occur with water. These represent the natural purification and redistribution of water. However, geographical, climatic and temporal factors only allow for a limited availability of fresh water. Water-rich regions often face management and protection challenges, while water-poor regions face access and sustainability issues.

1.3 Wastewater: Definition and Sources

Wastewater, or sewage, is a polluted water produced by human activities, which changes the initial parameters of the liquid, leading to an increase of the concentration of pollutants present, both organic and inorganic substances, which can be harmful to health and the environment. There are various categories of wastewater:

- **Civil wastewater:** from residential areas and deriving mainly from human metabolism and domestic activities, it includes water from sinks, showers, toilets, and laundry. It may contain a mixture of organic and inorganic matter, pathogens, nutrients, and various chemicals used in household products.
- **Industrial wastewater:** from industrial plants or commercial activities. It can vary significantly depending on the type of industry and its specific activities. Often contains high concentrations of hazardous substances, including heavy metals, solvents, and other toxic chemicals.
- **Zootechnical wastewater:** from livestock farms, including solid and liquid waste from domestic and farm animals.
- **Meteoric and runoff water:** originating from atmospheric precipitation which, not evaporated or absorbed by the soil, exerts a surface runoff action

There are different parameters that can characterise wastewater, but in detail the pollution loading present is characterised by five main parameters:

- **COD:** Chemical oxidation Demand, understood as the amount of oxygen expressed in mg required to chemically oxidise all substances present (organic and inorganic).
- **BOD:** Biochemical oxidation demand is defined as the amount of oxygen required by aerobic bacteria to oxidise the material in the effluent. The BOD/COD ratio is an index of the biodegradability of the effluent.
- **OD:** dissolved oxygen, is a parameter that indicates the amount of dissolved oxygen, a parameter closely related to the fitness for life of a water body.
- **ST:** Total solids is a parameter indicating the presence of suspended solids in solution. Divided in turn into settleable and dissolved solids.

1.4 Water Pollution: Causes and Consequences

Water pollution occurs when harmful substances, such as chemicals, microorganisms, or waste materials, enter water bodies and degrade water quality, rendering it unsafe for human consumption and harmful to the environment. The major sources of water pollution include agricultural runoff, industrial discharges, and untreated or partially treated urban wastewater. The impact of water pollution is far-reaching, affecting ecosystems, human health, and the economy.

The consequences of water pollution can be acute or chronic, depending on the nature and concentration of pollutants. Acute pollution events can cause immediate harm, such as fish kills or the contamination of drinking water supplies. Chronic pollution, on the other hand, may lead to long-term degradation of ecosystems, bioaccumulation of toxic substances in the food chain, and persistent public health issues.

1.5 Emerging Contaminants: A New Challenge

The development of new commercial products is often poorly connected to reducing the risks posed by the substances used and their potential impact on the environment. The increase in global production over the past centuries has frequently led to the introduction of pollutants, often Persistent Organic Pollutants (POPs), which have polluted local environments for many years before being recognized as potentially harmful substances and subsequently removed from the market or at least subjected to usage restrictions. Particularly, scientific attention has surged in recent years toward studying a novel category of contaminants known as "emerging contaminants" (ECs) (Kumar et al., 2023; Wickham et al., 2021; Yu et al., 2024). The term “emerging” is correlated with the increasing concerns about the environmental threats to fauna, flora and human beings. Over the years, the scientific community has thus began studying these substances, evaluating their possible impact. Emerging pollutants have been defined by the NORMAN network (Network of Reference Laboratories, Research Centres, and Related Organisations for Monitoring of Emerging Environmental Substances) as “Emerging pollutants can be defined as pollutants that are currently not included in routine monitoring programmes at the European level and which may be candidates for future regulation, depending on research on their (eco)toxicity, potential health effects and public perception and on monitoring data regarding their occurrence in the various environmental compartments” (*Emerging Substances, NORMAN, 2024*).

The presence of these substances in the environment is primarily due to direct or indirect release. For example, certain pesticides can directly reach groundwater after being applied to plants, infiltrating through the soil. The second reason is the incomplete removal of these substances from industrial or urban wastewater before its discharge into surface waters. This second reason is directly related to the design of treatment plants, which are closely tied to national and international regulations for wastewater discharge into surface waters. These regulations do not set legal limits for

such new substances, leading to irreversible pollution, often with severe consequences for local populations. A practical example of this is pollution from Perfluorooctanoic acid (PFOA), now considered a ubiquitous and persistent pollutant, present in very low concentrations in the blood of nearly the entire global population (Calafat et al., 2007; Lau et al., 2007; Nicole, 2013) due to pollution from companies that had no emission limits for surface water discharge (Wolfe, 2020).

To the “family” of emerging pollutants belong numerous substances pertaining to different categories.

1.5.1 Biocides (BIOCID) and Plant Protection Products (PPP)

Biocides are considered any toxic substances for microorganisms, such as insecticides and pesticides, that are not harmful to animal or plant tissues. Pesticides are defined as any substance used to prevent, destroy, repel, or mitigate any pest (Abubakar et al., 2020). The use of these substances is closely tied to technological innovation and the growth of the global population. Specifically, pesticides help achieve higher crop yields and prevent product loss due to infectious agents such as molds and insects. Pollution from these substances is strongly associated with leaching from agricultural soils (Flury, 1996). In fact, in most cases, only 1% of the active ingredient performs its intended function, while the remaining 99% disperses into the environment or degrades (Intisar et al., 2022), exerting its lethal effects on other species that are important for biodiversity and pollination (Ward et al., 2022; BBC NEWS | Americas | Vanishing Bees Threaten US Crops, 2007).

1.5.2 Drugs of Abuse (DOA)

The presence of illicit drugs in the environment is a topic of great interest to the scientific community. Various illicit drugs, such as cocaine, amphetamines, heroin,

and fentanyl, once consumed by users, are often excreted unchanged and can reach aquatic environments through wastewater discharge, as wastewater treatment processes often fail to completely remove them (Falfushynska et al., 2024). Furthermore, a European report on the illicit drug market has shown that the European market is constantly growing, reaching a total value of €30 billion (EU Drug Markets Report 2019). The presence of illicit drugs in surface waters poses a serious threat to aquatic species. A study conducted by Capaldo et al. (2018) demonstrated that even environmental concentrations of cocaine as low as 20 ng/L have harmful effects on the skeletal muscle of the European eel (*Anguilla anguilla*). Considering that new illicit drugs are introduced to the market each year, over time, new emerging pollutants from this category can reach surface waters, particularly impacting aquatic species.

1.5.3 Food Additives (FOODA)

Food additives are substances that are not consumed as such but are added to various consumer goods for industrial and technological purposes. A vast number of substances fall into this category, including colorants, preservatives, sweeteners, stabilizers, and antioxidants. Most of these substances, once ingested, undergo only partial biological modifications and are excreted unchanged, posing a serious threat to the environment (Lempart-Rapacewicz et al., 2023). Among these substances are artificial sweeteners, synthetic or natural compounds that impart a sweet taste to a variety of products, mimicking the flavour of traditional sugar. They are mainly used in food and beverages such as soft drinks, desserts, dairy products, sweets, chewing gum, and low-calorie or weight-control products. Many of these substances, particularly artificial ones such as sucralose, aspartame, and acesulfame-K, pose environmental risks due to their widespread presence in the environment (Scheurer et al., 2009; Yu et al., 2023), but also due to the potential formation of photoproducts generated by interaction with sunlight. These photoproducts can be up to 500% more toxic than the original compounds (Sang et al., 2014).

1.5.4 Metals and Their Compounds (MET)

Metals present in the environment, especially in aquatic ecosystems, are a key factor in the toxicity of surface waters, particularly impacting aquatic species. Heavy metals such as mercury, cadmium, copper, lead, and zinc tend to bioaccumulate in tissues, moving up the food chain, and pose significant health risks to fish and other aquatic organisms. They tend to weaken the immune system and cause severe pathological issues (Authman, 2015). A classic example of heavy metal pollution and its effects is the use of lead-based fuel additives. Lead was introduced into the atmosphere primarily due to its use in gasoline as tetraethyl lead, starting in the 1970s. This led to a significant increase in pollution and subsequent severe impacts on human health, as even low-dose exposure can induce various harmful effects (Javorac et al., 2021).

In recent years, technological advancements have led to a significant increase in the use of rare earth elements, specifically scandium, yttrium, and the lanthanides, which are highly valued for industrial, medical, agricultural, and zootechnical applications. The use of these substances and their continuous release into the environment have resulted in increased pollution levels, with these metals being detected even in biological samples from exposed individuals (Pagano et al., 2019). Moreover, preliminary studies have indicated that they may be potentially harmful to some living species (Pagano et al., 2015).

1.5.5 Plastic Additives (PLAST)

Plastic additives are substances used in the production of polymer plastics to enhance or modify their properties according to specific application requirements. Plastic additives are mainly used as plasticizers, flame retardants, stabilizers, antioxidants, and pigments. These chemicals are incorporated into plastics, from which they can leach out as most of them are not chemically bound. The primary substances in this

category include polybrominated diphenyl ethers (PBDEs), phthalates, nonylphenols (NPs), and bisphenols. Research conducted over the years has shown that many of these substances are endocrine-disrupting chemicals, leading to an increased risk of cancers, impaired immune function, early puberty, obesity, and diabetes (L. Zhang et al., 2024; Bridson et al., 2021). This has led to the removal from the market of certain plastic products associated with early childhood and close human contact (Directive 2011/8/EU, 2011; Jeon, 2022).

1.5.6 Per- and Polyfluoroalkyl Substances (PFAS)

PFAS are ubiquitous environmental contaminants, a family of fluorinated organic compounds of anthropogenic origin, now present in nearly every part of the globe due to pollution caused both by consumer use and industrial discharges. PFASs have gained global environmental concern because of their chemical stability and resistance to degradation (Brunn et al., 2023; Cousins et al., 2020). Direct emissions from industrial sources and indirect transport mechanisms, such as atmospheric deposition, contribute to the distribution of PFAS. As evidence of PFAS toxicity has emerged, many countries have developed regulatory measures to restrict PFAS levels in water. To date, the U.S. EPA has established health advisories for PFOA and PFOS in drinking water at 70 ng/L, while many other countries such as Canada, the UK, Sweden, Norway, and Australia have also set guideline values for PFAS in various environmental media (Kurwadkar et al., 2022).

1.5.7 Drinking Water Chemicals (DW)

The so-called drinking water chemicals are a class of substances not directly linked to their pre-existing presence in drinking water, but rather to their formation during water treatment processes, particularly disinfection, which is intended to eliminate harmful bacteria that could pose serious health risks. Water treatment is primarily carried out

using oxidizing and disinfecting agents, which, however, not only react with bacteria but also oxidize natural and anthropogenic molecules present in the water. A classic example of this is the formation of halomethanes because of the reaction between free chlorine disinfectant and mainly humic and fulvic acids. The presence of these substances in drinking water has been documented since the 1970s (Rook, 1972) and today they are included in the list of compounds under close surveillance by regulatory agencies at the national level, due to their harmful effects on human health.

Disinfection processes, however, do not only lead to the formation of halomethanes; given the presence of emerging pollutants in surface waters, water treatment can result in the formation of a plethora of new substances (Yang & Zhang, 2016; Shao et al., 2023), often significantly more toxic than their precursors, with unknown potential health impacts (Richardson & Plewa, 2020; Zarrelli et al., 2012).

1.5.8 Pharmaceuticals (PHARMA)

Numerous studies highlighted the presence of pharmaceuticals and personal care products in both wastewater treatment plant effluents and surface waters (Wilkinson et al., 2022), demonstrating the limited effectiveness of conventional treatment methods in removing these emerging contaminants (Deblonde et al., 2011).

PHARMA substances, as the name suggests, include a vast range of inorganic or organic, natural or synthetic compounds that induce functional modifications in living organisms, acting as remedies for pain or diseases through chemical, physico-chemical, or physical mechanisms. This category encompasses an overwhelming number of substances, such as analgesics (Paracetamol, Ibuprofen), antidepressants (Fluoxetine, Sertraline), antihypertensives (Losartan, Amlodipine), antidiabetics (Metformin, Glimepiride), gastro protectors (Omeprazole, Ranitidine), antihistamines (Loratadine, Cetirizine), and antibiotics (Ciprofloxacin, Amoxicillin).

Extensive literature highlights the presence of these substances in various water-based environmental matrices, including the sea (Tahrani et al., 2015), rivers (Wilkinson et al., 2022), and even drinking water (Charuaud et al., 2018). Their prevalence in the environment has become so widespread that they have been detected in living organisms, where bioaccumulation processes occur (Li et al., 2011).

The environmental contamination of PHARMA substances can be attributed to several factors. First, there is the direct discharge of these compounds from manufacturing plants (Cardoso et al., 2014; Bharti & Bora, 2023). Additionally, pharmaceutical waste from hospitals contributes significantly to this pollution. Other major sources include the use of antibiotics and hormones in livestock farming, runoff from agricultural fields fertilized with treated sewage sludge, human excretions, and improper disposal of expired medications in households.

A particularly noteworthy case within PHARMA is the widespread use of antibiotics, essential molecules for human survival, used to treat bacterial infections or as preventive measures in clinical interventions. However, the continuous and excessive use of these substances has led to the unnatural development of antibiotic-resistant species, especially those that survive in surface waters. This unintentional evolutionary pressure enables only resistant bacteria to thrive, increasing their numbers and posing a significant threat to human health (X. Zhang et al., 2009; Nwobodo et al., 2022; Muteeb et al., 2023). As a result, the pharmaceutical industry is currently focused on developing innovative antibiotics capable of eliminating these resistant strains (Terreni et al., 2021).

1.5.9 Personal Care Products (PCPs)

Personal care products are a category of substances to which belong a multitude of molecules used in formulations such as soaps, lotions, fragrances and sunscreens. Unlike the above-mentioned PHARMA products, PCPs, being mainly used for external use, are directly released into the environment without biological

modification (Ternes et al., 2004). The concern about these substances is closely related to their evident toxicity towards various living organisms (Molins-Delgado et al., 2014). UV filters have been shown to induce coral bleaching (He et al., 2023) and even inhibiting the growth of species at the very bottom of the food chain, by inducing developmental anomalies, altering the growth rate and reproduction, oxidative stress, neuronal defects (Prakash and Anbumani, 2021) accumulating along the food chain (Peng et al., 2017). Microplastics used as dispersing or abrasive agents (e.g. toothpaste, face scrubs) are also present within PCPs in some cases, in fact, it is estimated that from 1970 to 2019, approximately 3.00×10^5 tonnes of microplastics were released into the environment through PCP (Singh & Mishra, 2023).

1.6 The Reuse of Wastewater: Opportunities and Challenges

Given the growing scarcity of freshwater resources, the reuse of treated wastewater has become a critical strategy, particularly in agriculture. In Europe, the reuse of wastewater for irrigation has been formally addressed in Regulation (EU) 2020/741 of the European Parliament, which sets out minimum requirements for water reuse. This regulation emerged in response to recurrent and severe droughts that have significantly reduced agricultural productivity. For instance, the European olive oil production, a key agricultural sector, has seen a dramatic decline—from 2,271.7 tons in 2021/2022 to just 1,392.3 tons in 2022/2023, a reduction of approximately 38%. Reusing wastewater from treatment plants presents a promising solution to mitigate water scarcity. However, it also raises significant concerns. While these waters may meet legal standards, they often contain substances known as emerging contaminants, which are not currently regulated at either the European or national levels. Research is gradually revealing the presence of EPs in treated wastewater and their potential toxicity to both aquatic species and humans. This presents a complex challenge for water reuse strategies, as the long-term effects of EPs on ecosystems and public health are still not fully understood.

1.7 Water Framework Directive

In recent years, due to the growing interest of the scientific community in pollution caused by new substances, national and international legislations have begun to establish control systems for the evaluation of emerging pollutants, aiming to integrate them into regulatory watch lists. For example, Directive 2000/60/EC, known as the Water Framework Directive, introduces, from a legislative perspective, the concept of substances posing risks to the environment and human health, referred to as priority substances, which must be monitored. The first substances added to this list included benzene, polycyclic aromatic hydrocarbons, various pesticides, pharmaceuticals, and metals such as cadmium, lead, mercury, and nickel, for which Environmental Quality Standards (EQS) were established, specifying the concentration limits for water bodies. Through these European laws, such substances have been incorporated into state-level monitoring and control lists.

To monitor newly introduced substances in the environment, Directive 2013/39/EU, Article 8b, introduced a list known as the "Watch List" (Directive - 2013/39 - EN - EUR-LEX, 2013), wherein the European Commission identifies 10 substances to be monitored at the Union level to assess their potential hazards. Depending on the monitoring data collected, these substances may either be added to the priority substances list or excluded. Some of the first substances added to the initial "Watch List" were Diclofenac and 17- α -Ethinylestradiol, due to their already well-documented presence in the environment (Combalbert & Hernandez-Raquet, 2010; Kolpin et al., 2002)

1.8 Wastewater Treatment Plant

The increasing consumption of water by industrial facilities and the high rate of urbanization, resulting in a growing population density, have led to a significant rise in wastewater load over time. This has overwhelmed the natural capacity of surface

waters to self-purify. Wastewater treatment plants are designed to reduce the pollutant load in industrial or urban wastewater, making it safe for discharge into surface waters like rivers, lakes, and seas. The main goal of these plants is to produce effluent with pollutant concentrations that can be managed by the natural self-purification abilities of the receiving body of water. Otherwise, the water quality would be severely compromised.

Wastewater treatment processes are generally divided into pre-treatments, primary, secondary, and tertiary treatments.

1.8.1 Pre-and Primary and Treatments

Pre-treatment involves physical processes to remove larger, floating materials present in the wastewater to prevent damage to subsequent equipment and to avoid releasing coarse materials into surface waters. Common pre-treatment methods include:

- **Screening:** This process removes large solid waste such as plastics, wood, or gravel, which may have entered the wastewater unintentionally.
- **Grit removal:** A faster sedimentation process that helps eliminate heavier solid materials, such as glass, small stones, and sand, not removed by screening.
- **Oil and grease removal:** This takes place in a device called an oil separator, which removes oils, fats, and other substances lighter than water. These materials float to the surface, where they are skimmed off, as their presence could hinder subsequent treatment processes.

For specific industrial wastewater, additional pre-treatments like oxidation (for cyanide removal) or pH adjustment may be necessary to ensure efficient biological processes in the secondary treatment stage (Crini & Lichtfouse, 2018).

Primary treatment is mainly a physical process that removes suspended solids from the wastewater. The wastewater flows into sedimentation tanks where solid particles

settle by gravity, sometimes aided by chemical coagulants like iron or aluminium salts to help flocculate colloidal particles. This stage prevents anaerobic fermentation at the bottom of the tank.

1.8.2 Secondary Treatments

Secondary treatment is used to reduce biodegradable organic matter by aerobic or anaerobic bacteria, followed by secondary sedimentation processes (Zhang et al., 2016). Various biological oxidation processes exist:

- **Aerobic processes:** These involve "activated sludge," which is a suspension of active biomass (in the form of settleable flocs). Oxygen is supplied via compressed air or direct oxygen injection, and the bacteria oxidize the organic matter into CO_2 and H_2O , effectively reducing Biochemical Oxygen Demand and Chemical Oxygen Demand.
- **Anoxic processes:** In these, oxygen is absent, but other electron acceptors like nitrates are present. Denitrifying bacteria use organic matter to reduce nitrates (NO_3^-) into nitrogen gas (N_2).
- **Anaerobic processes:** Occurring in the absence of oxygen, these processes rely on anaerobic bacteria to break down organic matter through fermentation and digestion, converting it into smaller molecules like methane (CH_4) and carbon dioxide. Methane gases can even be utilized to generate electricity.

In biological oxidation processes, the biomass can either be suspended (as in activated sludge) or adhered to a surface (as in trickling filters). Trickling filters consist of large cylinders filled with porous materials (e.g., gravel, sand, or plastic packing media) through which wastewater percolates. In activated sludge systems, the biomass grows in suspension and is oxygenated, allowing aerobic oxidation of the wastewater. In the absence of oxygen, denitrifying bacteria thrive, reducing the total nitrogen content. After the secondary treatment stage, the biomass must be removed through secondary

sedimentation. The sludge is collected, and the clarified water undergoes further treatments before final discharge.

1.8.3 Tertiary Treatments

Tertiary wastewater treatment processes represent an advanced stage of purification, designed to remove additional pollutants and bacteria before the treated water is returned to surface water bodies such as rivers, lakes or seas. Here are some of the main processes used:

- **Adsorption:** Uses adsorbent materials, such as activated carbon, to retain unwanted substances in the effluent. Due to its large surface area, activated carbon is one of the most widely used adsorbents to remove a wide range of contaminants, from metals to chlorinated compounds.
- **Disinfection processes:** They use oxidising or disinfecting chemicals to drastically reduce the bacterial load in the effluent, ensuring that the discharged water does not present a microbiological risk.
- **Reverse osmosis:** This process uses a semi-permeable membrane that acts as a barrier, preventing the passage of impurities in the water. By applying pressure, water is forced through the membrane, retaining dissolved salts, particles, colloids and bacteria. However, the process generates a highly concentrated effluent of pollutants, which must be managed and disposed of.
- **Ozonisation:** This process uses ozone, a powerful oxidant, to degrade organic matter and kill bacteria. Ozone is generated on site using special generators, given its instability. It acts both through direct oxidation and through the production of *Reactive Oxygen Species* (ROS), which are also very oxidising. The main limitation of this technology is the high cost of installation and maintenance.
- **Advanced Oxidation Processes (AOP):** State-of-the-art technologies designed for the complete mineralisation of organic pollutants into CO₂ and H₂O. These processes create extremely reactive oxidant species to degrade

difficult-to-treat organic substances. They are currently used in very few plants worldwide due to their high costs.

These processes represent a crucial technological barrier to ensure the safety of water released into the environment, helping to reduce the impact of industrial and urban pollution.

1.8.4 Limitations and Drawbacks of Conventional Wastewaters Treatments Plants

Traditional wastewater treatment processes present various significant drawbacks. First, these systems are typically designed to meet only the regulatory limits set for surface water quality parameters, which understandably do not account for the control of emerging pollutants. Instead, they focus on meeting thresholds for COD which encompass all oxidizable organic matter present in the wastewater. This approach can allow potentially toxic substances to be released into the environment. Even when present in extremely low concentrations, such substances can have harmful effects on ecosystems, leading to bioaccumulation, which may ultimately pose risks to human health.

Moreover, as highlighted in previous sections and further demonstrated later, the use of conventional oxidants and disinfectants often results in the formation of highly toxic by-products. These include well-known low-molecular-weight organochlorinated compounds (e.g., halomethanes), which are frequently more toxic than the original contaminants and whose effects on the environment remain largely unknown.

For this reason, in recent years, scientific attention has increasingly shifted toward the study of new oxidation processes, known as Advanced Oxidation Processes. These methods show promise in reducing pollutant loads while promoting eco-friendly mineralization, offering a sustainable solution to environmental contamination.

1.9 Focus on Different Disinfection/Oxidation Processes

The disinfection process is the final stage that wastewater undergoes before being discharged into surface waters, with the primary objective of reducing the bacterial load. Various disinfection techniques exist, both chemical and physical. Among the physical methods are photochemical processes that use UV radiation to interfere with DNA and RNA molecules, thereby inactivating them. This method is advantageous from a chemical standpoint, as it avoids the addition of substances that could cause unpleasant odours or tastes in the water (making it a "green" method) while effectively eliminating bacteria and viruses. It disables pathogens, rendering them unable to grow and replicate by damaging their genetic material. However, this application requires high energy consumption, leading to elevated costs. Furthermore, suspended solid particles can shield germs from radiation (Cantwell & Hofmann, 2010). The maximum efficiency of this process has been observed when using high-intensity radiation at a wavelength of 254 nm (corresponding to UV-C rays). Nonetheless, some studies indicate that this method may promote the proliferation of UV-resistant species, leading wastewater bacteria to express increased robustness against irradiation (Süß et al., 2009).

Another physical method for disinfecting water is the use of heat sources; however, in this case, the high temperatures required also result in significant energy consumption and costs.

In addition to physical methods, there are chemical disinfection methods, which mainly rely on oxidizing agents capable of altering the chemical composition of treated water. These methods are broad-spectrum, with low selectivity, and can effectively reduce most microorganisms without significantly increasing the toxicity of the effluents.

Ozone is a highly unstable reactive species that reacts quickly with the organic matter present in the effluent. It is so unstable that it must be generated *in situ* by means of high-voltage electrical discharges in a special machine, known as an ozone generator, which presents considerably high costs for both production and maintenance. The reactivity of ozone is closely linked to its high standard reduction potential (+2.07 V) (Hoigné et al., 1985), but also to the formation of highly oxidising reactive oxygen species ROS, capable of further oxidising the organic matter present in the effluent. The absence of halogens makes it possible to avoid generating highly toxic species such as halomethanes that are normally formed during disinfection processes with chlorine, leading more to the formation of aldehydes and carboxylic acids, oxidising for example the fraction of humic acids presents in the water, tending to increase the biodegradability of these substances, and moreover, once decomposed, only releases oxygen from the water. Thus, in general, the process enables a considerable reduction in COD, BOD, improves the colour and UV absorbance of the products leading to an increase in dissolved oxygen in the effluent (Paraskeva & Graham, 2002).

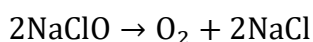
Peracetic acid (PAA) is another highly oxidising molecule currently used to disinfect wastewater (Kitis, 2003); it has a standard reduction potential (1.96 V) higher than that of chlorine 1.36 V, but lower than that of ozone (Ao et al., 2020). Several studies have shown that PAA produces almost no toxic or mutagenic disinfection by-products from the reaction with organic material present in the treated wastewater (Monarca et al., 2002), if not in some cases mostly carboxylic acids, aldehydes and a few disinfection by-products interacting with amino acids, phenols, and other aromatic substances (Booth & Lester, 1995), generally not mutagenic. One of the problems encountered with this disinfection technique is the downstream formation of acetic acid, which tends to increase the COD of the effluent, allowing the reformation of bacteria (Luongo et al., 2020).

Chlorine water treatment is the most widely used method for disinfection of both wastewater and potabilization processes; chlorine can degrade all species of

microorganisms present, it is the best disinfectant, germicide, algicide and anti-mildew agent. There are different oxidising agents that release active chlorine in solution, acting as disinfectants, e.g. sodium hypochlorite, chlorine dioxide, chlorine amines, chlorine gas. Although these substances are less oxidising than the previous methods, they still have a strong disinfecting power. The most widely used of these is sodium hypochlorite due to its efficiency and cost-effectiveness. It appears as a clear/yellowish liquid in commercial solutions between 6-14%. The most common method of synthesising sodium hypochlorite is based on the reaction between chlorine and sodium hydroxide, the by-products of which are sodium chloride and water.



Sodium hypochlorite solutions are unstable. Its degradation leads to the formation of sodium chloride, chlorates and oxygen according to the following reactions:



The storage stability of hypochlorite depends on various factors by physical and chemical conditions such as initial concentration, temperature, and exposure to UV light, pH, and the presence of heavy metal ions (Corporation, 2011).

Depending on the pH, one or more chlorine species predominate over others. At pH levels below 2, the formation of Cl_2 is favoured. Between pH 4 and 7, hypochlorous acid (HClO) is the dominant species, with a standard reduction potential of $E^\circ (\text{HClO}/\text{Cl}^-) = +1.49 \text{ V}$. At higher pH levels, the hypochlorite anion (ClO^-) prevails, with a standard reduction potential of $E^\circ (\text{ClO}^-/\text{Cl}^-) = +0.89 \text{ V}$. Thus, the oxidation potential of a hypochlorite solution varies depending on the pH (Liang et al., 2020).

Solutions at lower concentrations degrade more slowly, in fact under the classical storage conditions between pH 11-13 (minimum 11.86) the degradation of

hypochlorite follows a second-order reaction with respect to OCl^- concentration, with k the rate coefficient depending directly on temperature.

Table 1 Storage Temperature of Sodium Hypochlorite Solutions (Corporation, 2011).

Concentration (%)	Half-life (days)	
	25 °C	35 °C
15	144	39
6	360	97
3	720	194
1	2160	580

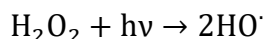
the presence of any trace metals as well as UV light tends to catalyse the degradation reaction, leading more to the formation of oxygen (Corporation, 2011).

1.10 Advanced Oxidation Processes

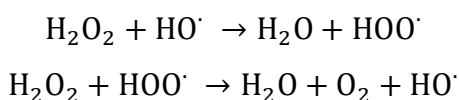
In recent years, new technologies for pollutant removal, have been developed, primarily due to the presence of refractory materials that resist traditional pollutant removal methods. AOPs are based on various reactions or different catalysts, but the central concept is the *in situ* generation of highly reactive species, such as hydroxyl radicals $\text{HO}^\bullet$, $E^0(\text{HO}^\bullet/\text{OH}^-) = 1.90\text{--}2.70\text{ V}$ (Lee et al., 2020). These radicals can oxidize organic matter present in wastewater and, in some cases, achieve complete mineralization into water and carbon dioxide. The radicals produced exhibit extremely high reactivity towards pollutants, with reaction rate constants ranging from $10^7\text{--}10^{10}\text{ M}^{-1}\text{s}^{-1}$ (Jin et al., 2015; Hoigné, 1997). In some cases, these processes are combined with biological oxidation, either before or after treatment. This combination can oxidize substances that are typically toxic and resistant to biological oxidation, converting them into more biodegradable species, with removal efficiencies exceeding 80% (Ponnusami et al., 2023; Fahmi et al., 2003; Del Moro et al., 2013).

AOPs are currently considered innovative technologies because they not only destroy a wide variety of contaminants but can also eliminate harmful microorganisms in wastewater. However, these advanced treatments are not yet widely implemented for wastewater treatment due to their high operational costs and the need for feasibility assessments (Garrido-Cardenas et al., 2019). The most studied AOPs for water purification include TiO_2 photocatalysis, UV/O_3 , $\text{UV/H}_2\text{O}_2$, Fenton, and photo-Fenton processes. Common oxidants used in these processes include hydrogen peroxide (H_2O_2), ozone (O_3), potassium persulfate ($\text{K}_2\text{S}_2\text{O}_8$), potassium peroxydisulfate (KHSO_5), and sodium percarbonate ($\text{Na}_2\text{CO}_3 \cdot 1.5\text{H}_2\text{O}_2$).

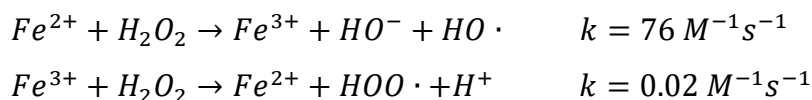
AOPs are classified based on the reactive phase (homogeneous/heterogeneous) or the methods for generating hydroxyl radicals (chemical, photochemical, sonochemical, and electrochemical). A classic example is the direct activation of hydrogen peroxide using UV light, which promotes the cleavage of the O-O bond in hydrogen peroxide:

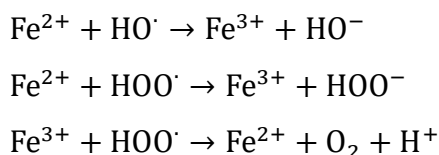


Further radical production can be achieved by the reaction of hydroxyl radicals with the remaining hydrogen peroxide:



The Fenton process, first developed by the chemist H.J.H. Fenton in the 1890s, has only recently been applied to wastewater pollutant removal. The process involves an autocatalytic reaction where Fe^{2+} ions react with hydrogen peroxide, producing Fe^{3+} and hydroxyl radicals. The Fe^{3+} then reacts with hydrogen peroxide to form perhydroxyl radicals ($\text{HO}_2^\cdot$) and regenerates Fe^{2+} , which is essential for continuing the hydroxyl radical formation (Huang et al., 2013).



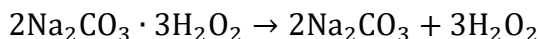


The second reaction in this process is slow, limiting the regeneration of ferrous ions (Fe^{2+}), which are necessary for further $\text{HO}^{\cdot}$ radical formation. To overcome this limitation, photo-Fenton processes have been studied, where radiation catalyses the reduction of Fe^{3+} back to Fe^{2+} (Mailhot et al., 1999), accelerating the overall reaction (Umar et al., 2010; Cai et al., 2023). The physical state in which metal ions are present (heterogeneous vs homogeneous) also significantly affects the reaction's efficiency. Many studies report that the Fenton reaction is limited by pH, not only due to the equilibria of secondary reactions but also because ferrous ions have low solubility in solutions with pH values greater than 4. Consequently, Fenton reactions show higher efficiency at acidic pH values. Chelating agents have been explored to keep $\text{Fe}^{2+}/\text{Fe}^{3+}$ in solution even at pH levels like those in wastewater. Studies have shown that using a non-toxic chelating agent like ethylenediamine-N,N'-disuccinic acid (EDDS) can enhance Fenton/photo-Fenton and Fenton-like reactions for the removal of common pollutants like bisphenol A, achieving pollutant removal efficiencies of up to 90%. The reaction was found to be much more effective at near neutral or basic pH than at acidic conditions. EDDS also acts as a superoxide radical-promoting agent, enhancing the regeneration of Fe^{2+} (the rate-limiting step) (Huang et al., 2013).

In summary, Fenton processes are influenced by several factors. pH is critical for the stability of metals in solution; at pH values greater than 3, ferric hydroxide ($\text{Fe}(\text{OH})_3$) precipitates form, reducing the concentration of Fe^{2+} in solution and, consequently, the number of hydroxyl radicals (Lu et al., 2001). Other factors, such as the concentration of hydrogen peroxide and ferrous ions, are also important. Excessive Fe^{2+} can lead to overproduction of hydroxyl radicals, but beyond a certain point, it reduces degradation efficiency due to the direct reactivity of $\text{HO}^{\cdot}$ radicals with Fe^{2+} .

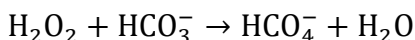
Similarly, increasing hydrogen peroxide concentration may result in unconsumed H₂O₂, leading to higher COD levels in the effluent.

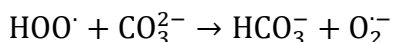
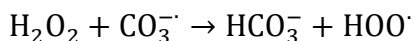
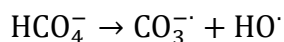
Sodium percarbonate (SPC) is a valuable alternative to hydrogen peroxide for treating wastewater and soil contaminated by organic compounds. This is due to its strong oxidative capacity, ease and safety of handling, and relatively low cost (Kornweitz and Meyerstein, 2016; Liu et al., 2021). SPC-based oxidative processes are typically catalysed by ultraviolet rays, metal ions (such as Fe²⁺ or Fe³⁺), or elemental metals dispersed on a nanoscale. These processes promote the production of hydroxyl radicals (HO[•]), superoxide radicals (O₂^{•-}), and carbonate anion radicals (CO₃^{•-}) (Zhou et al., 2020). Sodium percarbonate is a complex consisting of 32.5% hydrogen peroxide and 67.5% sodium carbonate by mass. When dissolved in water, SPC dissociates into Na₂CO₃ and H₂O₂ according to the reaction:



In this way, sodium percarbonate acts as a solid hydrogen peroxide source for Fenton-like processes. However, SPC differs from traditional Fenton processes in notable ways. In addition to generating hydroxyl radicals, it releases carbonate radicals (CO₃^{•-}), which are strong oxidants with a standard reduction potential of 1.78 V at pH 7.0. These radicals are especially reactive towards electron-rich groups, particularly those containing nitrogen and sulfur, as well as aromatic compounds (Gao et al., 2019).

Additionally, the combination of HCO₃⁻/CO₃²⁻ and H₂O₂ can lead to the formation of peroxymonocarbonate (HCO₄⁻), which is a more reactive, two-electron oxidant compared to hydrogen peroxide itself (Zhou et al., 2013):



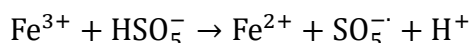
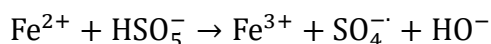


The homolytic cleavage of the O-O bond in HCO_4^- can generate a range of additional oxidizing species and radicals, such as $\text{HO}^\cdot$, $\text{O}_2^{\cdot-}$, $\text{CO}_3^{\cdot-}$, $\text{HO}_2^{\cdot-}$, and singlet oxygen ($^1\text{O}_2$) (Li et al., 2022; Zhou et al., 2013). This diversity of radicals enhances SPC's effectiveness, especially in the oxidation of complex and resistant organic compounds, making SPC a versatile and efficient option for environmental remediation applications.

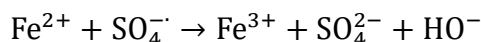
Advanced oxidation processes that utilize sulfate radicals have gained significant attention compared to those using hydroxyl radicals due to their higher redox potential and longer half-life, which makes them especially effective in treating persistent pollutants (Ghauch & Tuqan, 2011; Ahmed et al., 2012). One prominent sulfate radical source is peroxymonosulfate (PMS, HSO_5^-), which possesses standard reduction potential of 1.82 V, making it a potent oxidant. The sulfate radical ($\text{SO}_4^{\cdot-}$) offers a promising alternative to hydroxyl radicals ($\text{HO}^\cdot$) due to its comparable or even superior redox potential, ranging from 2.5 to 3.1 V, and a longer half-life of 30–40 μs , in contrast to $\text{HO}^\cdot$, which has a redox potential of 1.8–2.7 V and a much shorter half-life of approximately 20 ns (Lin et al., 2020)

However, for effective contaminant degradation in wastewater, PMS must be activated through various methods, including thermal, alkaline (Wacławek et al., 2022), UV light, activated carbon (Liu et al., 2020), transition metals (e.g., Fe^0 , Fe^{2+} , Cu^{2+} , Co^{2+} , Ag^+), or ultrasound (Wei et al., 2017). The potassium salt of peroxymonosulfate is commercially available as a triple salt ($2\text{KHSO}_5 \cdot \text{KHSO}_4 \cdot \text{K}_2\text{SO}_4$), known as OXONE®; this method of conservation is preferred as PMS is more stable over time.

Activation of sulfate radicals using transition metals, particularly Fe^{2+} , mirrors the Fenton process, where Fe^{2+} reacts with PMS to produce sulfate radicals, and with Fe^{3+} to produce less oxidant species like $\text{SO}_5^{\cdot-}$ and Fe^{2+} :



As in Fenton chemistry, optimal sulfate radical generation and, thus, contaminant degradation efficiency requires an appropriate Fe^{2+} dosage and an optimal pH range. While a minimum Fe^{2+} concentration is needed for PMS activation, excessive Fe^{2+} can induce side reactions that reduce efficiency:

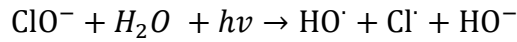
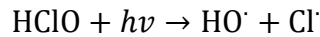


Studies have shown that a 1:1 ratio of ferrous ion to PMS maximizes contaminant removal efficiency (Ji et al., 2017). This balance is essential for minimizing waste and maximizing the oxidative potential of the sulfate radical system.

Among the latest-generation techniques for removing emerging contaminants, the combined UV-Chlorine process stands out. This technique uses UV radiation in the range of UV(A-B-C) (100-400 nm) combined with free chlorine in solution (typically NaClO) to produce highly oxidative *Reactive Chlorine Species* (RCS) such as $\text{Cl}^{\cdot}$ and $\text{OCl}^{\cdot}$, as well as the classic $\text{HO}^{\cdot}$. The RCS produced are often present at concentrations at least an order of magnitude higher than that of $\text{HO}^{\cdot}$ radicals. Moreover, radicals like $\text{Cl}^{\cdot}$ exhibit reactivity like $\text{HO}^{\cdot}$, while radicals like $\text{ClO}^{\cdot}$, though with lower second-order rate constants, show greater selectivity towards electron-rich groups. $\text{Cl}^{\cdot}$ primarily acts via hydrogen abstraction, except for olefins, where it undergoes addition reactions (Guo et al., 2022). RCS, particularly, appear to be selective oxidants for aromatic compounds, showing lower reactivity towards electron-poor benzene derivatives (e.g., nitrobenzene) (Kishimoto, 2019).

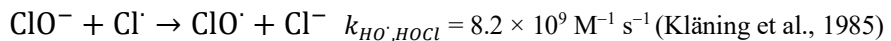
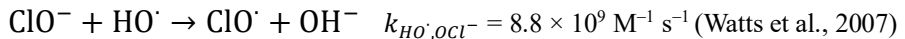
The coexistence of different oxidative species gives this process an advantage for oxidizing and disinfecting various water treatment matrices. Additionally, using two techniques that independently achieve microbial reduction allows further inactivation of bacterial loads when combined (Zhang et al., 2018).

The direct photolysis process occurs when UV light is absorbed by hypochlorous acid or hypochlorite molecules, generating reactive species:



These radicals can subsequently react with other species present to generate additional radical species, such as $\text{OCl}^\cdot$, enhancing contaminant removal efficiency based on the target substrate.

The specific reactions involved include:



Given the high difference in molar extinction coefficients at varying pH levels, the light used, and pH have a significant impact on process efficiency. HOCl has a

maximum absorbance coefficient of 98–101 $\text{M}^{-1} \text{cm}^{-1}$ at 235 nm, while OCl^- reaches a maximum absorbance coefficient of 359–365 $\text{M}^{-1} \text{cm}^{-1}$ at 292 nm. This difference in absorbance indicates variations in photolysis rates and reactive radical production (Remucal & Manley, 2016). The UV-Chlorine process achieves better contaminant degradation results compared to UV- H_2O_2 processes, especially due to the higher absorbance and quantum yield of chlorine relative to hydrogen peroxide (Fang et al., 2014). Various studies in the literature indicate that the formation of chlorine by-products in UV/chlorine processes is not significantly higher than in direct chlorine disinfection (Kishimoto 2019).

Chapter 2 Environmental Fate of Organic Sunscreens During Water Disinfection Processes: The Formation of Disinfection By-Products and Their Toxicological Profiles

2.1 Introduction

In recent years, the increasing awareness of the harmful effects of solar ultraviolet radiation on human health—such as DNA damage, the generation of reactive oxygen species, skin cancer, and photoaging—has led to a growing demand for sunscreen products incorporating both inorganic and organic UV filters (Sambandan & Ratner, 2011; Yeager & Lim, 2019; Zou et al., 2020; Santonocito et al., 2020).

UV Solar radiation reaching the Earth's surface is composed predominantly of UVA (~90%) and a smaller fraction of UVB (~10%) radiation. UVA rays penetrate deeper into the skin and are considered more harmful, leading to pathologies such as dermatitis and skin cancers. The concept of the 'Sun Protection Factor' (SPF) is crucial in this context, as it represents the ratio of the time required for skin to redden under solar exposure with sunscreen to the time needed without protection. For instance, applying a sunscreen with SPF 30 allows for approximately 30 times longer exposure before burning compared to unprotected skin. The ability of sunscreens to absorb UV radiation is closely linked to the conjugated arrangement of double and single bonds in their molecular structures, enabling the transition of molecules from ground to excited states upon UV absorption. Typically, the absorbed energy is harmlessly dissipated as heat or molecular vibrations. However, certain filters, such as avobenzene, can undergo structural changes when excited by UV light, losing their UV-absorbing capability. To counteract this, photo-stabilizers are added, capturing

and dissipating the excess energy before structural alterations occur, thus maintaining the protective function of the sunscreen (Wulf, 2009).

A sunscreen is a product that, by reflecting or transforming the sun's rays, protects skin structures from potential damage induced by ultraviolet UVA and/or UVB radiation. In this respect, sunscreens are products which, applied to the skin through cosmetic formulations, interfere with UV radiation, reducing its deleterious action on the health of the skin itself.

There are different types of sun filters:

- Chemical organic; they absorb harmful UV radiation, releasing most of the energy as heat. However, this type of solar filter can create radicals due to the transfer of energy from excited-state molecules to others. These molecules can be further divided into UVA, UVB, or complete filters, depending on their protection spectrum.
- Physical/Inorganic; they work in a very similar way, although their structure is different from that of organic sunscreens.

Inorganic filters like TiO_2 and ZnO reflect and scatter UV light, while organic filters absorb it, often ensuring broader-spectrum protection (Santonocito et al., 2020). The global regulatory framework has approved numerous UV filters as active sunscreen ingredients, with their concentrations ranging from 0.1 to 10% (Chavda et al., 2023). However, the large-scale production (about 10,000 tons per year) and widespread use of UV filters, especially during recreational activities in summer months, contribute to their extensive release into aquatic environments through untreated or treated wastewater discharges (Duis et al., 2022; Lorigo et al., 2023).

Among organic UV filters, Octocrylene (OCT) has gained particular attention. OCT primarily absorbs UVB radiation and short UVA wavelengths and is commonly found in various personal care products (Mokh et al., 2021; Sambandan & Ratner, 2011; Yeager & Lim, 2019; Zou et al., 2020). Despite concerns regarding its endocrine-disrupting potential and allergenic/photoallergic properties, its use remains

widespread, leading to its presence in different environmental compartments, including freshwater, seawater, and sediments (Balmer et al., 2005; Tang et al., 2018; Hu et al., 2021; D’Amico et al., 2024; Shetty et al., 2023). OCT and other organic filters such as Avobenzone, Homosalate, Octinoxate, and Oxybenzone have been detected at concentrations ranging from below 1 µg/L up to 10 µg/L in natural waters (Shetty et al., 2023). Their persistence and potential for long-range transport raise significant environmental concerns, as evidenced by the detection of UV filters in remote polar regions (D’Amico et al., 2024).

Octocrylene has emerged as a notable micropollutant in swimming pool waters, where it is directly released by consumers (Mokh et al., 2021). This places OCT in the category of emerging contaminants—similar to PFAS, cyanobacteria, hormones, pesticides, cosmetics, industrial additives, and drugs—that remain largely unregulated with no established maximum permitted values (Nakayama et al., 2019; Cheung et al., 2013; Vieira et al., 2020; Snow et al., 2012; Jean et al., 2012; Zarrelli et al., 2013; Rivera-Utrilla et al., 2013; Patel et al., 2019; Zhou et al., 2017; Mills & Chichester, 2005). The toxicity of these emerging contaminants to aquatic ecosystems and possibly human health, coupled with insufficient data on their long-term effects, has become a matter of growing concern.

Another emerging class of organic UV filters that has drawn attention is the triazine-based compounds, such as Ethylhexyl triazone. These filters have gained popularity due to increasing demands for environmentally friendly sunscreens. However, studies on their degradation, particularly under chlorination conditions typical of swimming pools, are relatively scarce compared to their environmental persistence (Romanucci et al., 2019; Romanucci et al., 2020; Zarrelli et al., 2012; Shetty et al., 2023; Luongo et al., 2020).

These active chemicals in sunscreens represent incredibly powerful hormones; they thus belong to the group of endocrine disruptors. Once freed into the organism, they can perform either like estrogen or testosterone, causing turmoil in natural hormonal

processes. Some studies found that with sunscreen components like 2-Benzoyl-5-methoxyphenol and 2-Ethylhexyl methoxycinnamate, given to rats, found that the weight of the rats' uteruses increased, which indicates that these substances exert an estrogenic action. The use of sunscreens has been so hyped in the USA that 2-Benzoyl-5-methoxyphenol is now found in the blood of 97% of Americans, including the 90% who say they have never used sunscreens. This substance has such widespread use that it has managed to invade our aqueducts, and phyto-purification plants are unable to eradicate it. As it is omnipresent in several products, it is currently omnipresent in the blood stream of several people, whether they use sunscreens or not (Barón et al., 2013; Tovar-Sánchez et al., 2013; Sánchez-Quiles & Tovar-Sánchez, 2015).

The danger of these products, however, does not stop at human life, but due to their dispersion in the environment, they tend to have harmful effects on the entire ecosystem, a classic example of which is the bleaching of corals (Moeller et al., 2021).

In addition to being released directly into the environment, these substances are also released into wastewater treatment systems, where, as a result of water disinfection processes using hypochlorite, numerous other by-products are generated whose toxicity is unknown, or almost unknown.

The formation of these by-products occurs mainly in wastewater following treatment with hypochlorite but, in many cases, these substances have been reported to be present directly in swimming pool water where users release them directly and a multitude of these toxic products can be formed as a result of water disinfection processes, remaining in close contact with humans potentially threatening their health (Teo et al., 2014). Many studies in the literature demonstrate the potential toxicity of disinfection by-products formed during the treatment of sewage polluted by sunscreens, and during the disinfection of swimming pool water. Manasfi et al. (2015) discovered up to 8 disinfection by-products formed by the treatment of Oxybenzone, in chlorinated seawater swimming pools, even resulting in brominated by-products.

Chlorination is commonly used as a tertiary treatment in wastewater treatment plants (WWTPs) and for disinfection in swimming pools. Although chlorination effectively inactivates pathogenic microorganisms, it can also trigger the formation of disinfection by-products due to reactions between chlorine and dissolved organic matter, including UV filters (Shetty et al., 2023; Luongo et al., 2020). These DBPs may be even more harmful than their parent compounds, posing additional risks to aquatic life and human health.

Major ecological concerns are associated with the effects of UV filters and their by-products on marine life, as they have been linked to coral bleaching (He et al., 2023), reduced growth and photosynthetic activity in primary producers, and various developmental and physiological anomalies in aquatic consumers (Prakash & Anbumani, 2021). Thus, there is a pressing need to understand the chemical fate and toxicity of these compounds and their by-products.

Advanced treatment technologies, including ozonation (Ibáñez et al., 2013), membrane filtration (Kim et al., 2017), adsorption (Yaghmaeian et al., 2013), and advanced oxidation processes (Kanakaraju et al., 2018), are under investigation to improve the removal of emerging contaminants like UV filters from wastewater. Implementing these treatments could mitigate the environmental impact of these substances, reducing ecological and human health risks.

In this chapter of the dissertation, the chemical fate and ecotoxicological effects of by-products generated by hypochlorite treatment of Octocrylene and Ethylhexyl triazone under conditions typical of swimming pool water disinfection will be analyzed. These environments represent major sources of exposure for these compounds. The study on OCT led to the identification of 11 DBPs, six of which were completely new. These DBPs were isolated by HPLC and characterized using mass spectrometry and NMR techniques. Proposed formation mechanisms aided in understanding their chemical structures. Ecotoxicological assessments, performed on organisms such as *Phaeodactylum tricornutum*, *Brachionus plicatilis*, and *Aliivibrio*

fischeri, indicated that these DBPs exhibit significant toxicity, thus potentially increasing environmental risks.

Similarly, EHT was investigated for its disinfection by-product formation under chlorination. The study identified 12 DBPs (DBP1–DBP12), 3 of which were reported for the first time. These by-products were fully characterized and their formation mechanisms elucidated. Ecotoxicological tests involving freshwater trophic chain organisms—*Raphidocelis subcapitata*, *B. calyciflorus*, *Thamnocephalus platyurus*, and *Heterocypris incongruens*—revealed lethal and sub-chronic effects at concentrations spanning from $\mu\text{g/L}$ to mg/L . Ostracods proved to be particularly susceptible, whereas algae were unaffected at the tested concentrations.

This integrated approach, combining analytical chemistry, reaction mechanism studies, and ecotoxicological evaluations, provides essential insights into the environmental behaviour of OCT and EHT and their chlorination by-products. Understanding their formation pathways, persistence, and toxicity will support future efforts to develop more effective removal strategies, inform regulatory guidelines, and ultimately help safeguard aquatic ecosystems and public health.

2.2 Octocrylene: Formation of By-products During Chlorination Processes

2.2.1 Introduction

Octocrylene (OCT), an ester formed by the condensation of 2-ethylhexyl cyanoacetate with benzophenone, was introduced in commercial sunscreens and anti-aging creams about 15 years ago. It is a viscous, oily liquid that is clear and colourless. The extended conjugation of the acrylate portion of the molecule absorbs UVB and short-wave UVA (ultraviolet) rays, with wavelengths from 280 to 320 nm (Berardesca et al., 2019), which promote tanning but also contribute to the onset of sunburn and skin cancer. It

is used in various cosmetic products (De Groot & Roberts, 2014; Bennàssar et al., 2009), in concentrations up to 10%, to provide an adequate sun-protection factor or to protect the cosmetic formulations themselves from UV radiation. This filter was recently indicted for the risk of inducing potential adverse effects on the endocrine system, as well as having an allergic and/or photoallergic potential (Carrotte-Lefebvre et al., 2003; Karlsson et al., 2011). In recent years, there has been an increase in the number of cases of photocontact allergic reactions to octocrylene, which has been referred to as an "emerging allergen". However, it has the advantage of working in synergy, allowing for wide and beneficial photoprotection; for example, it stabilizes avobenzene (butyl methoxydibenzoylmethane), a molecule present in the UVA filter. Like all chemicals used in cosmetics, the safety profile of octocrylene is constantly evaluated by the European Chemicals Agency (ECHA) as it is registered under European legislation.

In this work, the disinfection by-products of OCT were investigated under the same conditions as the chlorination process normally used in swimming pools to reduce similar emerging pollutants (Mokh et al., 2021; Teo et al., 2014); accordingly, two different experiments were carried out, one at concentrations comparable to those at which OCT is present in wastewater (10^{-5} M) and one at concentrations at least 100 times higher in order to isolate and identify the DBPs. The structures of 11 isolated DBPs, 4 of which were isolated for the first time, were determined by combining nuclear magnetic resonance (NMR) and mass spectrometry (MS), using matrix-assisted laser desorption/ionization as a source and a time-of-flight analyser (MALDI-TOF) for mass analysis. These DBPs were justified by a proposed mechanism of formation.

The spatial distribution of OCT is strictly connected to its presence in the marine aquatic environment; this presence is due to the anthropogenic activities responsible for its direct emission. For this reason, an ecotoxicological assessment was carried out with marine aquatic bioindicators such as *A. fischeri*, *P. tricornutum* and *B. plicatilis*.

2.2.2 Materials and Methods

2.2.2.I Reagents and Equipment

Reagents and Equipment are listed in supporting text ST1a

2.2.2.II Chlorination Experiments

A 10^{-5} M OCT solution was treated for 10 min with 10% hypochlorite (molar ratio OCT/HClO 2:1 concentration, spectroscopically determined $\lambda_{\max}$ 292 nm, ϵ 350 dm³/mol cm) at room temperature (Duis et al., 2021). The presence of OCT was quantified using a Lambda 12 UV-Vis spectrophotometer (Perkin Elmer, USA). Absorbance peaks were determined at 310 nm. The absorbance values were converted into a concentration using a calibration curve prepared from standard solutions with known OCT concentrations. DBP1–DBP11 were isolated from the methylene chloride extract of the aqueous solution and identified by comparing their retention times with those of commercially available standard compounds, or isolated by performing preparative experiments with a solution of OCT at a concentration higher than 10^{-3} M and treated with 6% hypochlorite at room temperature for 2 h. The pH of the solution, measured and recorded continuously using a pH-meter, increased immediately from the initial pH of 8.0 to 10.8, and the pH remained at this value during the reaction. An aliquot of the solution was taken every 15 min, quenched by sodium thiosulphate excess, filtered, and dried by lyophilisation, and the residue was dissolved in a saturated sodium bicarbonate solution and extracted with ethyl acetate. The course of the reaction was monitored using HPLC. The DBPs obtained were isolated using column chromatography and HPLC and were completely characterized using NMR and MS analyses.

2.2.2.III Chlorination Procedure and Product Isolation

Octocrylene (607 mg, 1.68 mmol) was dissolved in 22 mL of acetonitrile, and the solution was diluted with water until a final volume of 0.9 L was reached. A sodium hypochlorite solution (approximately 6% active chlorine, molar ratio OCT/HClO 1:25; concentration spectroscopically determined at $\lambda_{\max}$ of 292 nm, ϵ = 350 dm³/mol

cm) was added drop by drop to this solution under magnetic stirring at room temperature. The reaction was stopped after 2h with an excess of sodium thiosulphate and concentrated by lyophilisation. The residue was dissolved in water and pH-adjusted to 5.0, and this solution was extracted using methylene chloride. The crude organic fraction (835 mg) was chromatographed on silica gel CC, eluted with a gradient of chloroform:methanol (99:1 to 10:90, v/v) to yield 9 fractions. The fraction Fr. 2 (62 mg), eluted with chloroform:methanol (97:3), was chromatographed on silica gel CC, eluted with a gradient of petroleum ether:acetone (98:2 to 90:10, v/v) to yield DBP1 (27 mg). The fraction Fr. 4 (49 mg), eluted with chloroform:methanol (90:10), was chromatographed on silica gel CC, eluted with a gradient of petroleum ether:acetone (90:10 to 50:50, v/v) to yield DBP2 (29 mg). The fraction Fr. 7 (58 mg), eluted with chloroform:methanol (60:40), was separated by semipreparative HPLC using a reversed-phase column Phenomenex Gemini 10 μ m 110 Å C18 (250 $\times$ 21.20 mm) and eluted with a gradient of CH₃COONH₄ (A, pH 4.0; 10 mM) and methanol (B), starting with 30% B for 5 min and followed by the installation of a gradient to obtain 100% B over 30 min, at a solvent flow rate of 8 mL/min to yield DBP3 (7 mg) and DBP4 (3 mg). The fraction Fr. 8 (405 mg), eluted with chloroform:methanol (80:20), was chromatographed on silica gel CC, eluted with a gradient of methylene chloride:methanol (90:10 to 0:100, v/v) to yield 9 fractions.

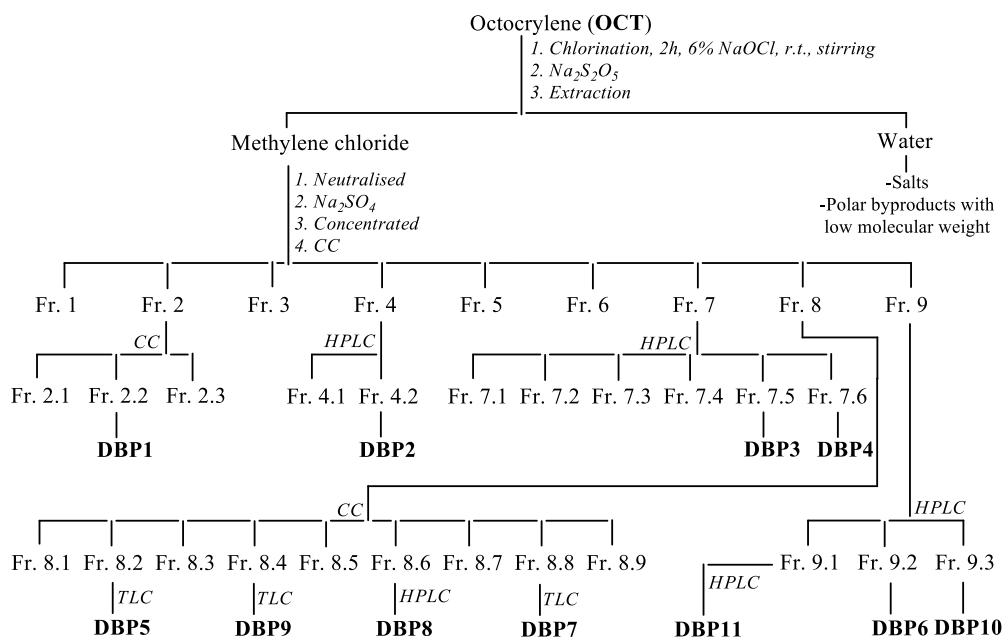


Figure 1 The isolation of the different identified compounds

The fraction Fr. 8.2 (61 mg), eluted with methylene chloride:methanol (90:10), was chromatographed on TLC, eluted with chloroform:methanol (80:20), to yield DBP5 (47 mg). The fraction Fr. 8.4 (131 mg), eluted with methylene chloride:methanol (80:20), was chromatographed on TLC, eluted with chloroform:methanol (70:30), to yield DBP9 (89 mg). The fraction Fr. 8.6 (43 mg), eluted with methylene chloride:methanol (65:35), was separated by analytical HPLC using a reversed-phase column Phenomenex Gemini 10 μ m 110 Å C18 (250 $\times$ 21.20 mm) and eluted with a gradient of CH₃COONH₄ (A, pH 4.0; 10 mM) and methanol (B), starting with 30% B for 5 min and followed by the installation of a gradient to obtain 100% B over 30 min, and eluted again with the same mixture for another 10 min, at a solvent flow rate of 8 mL/min to yield DBP8 (20 mg). The fraction Fr. 8.8 (53 mg), eluted with methylene chloride:methanol (60:40), was chromatographed on TLC and eluted with petroleum ether:acetone (65:35), to yield DBP7 (20 mg).

The fraction Fr. 9 (31 mg), eluted with chloroform:methanol (90:10), was separated by analytical HPLC using a reversed-phase column Phenomenex Kromasil 10 μ m 100 Å C18 (250 $\times$ 10.00 mm) and eluted with a gradient of CH₃COONH₄ (A, pH 4.0; 10 mM) and acetonitrile (B), starting with 20% B for 5 min and followed by the installation of a gradient to obtain 100% B over 30 min, at a solvent flow rate of 4 mL/min to give 3 fractions. The fraction 9.1 was purified by HPLC using a column Discovery RP-amide C16 (150 $\times$ 4.6 mm), 5 μ m, and eluted with 0.1% TFA in acetonitrile:water (25:75), at a solvent flow rate of 0.8 mL/min to give DBP11 (2 mg). The fractions Fr. 9.2 and Fr. 9.3 were identified as DBP6 (5 mg) and DBP10 (2 mg), respectively.

2.2.2.IV Ecotoxicity Data

The toxicity of OCT and its disinfection by-products was assessed regarding the following organisms: *A. fischeri*, *P. tricornutum* and *B. plicatilis*. A Microtox® acute ecotoxicity test was performed using the marine bioluminescent bacteria *A. fischeri* (NRRL-B-11177) to assess the toxicity of OCT and DBPs. The bacteria were supplied in a freeze-dried form by Aqua Science LLC (Newark, Delaware, USA) and were stored at -20 °C to preserve their microbial activity. The acute toxicity endpoint was determined after 30 min of exposure according to ISO 11348-3.

An algal growth-inhibition test was performed using benthic diatom *P. tricornutum*. The algal culture was kept at 20 $\pm$ 2 °C and 6000–10000 Lux light, to obtain a cellular density of 10⁶ cells/mL. Inocula were taken from pre-cultures set up three days before the experiment to adjust the initial cell density to approximately 10⁴ cells/mL. The test was carried out and miniaturized for 24-well sterile polystyrene micro-plates. The growth inhibition rate was calculated after 72h exposure using a UV-Vis spectrophotometer (Hach Lange DR5000) and a 5 cm cuvette.

The acute toxicity test with estuarine rotifer *B. plicatilis* was performed according to the standard procedure of Rotoxkit M® using certified dehydrated cysts (MicroBioTests Inc.). The test was conducted in multiwell plates with 300 μ L per well.

Six wells with ten rotifers each were filled to assess the toxicity of the parent compound and of its 11 DBPs. Incubation was carried out for 48h, at 25 °C, in darkness. The number of dead rotifers after the exposure period was observed under a stereomicroscope (LEICA EZ4-HD). The significance of the differences between the mean values of the different tests and controls was verified using Addinsoft XLSTAT (2016.02.27444 Version) by analysis of variance (ANOVA) with a 0.05 significance level. In addition, the post-hoc analyses were carried on with Tukey's test.

2.2.3 Results and Discussion

2.2.3.1 Chlorination Experiments

The chlorination experiments involving OCT were conducted using concentrations comparable to those at which this micropollutant has been observed in swimming pool water (Bedner & MacCrehan, 2005), approximately 10^{-5} . Specifically, sunscreen solutions were exposed to a 10% hypochlorite solution for 10 minutes, maintaining a molar ratio of OCT to hypochlorite of 2:1, under magnetic stirring and at room temperature. To facilitate the isolation and structural identification of by-products, additional experiments were carried out using significantly higher contaminant concentrations ($> 10^{-3}$ M) and a reduced OCT-to-oxidizing agent molar ratio (1:20).

The course of the reaction was monitored by HPLC, and the DBPs obtained were isolated by column chromatography and HPLC (Figure 1) and completely characterized using NMR and MS analyses. Finally, DBP1–DBP11 (Figure 2) were isolated at relative percentages of 1.12, 2.97, 0.89, 0.91, 1.15, 2.36, 1.08, 1.55, 6.39, 0.59, and 1.19. The proposed mechanism of their formation from OCT is shown in Figure 3, and DBP3–DBP5 were isolated for the first time.

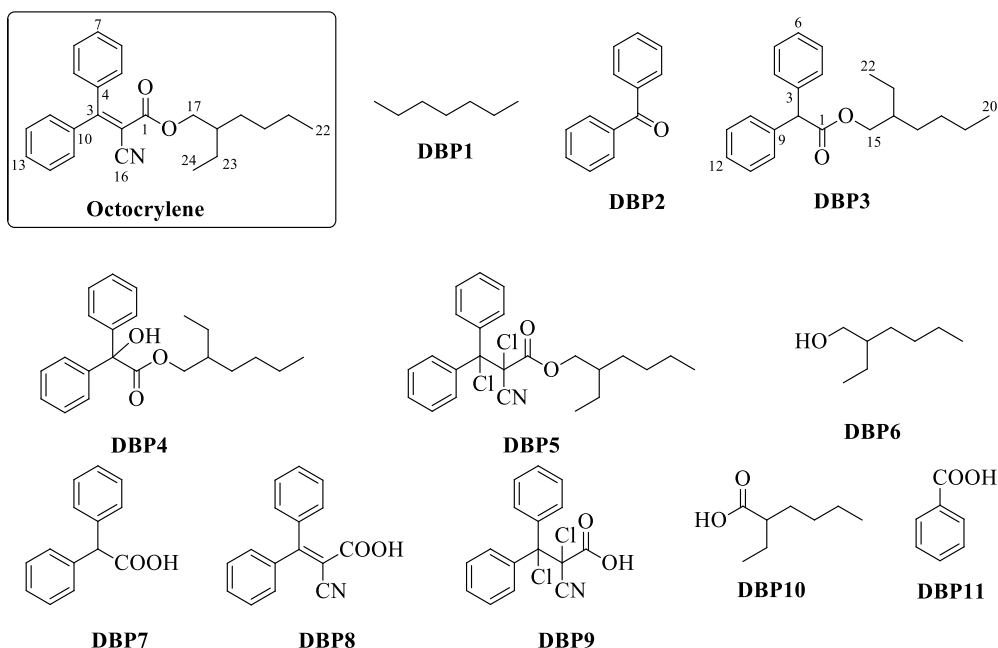


Figure 2 Chemical structures of octocrylene and its disinfection by-products DBP1–DBP11

2.2.3.II Structure Elucidation of Disinfection By-products DBP1–DBP9

In the OCT treatment, the concentration of DBP1–DBP11 reached its maximum after about 2 h, with a disinfection of 15% and a transformation of approximately 20%; percentages of by-products ranged from 0.59% for DBP10 to 6.39% for DBP9. In a basic environment, OCT can undergo a *retro-aldol* condensation, which leads to the formation of intermediate *I1* and the by-product DBP2. From this and other by-products, it is usually possible to obtain the by-product DBP11; the intermediate *I1*, identifiable with 2-ethylhexyl cyanoacetate, can undergo hydrolysis of the ester bond and lead to the formation of the by-product DBP10 and α -cyanoacetic acid, probably contained in the aqueous phase rich in salts and low-molecular-weight compounds. Finally, the DBP10 could decarboxylate to the DBP1 by-product. The by-product DBP10 is also obtained from the hydrolysis of the ester bond of the starting product, together with the by-product DBP8. The latter, by decarboxylation, could provide the intermediate *I2*, which, by the hydrolysis of the cyano group, provides the

intermediate *I3*. Considering the disinfection reaction of OCT in the presence of sodium hypochlorite, it can be assumed that a Weerman degradation takes place that leads to the formation of nitrene *I6*, through the deprotonation (*I4*) and chlorination (*I5*) of the amide nitrogen of the intermediate *I3*. The transposition of nitrene *I6*, or more probably the elimination of HCl from the intermediate *I5* with the concomitant transposition of the residue bound to the carbonyl, allows the obtainment of the isocyanate *I7*, from which alcoholises of the intermediate *I8* are obtained, from which, for the subsequent oxidation, the intermediate *I9* is obtained. The hydrolysis of the latter and the subsequent oxidation of the intermediate *I10* obtained provides the by-product DBP7. This can react with the DBP6 present in the solution and provide the by-product DBP3 and, for subsequent chlorination, create the by-product DBP4. The direct chlorination to the C-2/C-3 carbons of the starting product allows for and explains the obtainment of the by-product DBP5, from which DBP9 and DBP6 are obtained by the hydrolysis of the ester bond.

2.2.3.III Spectral Data

Octocrylene: *2-Ethylhexyl 2-cyano-3,3-diphenylacrylate*. Oily liquid. ^1H - and ^{13}C -NMR, see Table SMa1. MS-TOF (positive ions): m/z calculated for $\text{C}_{24}\text{H}_{27}\text{NO}_2$ m/z 361.20 $[\text{M}]^+$; found 362.47 $[\text{M} + \text{H}]^+$ (68%).

DBP1: *Heptane*. Identified by comparison with an authentic sample.

DBP2: *Benzophenone*. Identified by comparison with an authentic sample.

DBP3: *2-Ethylhexyl 2,2-diphenylacetate*. White powder. ^1H - and ^{13}C -NMR, see Table SMa2. MS-TOF (positive ions): m/z calculated for $\text{C}_{22}\text{H}_{28}\text{O}_2$ m/z 324.21 $[\text{M}]^+$; found 325.47 $[\text{M} + \text{H}]^+$.

DBP4: *2-Ethylhexyl 2-chloro-2,2-diphenylacetate*. White powder. ^1H - and ^{13}C -NMR, see Table SMa3. MS-TOF (positive ions): m/z calculated for $\text{C}_{22}\text{H}_{27}\text{ClO}_2$ m/z 358.17 $[\text{M}]^+$; found 361.82 $[\text{M} + \text{H}]^+$, 359.78 $[\text{M} + \text{H}]^+$.

DBP5: *(2S)-2-Ethylhexyl 2,3-dichloro-2-cyano-3,3-diphenylpropanoate*. White powder. ^1H - and ^{13}C -NMR, see Table SMa4. MS-TOF (positive ions): m/z calculated for $\text{C}_{24}\text{H}_{27}\text{Cl}_2\text{NO}_2$ m/z 431.14 $[\text{M}]^+$; found 436.33 $[\text{M} + \text{H}]^+$, 435.36 $[\text{M} + \text{H}]^+$, 434.38 $[\text{M} + \text{H}]^+$, 433.35 $[\text{M} + \text{H}]^+$, 432.36 $[\text{M} + \text{H}]^+$.

DBP6: *2-Ethylhexan-1-ol*. Identified by comparison with an authentic sample.

DBP7: *2,2-Diphenylacetic acid*. White powder. ^1H - and ^{13}C -NMR, see Table SMa5. MS-TOF (positive ions): m/z calculated for $\text{C}_{14}\text{H}_{12}\text{O}_2$ m/z 212.08 $[\text{M}]^+$; found 213.27 $[\text{M} + \text{H}]^+$.

DBP8: *2-Cyano-3,3-diphenylacrylic acid*. White powder. ^1H - and ^{13}C -NMR, see Table SMa6. MS-TOF (positive ions): m/z calculated for $\text{C}_{16}\text{H}_{11}\text{NO}_2$ m/z 249.08 $[\text{M}]^+$; found 250.19 $[\text{M} + \text{H}]^+$.

DBP9: *(S)*-2,3-dichloro-2-cyano-3,3-diphenylpropanoic acid. White powder. ^1H - and ^{13}C -NMR, see Table SMa7. MS-TOF (positive ions): m/z calculated for $\text{C}_{16}\text{H}_{11}\text{Cl}_2\text{NO}_2$ m/z 319.02 $[\text{M}]^+$; 324.18 $[\text{M} + \text{H}]^+$, 323.16 $[\text{M} + \text{H}]^+$, 322.19 $[\text{M} + \text{H}]^+$, 321.18 $[\text{M} + \text{H}]^+$, 320.21 $[\text{M} + \text{H}]^+$.

DBP10: *2-ethylhexanoic acid*. Identified by comparison with an authentic sample.

DBP11: *Benzoic acid*. Identified by comparison with an authentic sample.

2.2.3.IV Toxicity assessment

Octocrylene has relatively high environmental stability in aquatic environments and is hardly removed from wastewater treatment plants (Gago-Ferrero et al., 2013; Brausch & Rand, 2010). Previous studies showed that OCT was poorly removed from wastewater treatment plants (0-10% degradation in aerobic conditions) (Duis et al., 2021).

Toxicity data were reported in Figure 4(A, B and C) for *P. tricornutum*, *B. plicatilis* and *A. fischeri*, in that order, considering the effect of OCT and its by-products.

Chronic toxicity with *P. tricornutum* was also identified as 83% of DBPs showed growth inhibition effects ranging between 20% and 50% (OCT, DBP1, DBP3, DBP4, DBP6–DBP11). DBP5 has no toxicity, while DBP2 was the most toxic compound (Figure 4(A)).

The effects of this UV filter and its chlorinated derivatives on the acute toxicity of *B. plicatilis* change, probably due to the lower sensibility of the bioindicator. The toxicity has been evaluated by observing the mortality rate of *B. plicatilis* after 24h of exposure. As reported in Figure 4(B), no significant effect on mortality was observed in rotifers exposed to OCT and its disinfection by-products. In fact, all the samples investigated showed a toxicity ranging between 10% and 32%, with the exception of DBP3, which had a residual toxicity of approximately 47%. In the latter case study,

DBP2, on the other hand, has a toxicity of only 20%, while DBP3 appears to be the most toxic disinfection by-product for the bioindicator.

The acute toxicity of OCT and its chlorinated derivatives towards *A. fischeri* was shown in Figure 4(C). After 30 minutes of exposure, the bioluminescence inhibition swung between 14% and 90%, except for the parent compound OCT, which was the only compound to exhibit biostimulation behaviour.

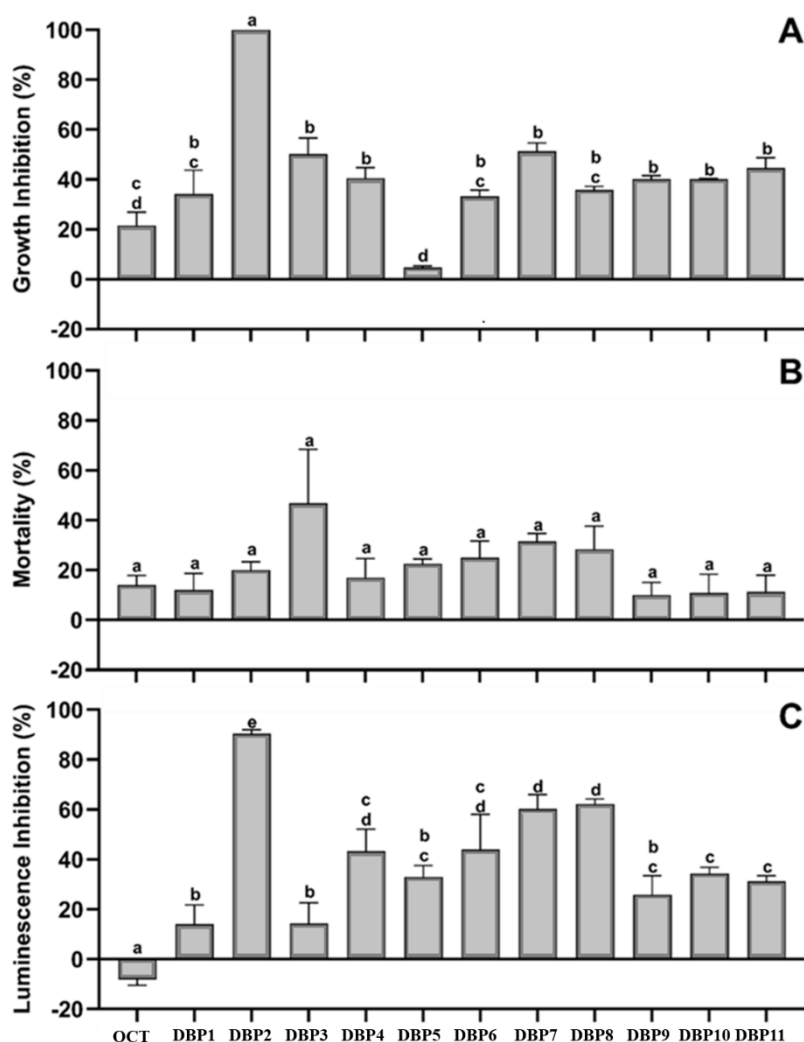


Figure 4 Toxicity data regarding exposure of *P. tricornutum* (A), *B. plicatilis* (B) and *A. fischeri* (C) to OCT and its by-products (DBP1–DBP11). Data with different letters (a–e) are significantly different (Tukey post hoc, $p < 0.05$).

So, 16% of the samples tested did not exceed 20% of the effect (DBP1 and DBP3), 33% of the disinfection by-products are included in the range between 26% and 33% of the effect (DBP5, DBP9, DBP10 and DBP11), while there was another 33% increase in toxicity up to 60% of the effect (DBP4, DBP6, DBP7 and DBP8). DBP2

(90% of the effect), once again, appears to be the most toxic product. The toxicity rate observed in *A. fischeri* is in good agreement with our previous results on *P. tricornutum*.

The discharge into the marine environment of chlorinated sewage effluents containing these disinfection by-products represents the worst scenario for environmental safety; indeed, in our study, these by-products could have negatively influenced the physiology of single-celled organisms such as *A. fischeri* and *P. tricornutum*, but they did not affect rotifers. This study highlighted the concerns and the potential risks from OCT by-products that may emerge and impact the quality of the marine ecosystem, especially concerning uncontrolled doses.

2.2.4 Conclusions

This study investigated the fate, following disinfection treatment by chlorination, of one of the most widely used sunscreens, namely octocrylene, conventionally considered an emerging micropollutant. The reaction was carried out by simulating the disinfection treatment employed in swimming pool waters, using excess sodium hypochlorite. After the chlorination treatment, chromatographic techniques were used to isolate eleven disinfection by-products, which were fully characterized by MS and NMR analyses and via comparison with a commercial standard. Four of them were isolated for the first time. Compared to the initial quantity considered, OCT was recovered unchanged for 45% and transformed into the corresponding by-products for 20%. A possible mechanism for the disinfection of OCT and its disinfection by-products has been hypothesized. Half of the investigated DBPs possessed anywhere from slightly to highly toxic effects. Thus, acute toxicity evaluation demonstrated that the presence of OCT in the water distribution system might pose a more significant threat to safety and water quality since disinfection increases the potential risk of OCT contamination.

2.3 Ethylhexyl Triazone Sunscreen and its Disinfection By-Products Obtained after Chlorine Treatment

2.3.1 Introduction

In recent years, there has been a growing focus on developing eco-compatible UV filters, referred to as "new generation filters," as a sustainable alternative to the "old generation filters" like Oxybenzone and Octinoxate, which are now banned in several regions worldwide, including Hawaii, Yucatan, and Thailand. Examples of these advanced UV filters include Diethylamino hydroxybenzoyl hexyl benzoate, Methylene bis-benzotriazolyl tetramethylbutylphenol, bis-Ethylhexyloxyphenol methoxyphenyl triazine, and Ethylhexyl triazone (EHT). This shift not only addresses environmental concerns but also leverages the superior properties of these compounds, such as lower effective dosages and broader industrial applications. The triazine compounds, can even used at lower dosages and have broad industrial applications. Ethylhexyl triazone has been commonly used since 1996 in the European Union and is listed as one of the most common UV products in sunscreens and cosmetics. The environmental fate and biological effects of the UV filters as well as their by-products which derive from photodegradation, photolysis and chlorination reactions and from interaction with other chemicals present in the environment, need to be investigated (Varrella et al., 2022).

Ethylhexyl triazone (tris(2-Ethylhexyl)-4,4',4''-(1,3,5-triazine-2,4,6-triyl)triimino) tribenzoate) has a central triazine ring that acts like a linker for three 2-ethylhexyl 4-aminobenzoate fragments (Nitulescu et al., 2023). The chemical-physical properties (<https://echa.europa.eu>) of this UVB filter are reported in Table 2.

Table 2 Chemical physical characteristics of EHT

Physical state	Solid
Molar mass	823.07 g/mol
Molecular formula	C ₄₈ H ₆₆ N ₆ O ₆
Density at 20 °C	1.1 g/cm ³
Water solubility (25 °C)	0.0007 mg/L
Melting point	114 °C
Boiling point	874.4 °C
Log K _{ow}	7

It is insoluble in water; therefore, it is not possible to obtain oil-free formulations (38% frequency in sunscreens for adults and almost 45% in sunscreens for children). EHT is highly photostable, with a strong absorption capacity due to the presence of three chromophore groups, exhibiting maximum absorption at 314 nm, surpassing all other currently available UVB filters. The skin does not absorb it, determining low irritation or photosensitization reactions. The absence of odour and colour makes it a suitable active ingredient in fragrance-free skin care products. EHT is included in the list of authorized sunscreen ingredients in Australia, European Union, and Japan (Sunscreens, 2005, 3rd edition), and in Europe their maximum permitted concentration is equal to 5% (Cosmetics directive, 2022) (Steinberg, 2005). Not surprisingly, EHT has been detected in sewage sludge, up to 27.7 ng/g dry weight (dw) (Plagellat et al., 2006; Rodil et al., 2009) and in sediments both Rhine-Meuse-Delta and the German Bight (Apel et al., 2018a) and of the Yellow Sea in China (Peng et al., 2017; Apel et al., 2018b). Kupper et al. (2006) reported EHT concentrations of 5720 ng/L in Swiss

wastewaters up to 20.8 mg/Kg in dry sludge. The presence of these substances in the environment is mainly due to the direct release by consumers and the inefficiency of wastewater treatment processes because of their recalcitrance especially for hydrophobic filters such as EHT (Fagervold et al., 2023). As shown by Ramos et al., 2016, EHT was found at concentrations ranging from 550 to 980 ng/L in influent wastewater, and 770 ng/L in effluent wastewater, demonstrating that wastewater treatment does not completely remove. EHT is not only exposed to disinfection processes in WWTPs but can also come into direct contact with disinfectants in recreational environments such as swimming pools. In these places, UV filters are released directly into the water by the swimmers through skin contact. The presence of free chlorine in swimming pool water is always kept at a minimum level and is even increased during maintenance periods when there are no swimmers, and these conditions allow the formation of disinfection by-products.

In this study, disinfection by-products (DBPs) of EHT have been isolated and structurally determined, mimicking the chlorination conditions used in swimming pool water disinfection treatment (Mock et al., 2022; Romanucci et al., 2019; Teo et al., 2015). Two distinct experiments were conducted: one at EHT concentrations of about 10^{-5} M and one at concentrations at least 100 times higher to isolate and identify the DBPs. The structures of 12 isolated DBPs, three of which discovered for the first time (DBP2–DBP4), were determined by combining nuclear magnetic resonance (NMR) and mass spectrometry (MS), using matrix-assisted laser desorption/ionization as the source and a time-of-flight analyser (MALDI-TOF) for mass analysis. A proposed formation mechanism elucidates the generation of these DBPs.

To the best of our knowledge, data about ecotoxicity of EHT, DBP1, DBP2, DBP3 and DBP4 (the last three newly discovered) on freshwater organisms belonging to different trophic levels are scarce or lacking. Thus, in this study, the acute, chronic and sub-chronic ecotoxicity of these compounds in organisms from two levels of the

freshwater aquatic chain, were investigated. A primary producer, the alga *R. subcapitata*, and three primary consumers, the rotifer *B. calyciflorus*, the anostracan crustacean *Thamnocephalus platyurus*, and the benthic ostracod *Heterocypris incongruens* were used.

2.3.2 Materials and Methods

2.3.2.1 Reagents and equipment

Ethylhexyl triazone (CAS: 88122-99-0, 97%) was purchased from Merck (Darmstadt, Germany). All the other chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany). Solvents were supplied by Merck, analytical grade or HPLC grade, and used as received.

Column chromatography (CC) was carried out with Kieselgel 60 (230–400 mesh, Merck, Darmstadt, Germany). HPLC was performed on a Shimadzu LC-8A system using a Shimadzu SPD-10A VP UV-VIS detector (Shimadzu, Milan, Italy). GC was performed on a Shimadzu 2010 series GC FID instrument with AOC-20i autosampler (Shimadzu, Milan, Italy). The ^1H and ^{13}C NMR spectra were recorded with an NMR spectrometer operated at 400 MHz and at 25 °C (Bruker DRX, Bruker Avance) and referenced in ppm to the residual solvent signals (CDCl_3 , at ppm_H 7.27 and ppm_C 77.0; and CD_3OD , at ppm_H 3.30 and ppm_C 49.0). The proton-detected heteronuclear correlations were measured using a gradient heteronuclear single-quantum coherence (HSQC) experiment, optimized for $^1J_\text{HC} = 155$ Hz, and a gradient heteronuclear multiple bond coherence (HMBC) experiment, optimized for $^nJ_\text{HC} = 8$ Hz. The MALDI-TOF mass spectrometric analyses were performed on a Voyager-De Pro MALDI mass-spectrometer (PerSeptive Biosystems, Framingham, MA, USA). The UV-VIS spectra were recorded with a Lambda 12 UV-Vis spectrophotometer (Perkin Elmer, Milan, Italy). A WTW pH 7110 pH meter (Xylem Analytics, Weilheim, Germany) equipped with a WTW SenTix41 electrode with a temperature sensor was used to record pH.

2.3.2.II Analytical experiments

A 5×10^{-5} M EHT solution was treated for 30 min with 10% sodium hypochlorite (molar ratio EHT/NaClO 2:1, Cl_2 concentration spectroscopically determined at λ_{max} 292 nm, ϵ 350 $\text{dm}^3/\text{mol cm}$), under stirring and at room temperature (Bedner & MacCrehan, 2006). The presence of EHT was quantified using a spectrophotometer with absorbance measures determined at 310 nm (Baker et al., 2017). The absorbance values were converted into a concentration using a calibration curve prepared from five standard solutions with known EHT concentrations.

Under these conditions, the by-products DBP1–DBP12 (Figure 5) were in insufficient quantities to proceed with their isolation. However, it was possible to compare their retention times with those of the by-products obtained in preparatory experiments, described in the next paragraph.

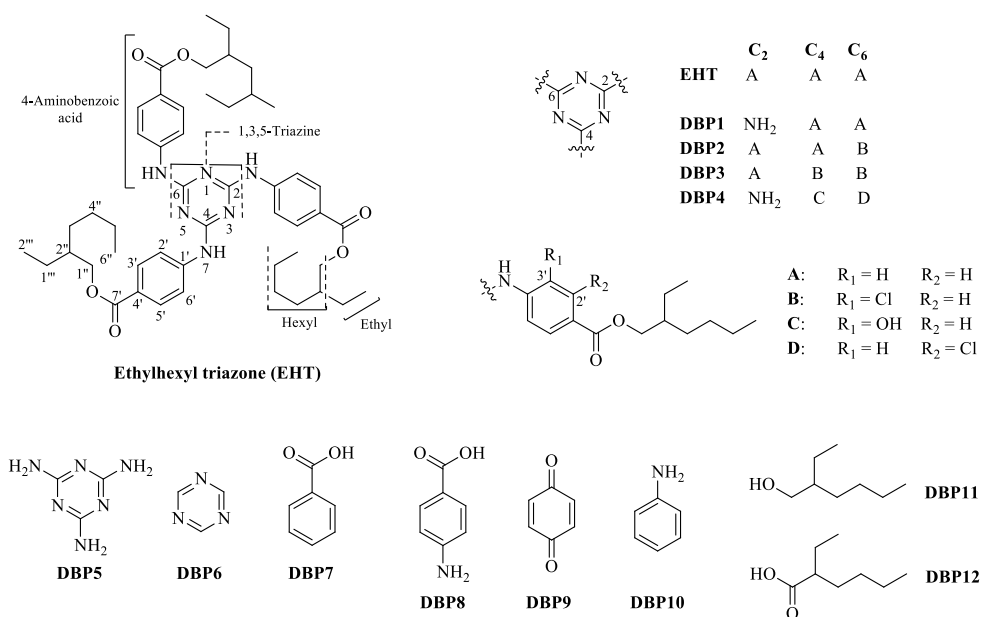


Figure 5 Chemical structures of Ethylhexyl triazone and its disinfection by-products DBP1–DBP12.

2.3.2.III Protocol for the disinfection and isolation of disinfection by-products (preparative experiment)

Ethylhexyl triazone (475 mg, 0.58 mmol) was dissolved in 10 mL of acetone, and the solution was diluted with water until a final volume of 0.9 L was reached. A sodium hypochlorite solution (approximately 10% active chlorine, molar ratio EHT/NaClO 1:25) was added drop by drop to this solution, under magnetic stirring at room temperature. The reaction progress was monitored by sampling the solution every 30 minutes, quenching with an excess of sodium thiosulphate, and drying under vacuum. The resulting residue was dissolved in water, pH-adjusted to 7.2, extracted with dichloromethane, and analysed by HPLC. Reaction was stopped after 3h with an excess of sodium thiosulphate and concentrated by lyophilisation. The residue was dissolved in saturated sodium bicarbonate solution and pH adjusted to 7.2, and this solution was extracted using dichloromethane. From this extract we then proceeded with the isolation (Figure 6) of the by-products having the same retention time as those obtained in the analytical experiments previously described. So, the crude organic fraction (632 mg) was chromatographed on silica gel CC, eluted with a gradient of petroleum ether:acetone (99:1 to 0:100, v/v) to yield 8 fractions.

The fraction Fr. 1 (55 mg), eluted with petroleum ether:acetone (99:1), was chromatographed on silica gel CC, eluted with a gradient of petroleum ether:acetone (98:2 to 90:10, v/v) to yield 4 fractions. Subfraction Fr. 1.1 (25 mg) was chromatographed on Strata FL-PR Florisil SPE (500 mg, 3 mL, Phenomenex, Bologna, Italy), eluted with hexane, to yield DBP6 (16 mg) and DBP9 (4 mg). Subfraction Fr. 1.3 (15 mg) was chromatographed on TLC, eluted with chloroform:methanol (98:2), to yield DBP3 (5 mg) and DBP4 (2 mg).

The fraction Fr. 3 (120 mg), eluted with petroleum ether:acetone (95:5), was chromatographed on silica gel CC, eluted with a gradient of petroleum ether:acetone (90:10 to 50:50, v/v) to yield DBP1 (6 mg) and DBP2 (46 mg).

A portion of fraction Fr. 6 (45 mg), eluted with petroleum ether:acetone (20:80) was dried, dissolved in an appropriate volume of acetone (100 μ L) and analyzed using GC FID instrument. The gas chromatograph was equipped with an Zebron™ ZB-FFAP, GC Cap. Column (30 m x 0.32 mm x 0.25 μ m film thickness, Phenomenex, Bologna, Italy). The following parameters were set during the experiments: 350 °C detector FID temperature, 1.5 mL/min constant flow helium (carrier gas), 1.0 μ L injected sample volume, 250 °C injector temperature, split ratio 20:1, oven profile 40 °C (hold 2 min), 8 °C/min to 250 °C (hold 10 min). DBP11 (t_R 20.2 min, 2 mg) and DBP12 (t_R 25.7 min, 3 mg) were identified by comparing the results with data on standard compounds and their relative calibration lines.

The fraction Fr. 7 (156 mg), eluted with petroleum ether:acetone (10:90), was chromatographed on silica gel CC, eluted with a gradient of chloroform:methanol:acetic acid (90:10:0.5 to 70:30:0.5, v/v/v) to yield 5 fractions. Subfraction 7.1 (33 mg) was separated by semipreparative HPLC using a column Phenomenex Synergi 10 μ m Fusion-RP 80 Å (250 $\times$ 10.00 mm) and eluted with HCOOH (A, 0.1% in H₂O) and acetonitrile (B), starting with 5% B for 5 min and followed by the installation of a gradient to obtain 100% B over 30 min, at a solvent flow rate of 5 mL/min to yield DBP5 (4 mg), DBP8 (9 mg), and DBP10 (7 mg). Subfraction Fr. 7.4 (40 mg), was separated by semipreparative HPLC using a column Phenomenex Kromasil 10 μ m 100 Å C18 (250 $\times$ 10.00 mm) and eluted with HCOOH (A, 0.1% in H₂O) and methanol (C), starting with 20% C for 5 min and followed by the installation of a gradient to obtain 100% C over 30 min, at a solvent flow rate of 5 mL/min, to yield DBP7 (17 mg).

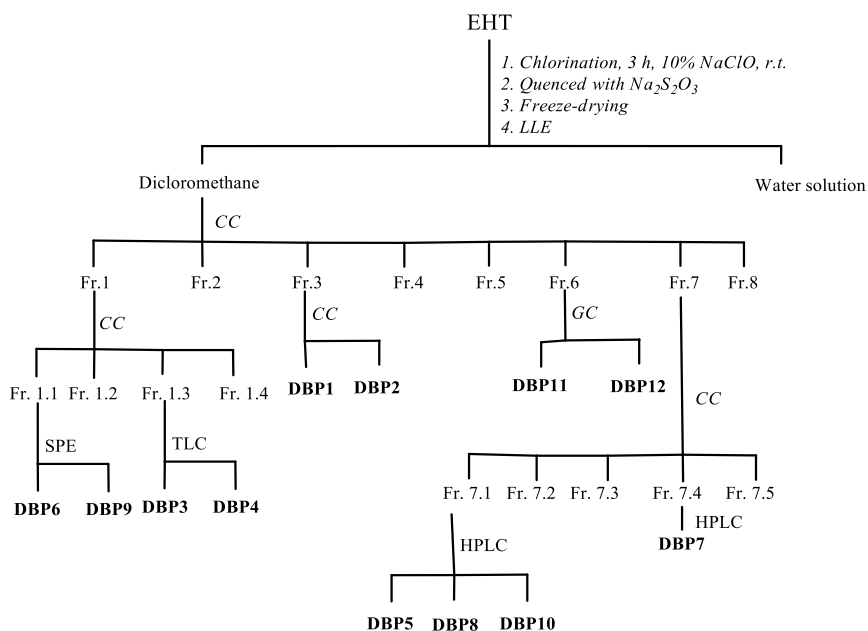


Figure 6 The isolation and identification of the different identified disinfection by-products.

2.3.2.IV Ecotoxicity tests

Ethylhexyl triazone and its by-products DBP1-DBP4 were dissolved in 100% acetone, sonicated for 30 min to obtain stock solutions and stored in the dark at 4 °C. Each test sample was obtained by mixing the proper volume of the stock solution and specific synthetic media for the considered organisms.

2.3.2.V Acute toxicity tests

The rotifer *B. calyciflorus*, the anostracan crustacean *T. platyurus* and the benthic ostracod *H. incongruens* were used in acute assays.

B. calyciflorus was employed for acute toxicity assay (ASTM E 1440–91, 2004) using organisms hatched from cysts obtained from MicroBioTest Inc. (Nazareth, Belgium) under continuous light exposure ranging from 3000 to 4000 lux for 16–18 h. The assays were conducted in a synthetic freshwater with a hardness of 80–100 mg/L CaCO₃, pH maintained at 7.5±0.3, maintained at 25±1 °C. The concentrations of the tested compounds ranged from 15 to 0.06 mg/L for EHT, from 5 to 0.03 mg/L for

DBP1, from 8 to 0.03 mg/L for DBP2, from 10 to 0.03 mg/L for DBP3, and from 9 to 0.03 mg/L for DBP4, following a dilution factor (DF) of 2. Subsequently, the test compounds were distributed into 36-well plates (0.3 mL/well), with five rotifers transferred to each well for six replicates per concentration. Negative control (NC), containing only synthetic freshwater, and solvent control (SC, acetone 0.025% v/v relative to the maximum concentration of each compound tested) were also included. Plates were then incubated for 24h at 25 °C in darkness.

Cysts of anostracan crustacean *T. platyurus* (MicroBioTest Inc., Nazareth, Belgium) were hatched in the same synthetic medium and light conditions of the rotifer for 20–22h of incubation at 25 °C following ISO 14380 (2011). At this first larval stage (instar I) nauplii live on their yolk reserves and do not have a fully developed digestive tract. The mouth is not open, and they do not ingest particles. The tested concentrations were the same utilized testing rotifers. Then, 1 mL/well of each dilution was put in 24-well plates and ten organisms per well were added to, for three replicates per concentration. NC was prepared using only the synthetic freshwater. Plates were incubated for 24h at 25 °C in the dark.

H. incongruens assay, following ISO 14371 (2012) guidelines, utilized ostracod neonates hatched from cysts (MicroBioTest Inc., Nazareth, Belgium). The hatching was conducted under 3000-4000 lux illumination for 52h in moderately hard synthetic freshwater at 25±1 °C. Pre-feeding with the blue-green *Spirulina* (MicroBioTest Inc., Nazareth, Belgium) occurred 4h before the test. In each well (6-well plates), ten organisms were exposed to 2 mL test solutions (ranging from 6 to 0.05 mg/L for EHT, 2 to 0.00005 mg/L for DBP1, 3 to 0.0007 mg/L for DBP2, 4 to 0.0002 mg/L for DBP3 and DBP4), 2 spoons (1728.88±48.30 mg dw) of reference sediment (river sand, particle size between 0 and 2 mm, MicroBioTest Inc., Nazareth, Belgium) and 2.0 mL of algal suspension (1.5×10^7 cells *R. subcapitata*/mL). The concentrations tested, followed a DF of 2, were determined after range finding tests. Tests were conducted in triplicate, exposing organisms for 6 days in darkness.

For each acute test, the negative control was performed exposing organisms to the appropriate test medium. Mortality was identified as the endpoint and calculated by comparing the number of organisms in the test dilutions of each compound with those in the negative control to determine the effective percentage. The effective percentages were analysed to determine the Median Lethal Concentration (LC₅₀) using non-linear regression (log agonist vs. normalized response-variable slope).

2.3.2.VI Chronic and sub-chronic toxicity tests

Chronic toxicity assays were performed in the rotifer *B. calyciflorus* and the green alga *R. subcapitata*, while sub-chronic toxicity tests in the ostracod *H. incongruens*.

The reproduction inhibition in *B. calyciflorus*, was determined following the ISO 20666:2008, in 48 h. The test was performed in less than 2h old organisms coming from cysts hatched as described above, conducted in 48-well plates, each well containing one rotifer and 0.9 mL of test solution. The concentrations tested, chosen after range finding tests, were from 1000 to 0.93 µg/L for EHT, from 1000 to 2.98 µg/L DBP1, from 3200 to 9.54 µg/L for DBP2, from 300 to 0.29 µg/L for DBP3 and DBP4, using a DF of 3.2. The organisms were fed with 0.1 mL of fresh suspension of 10⁷ cells/mL of the unicellular alga *R. subcapitata*. All sample dilutions were tested in eight replicates, and the plates were incubated in darkness at 25 °C for 48 h.

R. subcapitata growth inhibition test (OECD 201, 2011; Nugnes et al., 2024a and 2024b) was performed in 96-well plates in six replicates. The concentrations tested, chosen after appropriate range finding tests, were from 15000 to 0.4 µg/L for EHT, from 5000 to 0.14 µg/L for DBP1, from 8000 to 0.7 µg/L for DBP2, from 10000 to 0.3 µg/L for DBP3 and from 9000 to 0.25 µg/L for DBP4; for each sample a dilution factor of 3.2 was used. Continuous illumination (6000 lux) at 25±1 °C and a microplate shaker (450 rpm) were used to incubate the test solutions (200 µL) with 10⁴ cells/mL of algal suspension (100 µL). An automated microplate reader (Synergy H1, Biotek, Winooski, USA) was utilized to read the plates at 450 nm at time 0, after 24h, 48h and 72 h.

As regards the sub-chronic test in *H. incongruens*, it was set up in the same way as the acute assay following ISO 14371 (2012) guidelines. The growth inhibition, evaluated as variation in body length (test endpoint), was determined only when the mortality in the acute assay was below 30%. The live organisms were immobilized with Lugol's fixative to measure their length using a micrometre immediately before exposure to the samples and after 6 days of incubation at 25 °C in darkness.

Negative control (only medium) was performed for each chronic/sub-chronic test. The rotifer offspring, the algal growth inhibition and the ostracod length findings obtained in test solutions were compared to those obtained for the respective negative controls to determine the effective percentages.

2.3.2.VII Data analysis

To measure acute and chronic toxicity, three experiments were conducted, and the effect percentages were determined by comparing the results of the samples to each negative control.

The concentrations that determine X% effect (L(E)Cx) were estimated through non-linear regression (log agonist versus normalized response-variable slope) using Prism 9 software (GraphPad Inc., CA, USA) by pooling effect percentages.

The median lethal concentration (LC₅₀) and the concentration giving the 20% lethality (LC₂₀) were the endpoint of all acute toxicity tests. The EC₅₀, EC₂₀ and EC₁₀, were the concentrations giving 50%, 20% and 10% inhibition of reproduction of rotifers, the inhibition of growth in algae and ostracods.

2.3.3 Results and Discussion

2.3.3.I Disinfection experiments

The EHT chlorination experiments were performed in concentrations of about 5×10^{-5} M. Specifically, the sunscreen solutions were treated for 30 min with sodium hypochlorite at 10% (EHT:NaClO molar ratio 2:1), under magnetic stirring and at

room temperature. The presence of EHT was quantified using a spectrophotometer with absorbance measures determined at 310 nm (Baker et al., 2017). The absorbance values were converted into a concentration using a calibration curve prepared from five standard solutions with known EHT concentrations. At these starting EHT concentration values it was not possible to isolate the disinfection by-products, because they were present in too small quantities. The experiments were repeated at higher EHT concentrations, treated with 10% sodium hypochlorite (EHT:NaClO molar ratio 1:25) for 3h, at room temperature. The pH of the solution, measured and recorded continuously using a pH-meter, increased immediately from the initial value of 8.0 to 10.8 and remained at this value during the reaction. In this last conditions it was possible to have enough amounts of disinfection by-products to proceed with their isolation. The course of the reaction was monitored by HPLC using an aliquot of the solution quenched by sodium thiosulphate excess, filtered, and extracted with methylene chloride.

The DBPs (Figure 5), isolated by column chromatography and HPLC (Figure 6), were completely characterized using NMR and MS analyses. Finally, DBP1–DBP12 were isolated at relative percentages of 1.26, 9.68, 1.05, 0.42, 0.84, 3.37, 3.58, 1.89, 0.84, 1.47, 0.42, and 0.63. DBP2–DBP4 were isolated for the first time and a proposed mechanism of formation of all DBPs from EHT is shown in Figs. 2 and 3. Spectral data for Ethylhexyl triazone and its disinfection by-products are listed in chapter supplementary materials b SMb.

2.3.3.II Structure elucidation of disinfection by-products DBP1-DBP12

From the chlorination of EHT, 12 disinfection by-products have been isolated and identified, of which the first four retain the central core consisting of the 1,3,5-triazine ring, with at least two of the three side chains. Precisely, the by-product DBP1 differs from the starting product in having suffered the loss of one of the three side (pathway *a* Figure 7) chains and in presenting a free amino function at the carbon C-2 of the triazine ring. The by-product DBP2 retains all three side chains of the triazine core

but one of the three was chlorinated at the position C-2' (pathway *b* Figure 7). The by-product DBP3 must be considered a derivative of the previous one since it presents two (and not one) of the three side chains chlorinated at the positions C-2'. Finally, the by-product DBP4 presents: *i*) the loss of one of the three side chains (pathway *a*), with the presence of a free amino function on the carbon C-2 of the central ring, *ii*) chlorination on the carbon C-3' (and not C-2') of the aromatic ring of one of the two remaining side chains (pathway *c* Figure 7), and *iii*) the oxidation of the phenyl ring of the third side chain, which in fact has a hydroxyl in the position C-2' (pathway *d* Figure 7).

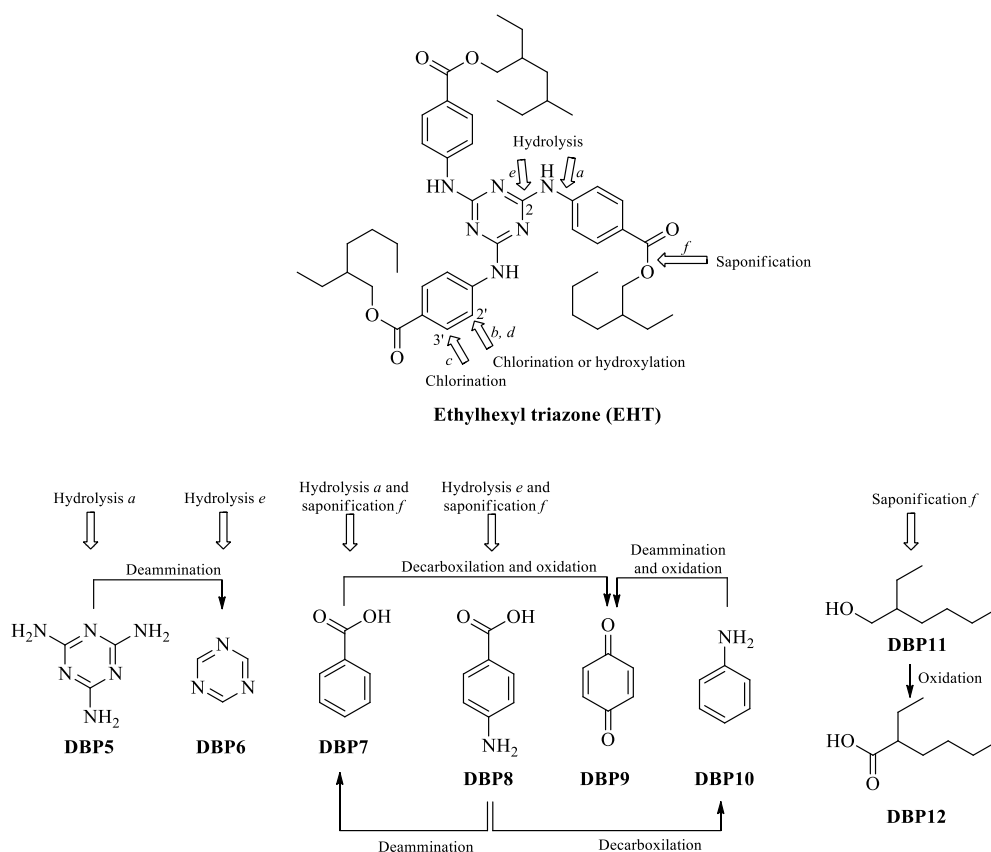


Figure 7 Mechanistic pathways for the formation of EHT disinfection by-products

Then, two by-products were also isolated which retain the 1,3,5-triazine nucleus, namely DBP5, with the three free amino groups linked to carbons C-2, C-4 and C-6, and the simpler DBP6 which coincides precisely with the simple 1,3,5-triazine, that can be considered obtained from the previous by-product by reductive deamination or by cleavage of the bond between the amino functions and the triazine ring (pathway *e* Figure 7).

Four by-products with a phenyl aromatic nucleus were also isolated, evidently deriving from one of the three side chains, namely benzoic acid (DBP7), its *p*-amino derivative (DBP8), quinone (DBP9), and aniline (DBP10). The first is the by-product that would be obtained during the formation of the by-product DBP5 (pathway *a* Figure 7), by splitting the bonds between the amine functions and the phenyl aromatic rings. The by-product DBP8 could be obtained from the hydrolysis of the bonds between the amino functions and the triazine ring (and the simultaneous formation of DBP6) (pathway *e* Figure 7). Quinone is the oxidation product that can be obtained from the oxidation of benzene, which in turn can be easily obtained from the decarboxylation of benzoic acid DBP7. It is easy to hypothesize that the latter occurred through decarboxylation of *p*-aminobenzoic acid DBP8.

The last two isolated by-products are the alcohol DBP11, obtained from the saponification (pathway *f* Figure 7) of one of the three ester functions of the side chains, and its corresponding carboxylic acid DBP12.

2.3.3.III Proposed mechanism for the formation of disinfection by-products DBP1–DBP12

The EHT molecule has a 1,3,5-triazine nucleus and three amine functions at the C-2, C-4 and C-6 positions. Each of these is involved in the formation of a bond with the C-4 carbon of a benzoic acid residue, esterified with a 2-ethylhexyl chain. It is easy to hypothesize that under the conditions of basic reactions used, an aromatic residue can undergo saponification and supply the alcohol DBP11, which in turn by oxidation, can form the acid DBP12, and the intermediate *I1* (Figure 8). This last one, then, gives the intermediate *I2* by decarboxylation. Intermediate *I2* could lead to intermediate *I5*,

by oxidation of the phenyl ring in para position with respect to the amino function and by chlorination of the amino function itself.

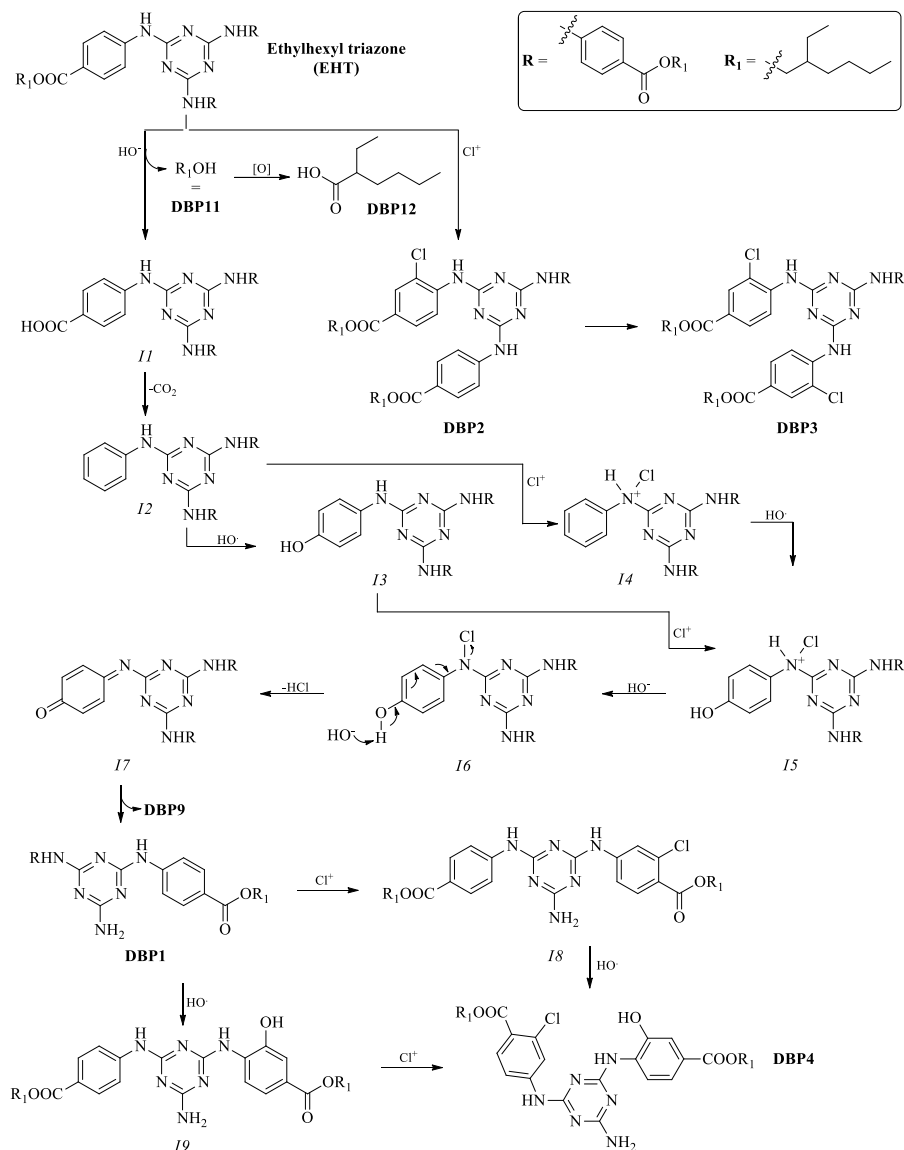


Figure 8 Plausible mechanism for the formation of DBP1–DBP4, DBP9, and DBP11–DBP12.

Probably the formation takes place through the intermediate **I3** and not that **I4**, the latter in fact due to the inductive effect of the positively charged nitrogen could preferably be hydroxylated in meta (and not in para) to the amino function itself.

However, intermediate *I5*, via the deprotonated intermediate *I6*, could undergo dehydrochlorination to form a 4-iminocyclohexa-2,5-dienone type derivative (*I7*). Hydrolysis of the imine function of *I7* would provide the products DBP9 and DBP1. From this, then, the DBP4 product would be obtained by hydroxylation on the C-2' of one of the two remaining phenolic rings and chlorination on the C-3' carbon of the different phenyl ring, through the possible intermediates *I8* or *I9*, respectively. Finally, the chlorination reaction on the C-2' position of one of the three phenyl rings of the starting product would give the product DBP2 and, by subsequent chlorination of this product on one of the two remaining phenyl rings, DBP3 would be obtained.

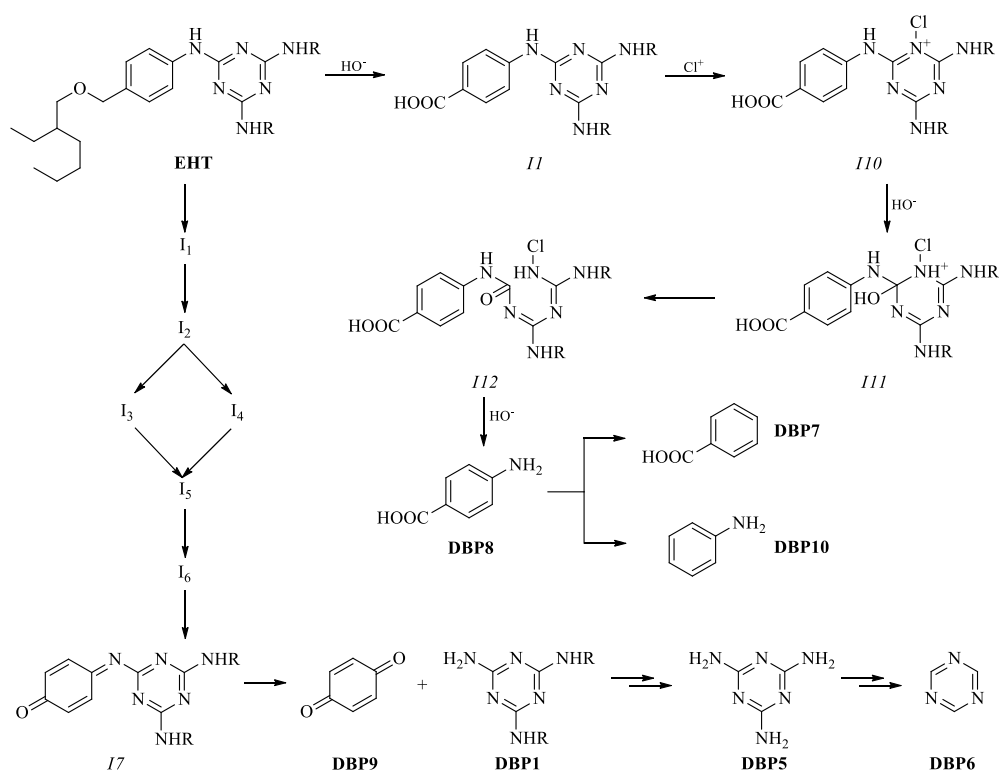


Figure 9 Plausible mechanism for the formation of DBP1 and DBP5–DBP10

Repeating the reaction sequence that leads to the formation of DBP1 two additional times would produce DBP5 (Figure 9). Reductive deamination of the latter would lead

to the formation of DBP6. The intermediate *II* could also undergo chlorination at one of the two N atoms of the triazine ring, adjacent to the C-2 carbon, and lead to the formation of the intermediate *II0*. The latter could first be hydroxylated at carbon C-2, to form the corresponding intermediate *III*, and then be further oxidized at intermediate *II2*, which would show triazine ring opening. The hydrolysis of the amide bond would lead to the formation of DBP8, from which DBP7 could then be obtained by reductive deamination and DBP10 by decarboxylation (Figure 9).

2.3.3.IV Ecotoxicity tests

To evaluate the ecotoxicological effects of Ethylhexyl triazone and its by-products under investigation, acute and chronic assays were conducted using freshwater organisms belonging to different trophic levels of the freshwater chain: the unicellular alga *R. subcapitata* as producer, the rotifer *B. calyciflorus*, the anostracan crustacean *T. platyurus*, and the ostracod crustacean *H. incongruens* as primary consumers.

2.3.3.V Acute toxicity tests

Acute toxicity results were obtained exposing *B. calyciflorus* (24h-exposure), *T. platyurus* (24h-exposure) and *H. incongruens* (6 day-exposure) to EHT and its DBPs. Data were reported or as LC₅₀, the concentration (mg/L) determining the death of 50% of the exposed organisms, or as mortality percentages at the maximum concentration tested (chosen according to the percentages of no statistically significant effect of acetone solvent, equal to 0.025% for *B. calyciflorus* and *T. platyurus* and 0.01% for *H. incongruens*, compared to negative control, Dunnett's multiple comparison test) if the LC₅₀ values were not reached, Table 3.

Table 3 Acute toxicity results of EHT and DBPs, expressed as LC₅₀ values [mg/L] or effect percentages (mean ±SD), with 95% confidence intervals and maximum tested concentrations reported in brackets.

EHT	DBP1	DBP2	DBP3	DBP4
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<i>B. calyciflorus</i> (24h)	12.86 (8.70–30.26)	43.35%±3.35 (5 mg/L)	34.80%±2.54 (8 mg/L)	46.70%±0.01 (10 mg/L)	36.65%±3.35 (9 mg/L)
<i>T. platyurus</i> (24h)	37.50%±2.51 (15 mg/L)	33.30%±0.01 (5 mg/L)	15.85%±0.85 (8 mg/L)	10.00%±0.01 (10 mg/L)	11.12%±1.90 (9 mg/L)
<i>H. incongruens</i> (6 d)	42.52%±7.68 (6 mg/L)	45.00%±0.01 (2 mg/L)	27.50%±10.60 (3 mg/L)	42.50%±3.53 (4 mg/L)	37.50%±3.53 (4 mg/L)

The parent compound EHT revealed to be the only molecule for which it was possible to obtain the LC₅₀ value (12.86 mg/L) when tested in *B. calyciflorus*. Considering this value, EHT is recognized to be harmful (EC₅₀ value between 10 and 100 mg/L) for the rotifer, according to EU Directive 93/67/EEC and the Globally Harmonized System of Classification and Labelling of Chemicals used to classify chemical substances based on the EC₅₀ values. EHT, at the maximum concentration tested (15 mg/L) on the *T. platyurus* induced a mortality of 37.50% highlighting the greater sensitivity of the rotifers than the anostracan crustaceans after the same time of exposure (24h). Although *T. platyurus* was in a non-feeding early life stage, a crucial stage for its sensitivity (Yang et al., 2024), it was the least affected organism during the acute exposure.

The rotifer seems to have a higher level of sensitivity to EHT also than the ostracod, which has a maximum percentage effect of 42.52%±7.68 when tested at a maximum concentration of 6 mg/L.

Regarding the DBPs, the percentages of mortality (~30 - 40%) were overlapping when tested at units of mg/L in the ostracod and the rotifer (only 10 mg/L for DBP3 in rotifer). The lowest effect percentages (< 20%) were observed testing DBP2, DBP3 and DBP4 in the anostracan crustaceans, proving to be the most resistant organism at the investigated molecules after the acute exposure. For the same organism, the highest percentage of effect was shown by DBP1 (33.30%±0.01) when tested at 5 mg/L.

Going deeply, to facilitate a comparison among all the tested molecules, the concentrations (mg/L) of each compound inducing the death of the 20% of organisms exposed (LC_{20S}) were estimated (Table 4).

Table 4 Acute toxicity results expressed as LC₂₀ [mg/L] with 95% confidence intervals in brackets obtained by exposing consumers to EHT and its DBPs.

	EHT	DBP1	DBP2	DBP3	DBP4
<i>B. calyciflorus</i>	3.71	2.52	3.38	1.07	2.08
(24h)	(1.86–6.01)	(2.07–2.95)	(1.77–5.60)	(0.001–1.770)	(1.33–2.96)
<i>T. platyurus</i>	4.34	3.08	n.e.	n.e.	n.e.
(24h)	(1.82–7.96)	(2.34–3.96)			
<i>H. incongruens</i>	0.15	0.01	0.24	0.04	0.07
(6 d)	(0.03–0.50)	(0.004–0.020)	(0.05–1.34)	(0.02–0.08)	(0.02–0.18)

n.e.: no effect ($\leq 10\%$)

What clearly emerges from Table 4 is that LC₂₀ values, obtained by exposing *B. calyciflorus* to the investigated molecules, were always in the order of magnitude of units of mg/L, without statistically significant differences between the parent compounds and its DBPs. Similarly, exposing *T. platyurus* to EHT and DBP1, LC₂₀ values were at units of mg/L but the remaining DBPs determined a mortality lower than or equal to 10%, confirming the least sensitive of this organism than the other tested consumers. Interestingly, the ostracod proved to be the most sensitive organism, for which LC₂₀ values (Table 4) were estimated in the order of tens (DBP1, DBP3 and DBP4) and hundreds (EHT and DBP2) of µg/L, very close to the most investigated sun filters environmental concentrations of (ng-µg/L) (Tsui et al., 2014). In the light of above, DBP1, DBP3 and DBP4 showed the greatest acute toxicity, and the ostracod was the most sensitive consumer among those tested.

To the best of our knowledge, little data is present in literature regarding the aquatic toxicity of EHT in freshwater non-target organisms; the studies found in literature mainly concern ecotoxicological investigations in sea water. In addition, to the best of our knowledge, this work is the first to introduce the study relating to the identification and ecotoxicology of the EHT chlorinated by-products obtained. Thorel and collaborators (2020) evaluated the acute toxicity of Ethylhexyl triazone in the saltwater crustacean *Artemia salina* (primary consumer) and similarly to the results obtained in this study, EHT did not determine an effect of 50% up to the maximum

concentration tested (2 mg/L). Looking for other UV-B filters ecotoxicity, DePaula and collaborators in 2022 evaluated the effect of 2-Ethylhexyl-4-methoxycinnamate (EHMC) in the freshwater crustacean *D. magna* and in the saltwater crustacean *A. salina*, reporting LC₅₀ values of 0.5 mg/L and 0.37 mg/L, respectively. In 2023, Marcin and Aleksander tested the effect of Octocrylene (OCT) in *A. salina* and *D. magna*, reporting acute toxicity values corresponding to LC₅₀ values of 0.55 mg/L and 2.66 mg/L, respectively.

2.3.3. VI Chronic toxicity testing

Chronic toxicity tests were conducted by exposing the microalgae *R. subcapitata*, the rotifer *B. calyciflorus* and the ostracod *H. incongruens* to the selected molecules over an extended period to simulate real aquatic conditions to which organisms are exposed to residues of chemical compounds for a prolonged period and at concentrations lower than those causing acute toxicity. As regards results obtained exposing the producer *R. subcapitata* to the selected molecules, no chronic effect was observed for all of them after 72h (algal growth inhibition < 10%) at the highest tested concentration (15 mg/L for EHT; 5 mg/L for DBP1; 8 mg/L for DBP2; 10 mg/L for DBP3; and 9 mg/L for DBP4) prepared in 0.025% acetone, percentage that induced effects not statistically different from those of the negative control (*Dunnett's multiple comparison test*). Differently, by exposing *B. calyciflorus* and *H. incongruens* consumers to EHT and its disinfection products, inhibition of reproduction and inhibition of growth (variation in length) were respectively observed after 48h and 6 days of exposure. Results, expressed as EC₅₀, EC₂₀ and EC₁₀, i.e. the concentrations of the molecules determining the 50%, 20% and 10% of effect respectively, were shown in Tables 5-6.

Table 5 Chronic toxicity results expressed as EC_{50} , EC_{20} and EC_{10} ($\mu\text{g/L}$) and effect percentage of EHT and DBPs obtained in the rotifer *B. calyciflorus*. 95% confidence intervals and maximum concentrations tested are reported in brackets.

	EC_{50}	EC_{20}	EC_{10}
EHT	46.28% $\pm$ 12.43 (1000 $\mu\text{g/L}$)	40 (20–90)	6 (2–20)
DBP1	39.67% $\pm$ 10.94 (1000 $\mu\text{g/L}$)	70 (30–130)	8 (4–26)
DBP2	1970 (1160–3320)	150 (70–270)	30 (10–90)
DBP3	200 (120–340)	6 (3–12)	1.8 (0.1–2.5)
DBP4	39.03% $\pm$ 10.51 (310 $\mu\text{g/L}$)	40 (10–70)	6 (1–200)

Table 6 Sub-chronic toxicity results expressed as EC_{50} , EC_{20} and EC_{10} ($\mu\text{g/L}$) and effect percentage of EHT and DBPs obtained in the ostracod *H. incongruens*. 95% confidence intervals and maximum concentrations tested are reported in brackets.

	EC_{50}	EC_{20}	EC_{10}
EHT	210 (120–470)	19 (7–37)	5 (1–14)
DBP1	9 (6–10)	2 (1–3)	0.8 (0.03–2.1)
DBP2	1620 (1050–2760)	70 (30–130)	10 (3–30)
DBP3	42.37% $\pm$ 9.30 (110 $\mu\text{g/L}$)	10 (5–22)	2 (0.6–4)
DBP4	20 (10–30)	3 (2–5)	1 (0.5–2)

The rotifer exposed to the parent compound and its DBPs, showed the highest activity with EC_{50} s at hundreds of $\mu\text{g/L}$ for DBP3. DBP4 exhibited 39.03% di effect at 310

µg/L. Less chronically toxic was DBP2 with EC₅₀ in the order of thousands of µg/L. The ostracod was the most sensitive consumer with EC₅₀ values of units for DBP1, tens for DBP4 and hundreds of µg/L for EHT. In line with the EU Directive 93/67/EEC, considering EC₅₀ values < 1 mg/L, DBP1, DBP4, EHT resulted very toxic for crustaceans while DBP3 resulted very toxic for rotifers. With EC₅₀ values from 1 to 10 mg/L, DBP2 resulted toxic for both organisms for long term exposure. As regards the EC₂₀, the concentration giving the 20% effect, particular attention is necessary. This value is to be assumed as the concentration giving the lowest effect, extrapolated from a nonlinear regression (log agonist vs. normalized response-variable slope) by the software Graphpad prism 9 and not dependent on the test concentrations chosen by the operator. Thus, in the present study, DBP1 and DBP4 were active in ostracod crustaceans already at units of µg/L (2 and 3 µg/L, respectively, Table 6), exactly as DBP3 (6 µg/L, Table 5) in rotifers showing greater toxicity than EHT showing EC₂₀s at tens of µg/L for both organisms.

Differently from EC₂₀, the EC₁₀ values (the concentration causing a 10% effect, not significantly different from that of the negative control) were found in the range of units of µg/L for EHT, DBP3 and DBP4 for both consumers. Like acute toxicity assays, also for chronic toxicity assays, to the best of our knowledge, literature data regarding the toxic effects of EHT and its DBPs in freshwater organisms are scarce. The chronic effect of Ethylhexyl triazone was studied by Thorel and collaborators in 2020 in the sea microalgae *Tetraselmis spp*, and no inhibition of growth was observed up to 1 mg/L, in line with the results found in this study. In 2020, Stien and collaborators observed no effect on coral metabolome exposed to EHT up to the same order of concentration detected in this study. Fel et al. (2019) studied the chronic effects of EHT on the maximal photosynthetic efficiency of the symbionts associated with the scleractinian coral *Stylophora pistillata* with no effect concentration up to 5 mg/L. Szczesny and collaborators, in 2010, tested the activity of another UV filter, the Ethylhexyl Methoxycinnamate, on *B. calyciflorus*, with a chronic EC₅₀ at hundreds of µg/L, more active than Ethylhexyl triazone investigated in this study.

An overall comparison between the effective concentrations of EHT and its by-products detected in test organisms and the occurrence of such xenobiotics in aquatic systems is essential to comprehend their real environmental impact. To the best of our knowledge, environmental occurrence of EHT is scarce. In 2018, Apel and coauthors quantified EHT in the Rhine-Meuse-Delta and the German Bight at concentrations up to 2.0 ng/g dry weight sediment (Apel et al., 2018a).

Considering our results and the environmental occurrence, we estimated the environmental risk posed by EHT, following standard guidelines (EU TGD, 2003; EMA, 2024; ECHA, 2013).

Since EHT is a hydrophobic compound ($\text{LogK}_{\text{ow}} = 7$), the Risk Quotient (RQ) was calculated as the ratio between the measured environmental concentration (MEC) in freshwater sediments (0.31-2 ng/g dw, Apel et al., 2018a) and the predicted no-effect concentration for sediments (PNEC_{sed}). The PNEC_{sed} was calculated using the EC_{10} value of ostracods (Table 6) after its conversion into ng/g dw and applying an assessment factor (AF) of 100, as only one trophic level was used. The resulting RQ values ranged from 5.36 to 34.6, which exceed the threshold value of 1, indicating potential environmental concern.

2.3.4 Conclusions

The present study focused on the environmental fate of one of the most used UV filters, the Ethylhexyl triazone, following the disinfection treatment by chlorination, commonly used in the disinfection of swimming pool water. After chlorination, chromatographic techniques were used to isolate twelve disinfection by-products, three of which for the first time, and fully characterized by MS and NMR analysis. After the chlorination treatment, approximately 62% of EHT was recovered unchanged, and the 25% constituted the disinfection products which formation was proposed through a possible reaction mechanism. Toxicity tests highlighted that the consumers, particularly the benthic ostracods, were the most sensitive organisms to

EHT and its by-products. At the end of this study, we assessed the environmental risk of EHT using its occurrence in freshwater sediments and the EC₁₀ value in ostracods obtaining a risk quotient greater than the threshold value of one, with environmental concern.

Chapter 3 Oxidative Techniques for Pharmaceutical Pollution

3.1 Efficient Nimesulide Removal via Chlorination and Sun-Simulated Radiation: Kinetic Insights, Reactive Species Formation, and Application to Real Wastewater

3.1.1 Introduction

Nimesulide (*N*-(4-Nitro-2-phenoxyphenyl)methanesulfonamide) has been used since 1985 under the brand names Mesulid or Aulin. It is primarily used to treat acute pain conditions such as dysmenorrhea and osteoarthritis. In 2007, Nimesulide came under investigation by the European Medicines Agency (EMA) following its withdrawal from the Irish market due to potential liver damage issues. Despite this, considering that the benefits outweighed the risks, the EMA allowed the commercialization of Nimesulide in Europe, and many member states, including France, Italy, and Greece, permit its use. However, the continued global use of this drug and its improper disposal as common household waste (Salgado et al., 2021) have contributed to increase surface water pollution (Caldas et al., 2013; Lacey et al., 2012; Papageorgiou et al., 2016; Paiga et al., 2016), classifying it as an emerging contaminant. Several studies in the literature highlight the potential removal of Nimesulide through advanced oxidation treatments using activation of H_2O_2 (da Silva et al., 2018), peroxydisulfate (Xin et al., 2021), peroxymonosulfate (Liu et al., 2021).

The present investigation aims to demonstrate an effective method for degrading an emerging pollutant like Nimesulide during conventional oxidation processes used in wastewater treatment. The proposed approach involves specifically the use of hypochlorite, typically employed for bacterial disinfection. However, in this research study, the oxidizing agent is activated using polychromatic light emitting from 290

nm or, more simply, natural sunlight. Through this process, high reactive species such as $\text{HO}^\bullet$ and $\text{Cl}^\bullet$ are generated, capable of further oxidizing the analysed pollutant, significantly improving its removal rate and enhancing the production of by-products.

3.1.2 Materials and Methods

3.1.2.I Chemicals

Nimesulide (NIM), sodium hydroxide (NaOH), sulfuric acid (H_2SO_4), benzoic acid, phenol, 1,4-dimethoxybenzene (DMOB), sodium thiosulfate were obtained from Sigma Aldrich (Milan, Italy). NaOH and H_2SO_4 were used to adjust the pH of solutions. All chemicals were used without further purification. Unless otherwise specified, Milli-Q water (18 M Ω cm) was used in all experiments.

3.1.2.II Degradation experiments and kinetics

Degradation experiments were conducted using a 100 mL Pyrex reactor maintained at a constant temperature of 20 °C. Four fluorescent lamps (F15W/350BL, Sylvania blacklight, Germany) emitting light within the range 290 – 410 nm (UVB-UVA region) with a total energy of 18.9 W m⁻² measured with a calibrated Ocean Optics QE65000 spectrophotometer were positioned on the top of reactor. The emission spectrum of adopted irradiation system termed as sun-simulated radiation (SSR) is illustrated in Figure 10, alongside the emission spectrum of solar radiation (SUN) taken on 15th June 2023 during the photochemical experiment conducted under real sunlight exposure. Throughout the entire reaction duration, solution homogeneity was maintained by a magnetic bar stirring mechanism.

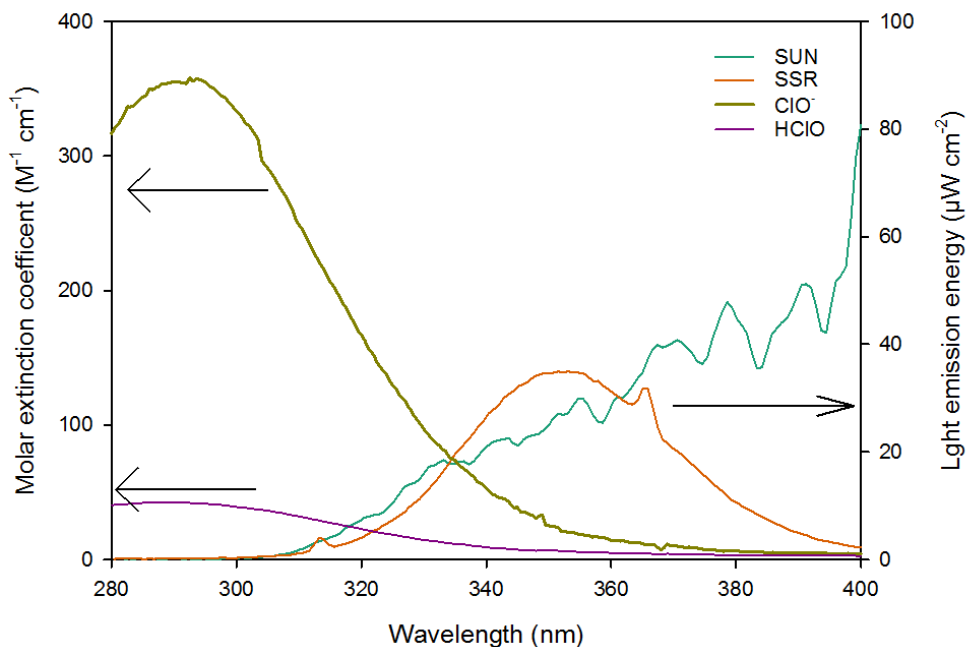


Figure 10 Molar absorption coefficients of HOCl and ClO*. Emission spectrum of the adopted lamp (SSR) and solar spectrum (SUN) recorded on 15th June 2023 on a sunny day in Clermont-Ferrand, France

Stock solutions of NaClO and NIM were prepared in Milli-Q water and stored at 4 °C until utilization in the experiments. Solutions containing 25 μM of NIM were prepared in the presence of different concentration of NaClO expressed as μM of Free Active Chlorine (FAC). Scavenging species were introduced immediately before each experiment, as required. In all experiments, the pH was adjusted using NaOH and H₂SO₄.

At predetermined intervals, 1 mL of aliquot was withdrawn and mixed with 20 μL of 0.1 M Na₂S₂O₃ to trap hypochlorite and prevent oxidation before analysis. The analysis of samples was conducted utilizing ultra-high performance liquid chromatography (UHPLC, Nexera XR, Shimadzu, France) coupled with a photodiode array detector (PDA). The flow rate was 1 mL min⁻¹ and the mobile phase was a

mixture of water (0.5% HCOOH) and acetonitrile (50/50, v/v). The column employed was a Nucleodur 100-3 C18 column (150 × 4.6 mm with particle size of 3 µm), thermostated at 40 °C. The retention time of NIM, monitored at 298 nm, was 7.91 min.

The pseudo-first order decay of NIM (k'_{NIM}) was calculated using the following equation 1:

$$\ln \frac{[NIM]}{[NIM]_0} = -k'_{NIM}t \quad (\text{eq. 1})$$

where $[NIM]_0$ and $[NIM]$ represent the initial and residual concentrations of NIM at time t , respectively, and k'_{NIM} is the pseudo-first-order apparent rate constant in s^{-1} .

The synergistic effect of solar simulated radiation (SSR) was estimated calculating the Enhanced Efficiency (EE) value determined as following:

$$EE = \frac{k'_{NIM,comb}}{\sum_i k'_{NIM,exp}} \quad (\text{eq. 2})$$

where $k'_{NIM,comb}$ is the kinetic constant determined in the combined experiment, while $\sum_i k'_{NIM,exp}$ is the sum of the kinetic constants from each individual process involved (i.e. direct oxidation and photolysis).

The total concentration of FAC was determined increasing the pH of sample to 12 and subsequently measuring ClO^- by UV-vis spectroscopy ($\epsilon_{292.5nm} = 360 \text{ M}^{-1} \text{ cm}^{-1}$) (Hussain et al., 1970).

Radical species generation and their contribution to NIM removal were investigated using a competition kinetic approach in the presence of specific organic scavengers.

The mineralization efficiency of NIM in water was assessed by monitoring the decrease in the total organic carbon (TOC) utilizing a TOC-L CSH/CSN, Shimadzu instrument.

Sewage treatment plant water (STPW) obtained from an urban sewage treatment plant located in Clermont-Ferrand, France (October, 2022) was used for photochemical experiments immediately after PTFE (0.45 μm size pore) filtration to prevent bacteriological and chemical alterations. The main physico-chemical characteristics are reported in chapter Supporting materials Table SM1c.

3.1.2.III Transformation products identification

The transformation products resulting from NIM disinfection were determined by UHPLC coupled to high-resolution mass spectrometry (HRMS) performed on an Orbitrap Q-Exactive (Thermo scientific). The chromatographic separation was achieved using a Phenomenex Kinetex C18 column (1.7 μm $\times$ 100 $\text{\AA}$; 100 $\times$ 2.1 mm), thermostated at 30 $^{\circ}\text{C}$. The initial mobile phase was a mixture 5% CH_3CN and 95% water with 1% formic acid, followed by a linear gradient to 99% CH_3CN within 8.5 min and kept constant during 1 min. The flow rate was 0.45 mL min^{-1} and the injection volume was 5 μL . Ionization parameters were set to 3.2 kV (ESI^+) and 3.0 kV (ESI^-).

For sample preparation, 20 mL of solutions under SSR and Dark were withdrawn at time corresponding to the about 40% of NIM removal and preconcentrated using solid phase extraction (SPE) with an Oasis HLB cartridge (Waters, Milford, MA. U.S.A.). The cartridge was conditioned with 3 mL of CH_3CN , equilibrated with 3 mL of ultrapure water and eluted to a final volume of 1 mL of CH_3CN .

The identification of short-chain carboxylic acids and inorganic anions was performed using ion chromatography-mass spectrometry (IC-MS) with a Dionex ICS-6000 system interfaced with a simple quadrupole mass spectrometer (Thermo Scientific, WALTHAM, MA. U.S.A.). A Dionex IonPac AS-11-HC column (4 μm 2×250 mm) was employed. The mobile phase consisted of a gradient starting with 1 mM of KOH from 0 to 5 min, followed by a linear increase to 30 mM within 25 minutes and to 60 mM from 25.1 to 31 min. The flow rate was maintained at 0.36 mL min^{-1} and column temperature was set at 40 $^{\circ}\text{C}$.

Negative ion mode using electrospray ionization (ESI capillary voltage of 3000 V) was applied on the simple quadrupole mass spectrometer. Full scan analysis was conducted within the m/z range of 10 to 500 with a resolution of ± 0.5 amu.

3.1.3 Results and Discussion

3.1.3.1 NIM removal by FAC/SSR process

To evaluate the stability of Nimesulide under sun-simulated radiation and in the presence of 0.5 mM of FAC, experiments were conducted at pH 7.5, corresponding to the pKa of HOCl/ClO⁻ (Figure 12). Interestingly, in the presence of 0.5 mM of FAC, only 10% degradation of NIM was observed, yielding a degradation constant (k'_{NIM}) of $7.91 \pm 0.40 \times 10^{-5} \text{ s}^{-1}$. In contrast, when SSR was employed with FAC, the degradation constant significantly increased reaching $1.36 \pm 0.07 \times 10^{-4} \text{ s}^{-1}$ (Figure 11). This augmentation in the degradation rate led to nearly 80% removal of NIM within a 3h timeframe. Notably, this substantial improvement in the enhancement efficiency (EE) when employing combined SSR and FAC direct oxidation was estimated to be 1.7. It is noteworthy that under direct irradiation alone, no significant NIM photolysis was observed.

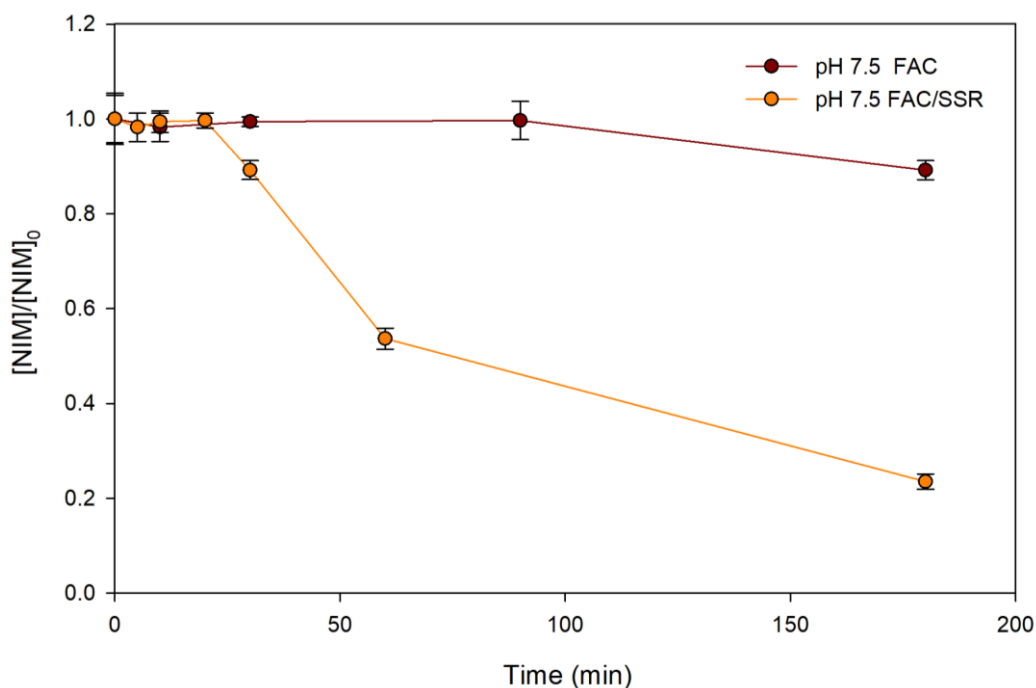


Figure 11 NIM removal at pH 7.5 in dark and sun-simulated irradiation. Initial conditions for both experiments are: $[NIM] = 25 \mu M$ and $[FAC] = 0.5 mM$.

Figure SM1c illustrates a substantial decline in FAC concentration during irradiation in the presence of NIM. In fact, after 1h of reaction, a conversion of 22% of FAC was observed, corresponding to a degradation constant of $5.08 \pm 0.37 \times 10^{-5} s^{-1}$. This observation clearly indicates the evident consumption (i.e. activation) of FAC as it actively participates in the degradation of NIM. To assess the oxidation efficiency of this process, it was calculated the reaction stoichiometric efficiency (RSE) (Ghauch et al., 2015). RSE serves as a pivotal parameter quantifying the ratio between the moles of organic pollutant (NIM) and the number of moles of oxidant consumed within a specific timeframe.

At pH 7.5, the calculated RSE was determined to be 0.37, indicating a good efficiency of the combined process employing FAC and solar-simulated irradiation. For instance,

RSE values for other light or thermally activated processes such as hydrogen peroxide and persulfate-based AOP, typically fall within the range of 0.08 to 0.4 (Gabet et al., 2021; Sonowane et al., 2022). This observation underscores that the investigated combined process outperforms several existing methods in terms of oxidation efficiency.

To further investigate the impact of pH on the reactivity of both forms of chlorine (HOCl and ClO^-), experiments were conducted at pH 3 and 10, and the results were compared with those obtained at pH 7.5 (Figure 12).

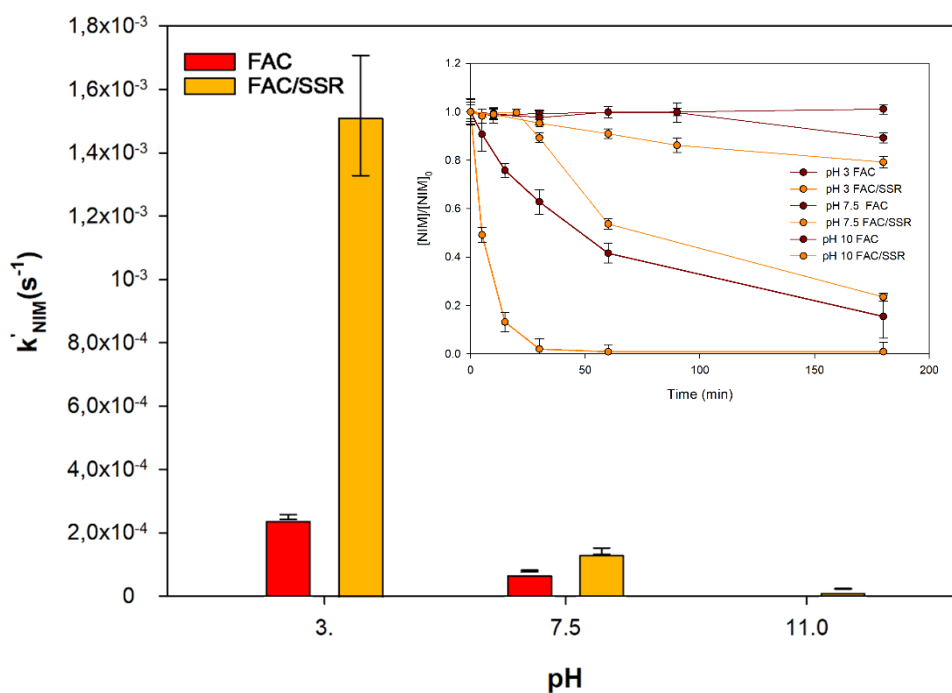


Figure 12 Degradation constant of NIM under dark and irradiation at different pH values. Conditions are: $[\text{NIM}]$ 25 μM , $[\text{FAC}]$ = 0.5 mM.

At pH 3.0, direct oxidation of NIM exhibited a k'_{NIM} of $2.50 \pm 0.13 \times 10^{-4} \text{ s}^{-1}$ corresponding to a remarkable 59% degradation after 1h, which was about three times

higher than that observed at pH 7.5. In the other hands, at pH 10, negligible degradation of NIM was noted, with a low degradation constant of $3.91 \pm 0.20 \times 10^{-7} \text{ s}^{-1}$. The variances in NIM degradation can be explained by considering the distinct standard reduction potentials of the two chlorine forms, with $E^0 (\text{HClO}/\text{Cl}^-) = + 1.49 \text{ V}$ and $E^0 (\text{ClO}^-/\text{Cl}^-) = + 0.89 \text{ V}$ (Liang et al., 2020).

Under irradiation, NIM degradation was significantly accelerated, especially at pH 3.0. At this pH, the degradation constant increased to $1.53 \pm 0.03 \times 10^{-3} \text{ s}^{-1}$, corresponding to an EE value of 8.6. Almost complete disappearance of NIM was achieved within just 30 minutes. This noteworthy enhancement in NIM degradation can be attributed to the increased formation of oxidant species under light irradiation, leading to the generation of additional reactive species, thereby facilitating a more efficient and rapid degradation process.

In summary, the observed pH-dependent effect on NIM degradation highlights the influence of the predominant chlorine species and their standard reduction potentials. Furthermore, the remarkable acceleration of NIM degradation under sun-simulated conditions reveals the synergistic effect of light irradiation in promoting the formation of reactive species, thereby enhancing the efficiency of the degradation process.

At pH 10, where ClO^- is the predominant species, no significant degradation of NIM was observed under dark conditions. However, under sun-simulated conditions, 10% of NIM degradation was achieved within 60 minutes yielding a degradation constant of $2.31 \pm 0.11 \times 10^{-5} \text{ s}^{-1}$.

In all experiments, the significant increase in NIM degradation under irradiation can be attributed to the formation of additional oxidation species, such as hydroxyl radical ($\text{HO}^\bullet$) and chlorine radical ($\text{Cl}^\bullet$) which have higher reduction potential ($E^0 (\text{HO}^\bullet/\text{H}_2\text{O}) = 2.7 \text{ V}$ and $E^0 (\text{Cl}^\bullet/\text{Cl}^-) = 2.4 \text{ V}$) compared to the predominant chlorine species (Amstrong et al., 2015).

3.1.3. II Reactivity between NIM and oxidant species

To gain insights into the formation and impact of reactive species on the NIM degradation, it was determined the second-order rate constants of NIM with HOCl, HO[•] and Cl[•] by using competition kinetics with chemical probes. Considering that NIM has a pKa of 6.6 (Goswami al., 2017), the reactivity constants were determined for both molecular forms.

For HO[•] generation (R1), 10 mM of hydrogen peroxide were irradiated under SSR in the same system used for FAC experiments. For Cl[•] generation (R2), chloroacetone (ClAc) was used as a source and UVB radiation from two fluorescent lamps G15T8E (Sanyo Denki, Japan with $\lambda_{\text{max}} = 308$ nm), was employed. No direct photolysis of NIM was observed under both irradiation systems.



In both systems involving HO[•] and Cl[•] radicals, two different competition reactivity experiments were conducted to quantify the second-order rate constants for the reaction of NIM with these oxidant species ($k_{NIM,Ox}$). The competition reactivity equation used is as follows (Eq. 3):

$$\ln \frac{[NIM]_0}{[NIM]} = \frac{k_{NIM,Ox}}{k_{probe,Ox}} \times \ln \frac{[Probe]_0}{[Probe]} \quad (\text{eq. 3})$$

where Ox represents the oxidant species (either HO[•] or Cl[•]). The slope of the linear fit of the experimental data reported in Figure SM2c provides the ratio between the second-order rate constants $k_{NIM,Ox}$ and $k_{Probe,Ox}$.

For HO[•], the determined second-order rate constant, the experiments were conducted in the presence of 25 μ M of NIM and phenol (Ph) ($k_{Ph,HO^{\bullet}} = 1.40 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$) (Land et al., 1967) at pH 4.0 or Benzoate ion (Bz⁻) ($k_{Bz^-,HO^{\bullet}} = 5.90 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$) (Buxton et al., 1988) at pH 9.4. under adopted experimental conditions, the reactivity

was determined to be $1.48 \pm 0.05 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ for the acidic form of NIM while the value determined at high pH (anionic form) was $9.06 \pm 0.64 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$.

For $\text{Cl}^\bullet$, the reactivity constant with NIM at acidic pH was not determined probably due to secondary reactions between chloroacetone involving at acidic pH values that inhibited the NIM degradation. However, the value at high pH value was determined to be $1.48 \pm 0.08 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$.

Despite a negligible reactivity between NIM and ClO^- (as depicted in Figure 12), the reactivity with HOCl was determined using the following reaction model:

$$\frac{d[\text{NIM}]}{dt} = -k_{\text{NIM},\text{HOCl}} [\text{HOCl}]_T [\text{NIM}] \quad (\text{eq. 4})$$

Considering the negligible consumption of HOCl during the initial minutes of the reaction (as indicated in Figure SM1c), equation 4 can be rearranged as :

$$\frac{d[\text{NIM}]}{dt} = -k'_{\text{NIM}} [\text{NIM}] \quad (\text{eq. 5})$$

The second-order rate constant $k_{\text{NIM},\text{HOCl}}$ was determined from the slope of the linear fit of k'_{NIM} as a function of HOCl concentration (see Figure SM3c) yielding a value of $0.71 \pm 0.04 \text{ M}^{-1} \text{ s}^{-1}$. This value, while relatively low, is compared to the reactivity constants observed for amides, which typically range from 10^{-4} to $10^{-1} \text{ M}^{-1} \text{ s}^{-1}$. This observation has been attributed to the less electron-donor character of the nitro substituent.

While HOCl photolysis leads directly to the formation of $\text{HO}^\bullet$ and $\text{Cl}^\bullet$ with a photolysis quantum yield of 1.0 at 311 nm (eq. R3), the photoreactivity of ClO^- follows a more complex mechanism. The primary products include $\text{HO}^\bullet$, $\text{Cl}^\bullet$ and O_3 with quantum yields ranging from 0.075 to 0.127 (Buxton et al., 1972; Bulman et al., 2019) (eq. R4-R5-R6). Moreover, in the presence of ClO^- , it can be expected to have higher reactivity with photogenerated $\text{HO}^\bullet$ and $\text{Cl}^\bullet$ leading to the formation of $\text{ClO}^\bullet$ (see Table SM2c).



Notably, pH reduction has a strong influence on the overall degradation processes enhancing oxidation process by promoting higher reactivity of HClO and a great generation of the radical species during exposure to sun simulated irradiation (Kishimoto et al., 2019).

To gain deeper insights into the specific reactive species involved during NIM degradation, experiments using chemical probes were conducted to evaluate the cumulative effects of each oxidant species that are present in the solution. Using 10 mM of Terbutanol (TBA), a known efficient quencher of $HO^\bullet$ and $Cl^\bullet$, in the presence of 0.5 mM of FAC at pH 7.5 and 25 μ M of NIM, it was observed a significant decrease in the k'_{NIM} from $1.42 \pm 0.10 \times 10^{-4} \text{ s}^{-1}$ to $9.12 \pm 0.78 \times 10^{-5} \text{ s}^{-1}$, representing a reduction of approximately 35% (Figure 13).

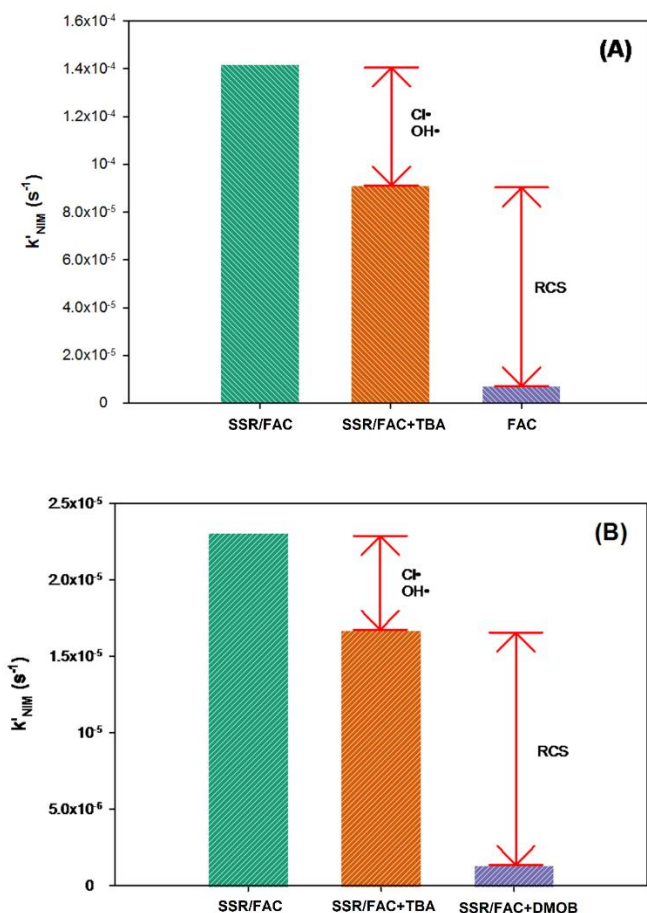


Figure 13 (A) Kinetic constant of NIN degradation in the presence of tert-butyl alcohol in the dark and under sun simulated irradiation at pH 7.5 and (B) kinetic constants at pH 10 using TBA and DMOB.

In experiments performed to assess the presence of $ClO^{\bullet}$ in solution, 1,4-dimethoxybenzene (DMOB) was employed as a classic chemical probe due to its high reactivity constant of $2.1 \pm \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ (Alfassi et al., 1988). Initially, when only a 10 mM TBA were used in the presence of 0.5 mM of FAC at pH 10, it was observed limited inhibition of NIM degradation, suggesting the potential presence of other reactive species generated in solution under these conditions. However, upon repetition of the experiment with the addition of 1 mM DMBO, a remarkable decrease

in NIM degradation was observed. This result indicates the presence of $\text{ClO}^\bullet$ as an oxidizing species in the solution. Notably, these experiments were not conducted at pH 7.5 and 3.0 since at these pH values, HOCl predominates due to its direct reaction with DMOB (Lei et al., 2023).

3.1.3.III By-products identification and mineralization efficiency

High-resolution mass spectrometry (HPLC-MS) was employed to identify and characterize the various NIM by-products generated during the oxidative treatment while ion chromatography mass spectrometry (IC-MS) was used to detect the formation of short-chain carboxylic acids and inorganic ions. Total organic carbon (TOC) analysis was performed to determine the degree of mineralization, which indicates the level of complete oxidation and breakdown of the NIM molecule.

In the literature, there are no studies evaluating the degradation of NIM in the presence of FAC and UV light. De Silva et al., 2018, presented excellent degradation results with higher concentrations of oxidant like H_2O_2 , around 1.5 mM - 4.5 mM, and UV-ABC light, more energetic than that used in this study. They managed to degrade approximately 90% of Nimesulide in about 20 minutes, leading to a 70% mineralization. They also indicated that dissolved organic matter tends to reduce efficiency in wastewater treatment, a finding that is also observed in this study. Xin et al. 2021, obtained similar results for Nimesulide degradation using catalytic degradation of persulfate in the presence of alfalfa-derived nitrogen self-doped porous carbon supported CuFeO_2 . They achieved complete degradation of Nimesulide in 30 minutes and a TOC mineralization similar to the one obtained in this study, at 30.2%.

In the presence of 0.5 mM of FAC at pH 7.5, the degradation of NIM resulted in the generation of numerous by-products both in the dark and under sun-simulated irradiation.

The main by-products detected exhibited a carbon skeleton formed by the two aromatic rings connected by an ether bridge, like the structure on Nimesulide

molecule. In dark condition, the analysis revealed the presence of mono-, di-, and tri-chlorinated by-products, with a higher abundance of the monochlorinated product. However, under irradiation conditions, a significant decrease in chlorinated by-products was observed, concomitant with a decrease in the concentration of the initial NIM molecule (Figure 14).

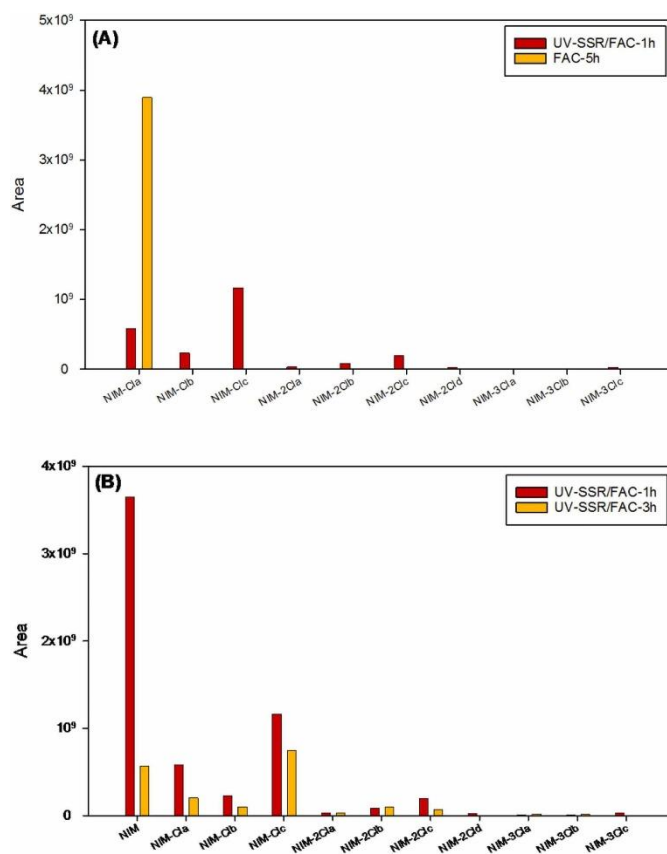


Figure 14 | Main NIM degradation products observed in the dark and by irradiation in the presence of 0.5 mM of FAC at pH 7.5. A: products generated after 1h of irradiation (SSR-1h) and 5h of Dark (DARK-5h) corresponding to 20% of NIM transformation. B: comparison

It was observed the formation of similar products in the treatment without light, alongside others displaying different retention times but sharing the same mass. These additional products are likely constitutional isomers, containing one chlorine atom, such as NIM-Cl_{a,b,c}, or multiple chlorine atoms, like NIM-2Cl_{a,b,c,d} and NIM-

3Cl_{a,b,c}, bound to the starting molecule at different positions. Under irradiation and in the presence of 0.5 mM of FAC at pH 7.5, different degradation pathways are proposed considering the identification of NIM products with characteristic hydroxyl and chloro functional groups, suggesting their formation from the attack by HO[•] and Cl[•] as reported in the figure 15 and Table SM3c. Notably, the functional groups originally present in the NIM molecule, such as the methanesulfonamide and nitro groups, were oxidized and transformed into amine and hydroxyl functional groups.

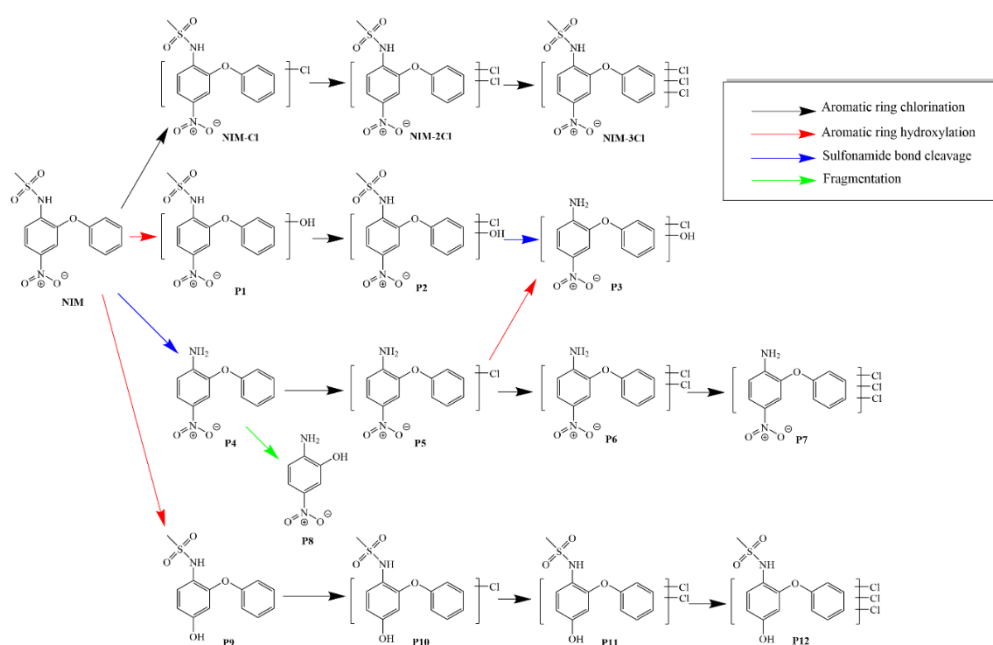


Figure 25 Proposed transformation pathway of NIM into primary products.

These by-products indicate that might-assisted treatment not only significantly reduces the concentration of NIM in water but also generates by-products in advanced oxidation states compared to the initial molecule.

IC-MS analysis revealed the formation and gradual accumulation under irradiation of short-chain carboxylic acids, oxalic and formic acids (Figure SM4c) as well as nitrate ions (NO₃⁻) and methanesulfonic acid (CH₃SO₃H, MSA) (see Figure 16) (Cai et al., 2023). The formation of these products highlights the efficiency of the treatment

processes. TOC quantification was employed to estimate the mineralization efficiency of our systems. The mineralization of 25 μM of NIM using 0.5 mM of FAC in the dark and under sun-simulated irradiation was compared and reported in Figure 15. The results clearly indicate that after 5h of irradiation 32% mineralization was reached, whereas no significant changes were observed in dark condition.

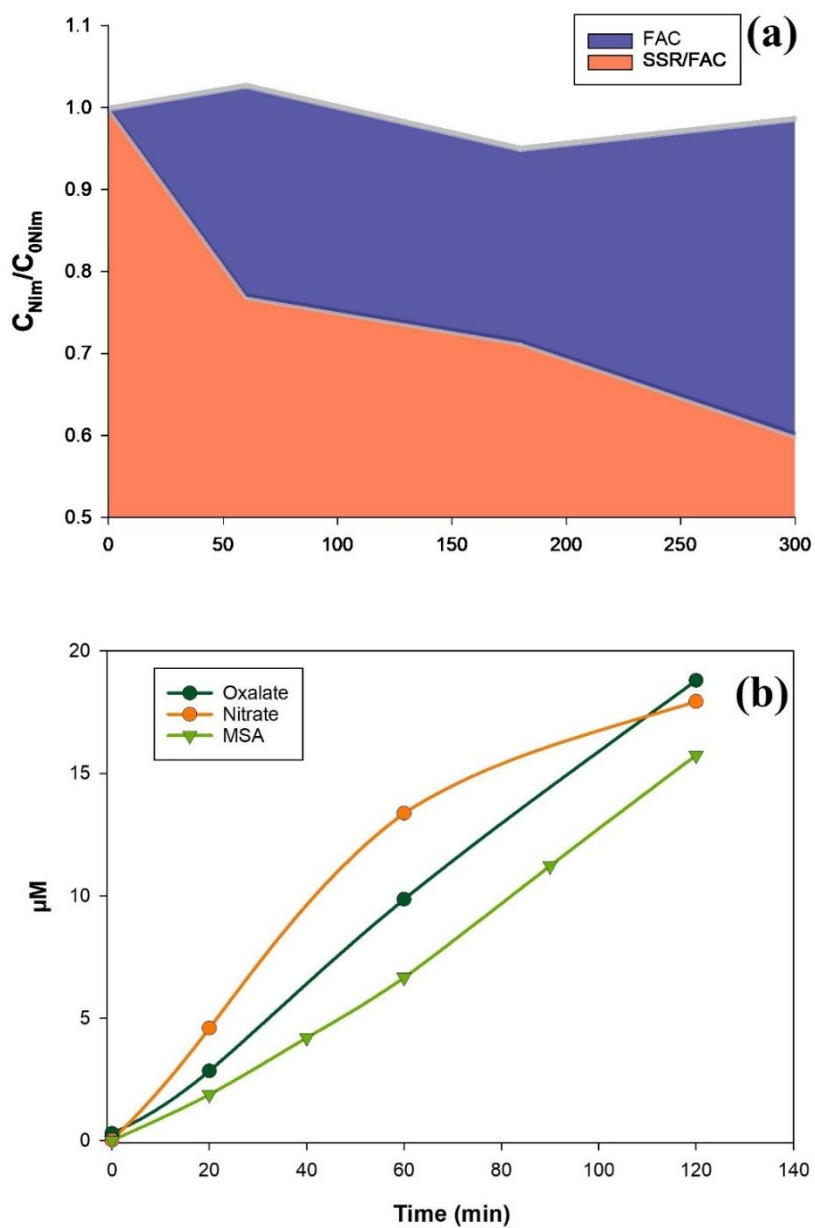


Figure 36 TOC analysis (a) and ICP-MS analysis (b) for Oxalate, Nitrate and methanesulfonic acid analysis.

3.1.2 IV Degradation efficiency in real STPW and using solar radiation

To explore the practical application of FAC/SSR process, the degradation of NIM was evaluated in real wastewater treatment plant and using solar radiation instead of simulated sunlight. The NIM solution was directly prepared in STPW and results are presented in Figure 17 at pH 7.5 with a concentration of 10 μM [NIM], in the STPW using sunlight and FAC the nim concentration reached 8.9 μM , on the other hand with SSR/FAC in STPW it reached 3.6 μM . Different trends can be observed: i) the decline in degradation efficiency compared to pure water can be attributed to the competition between dissolved organic matter present in wastewater and the pollutant NIM for reactive species generated during light assisted FAC treatment; ii) a decrease from $4.68 \pm 0.12 \times 10^{-4} \text{ s}^{-1}$ to $2.56 \pm 0.19 \times 10^{-4} \text{ s}^{-1}$ corresponding to a 46% decrease was observed when real sun radiation was used compared to SSR which is also attributed to the differences of energy and wavelength as reported in Figure 10; iii) under irradiation, the NIM degradation decrease observed when SUN was used could be also attributed to the competition with dissolved organic matter for the radiation absorption with hypochlorite. Finally, the half-life time of NIM calculated as $t_{1/2} = \frac{\ln 2}{k}$ varied from 46 minutes in Milli-Q water to 4.8h when the system was used in STPW in the presence of sun radiation.

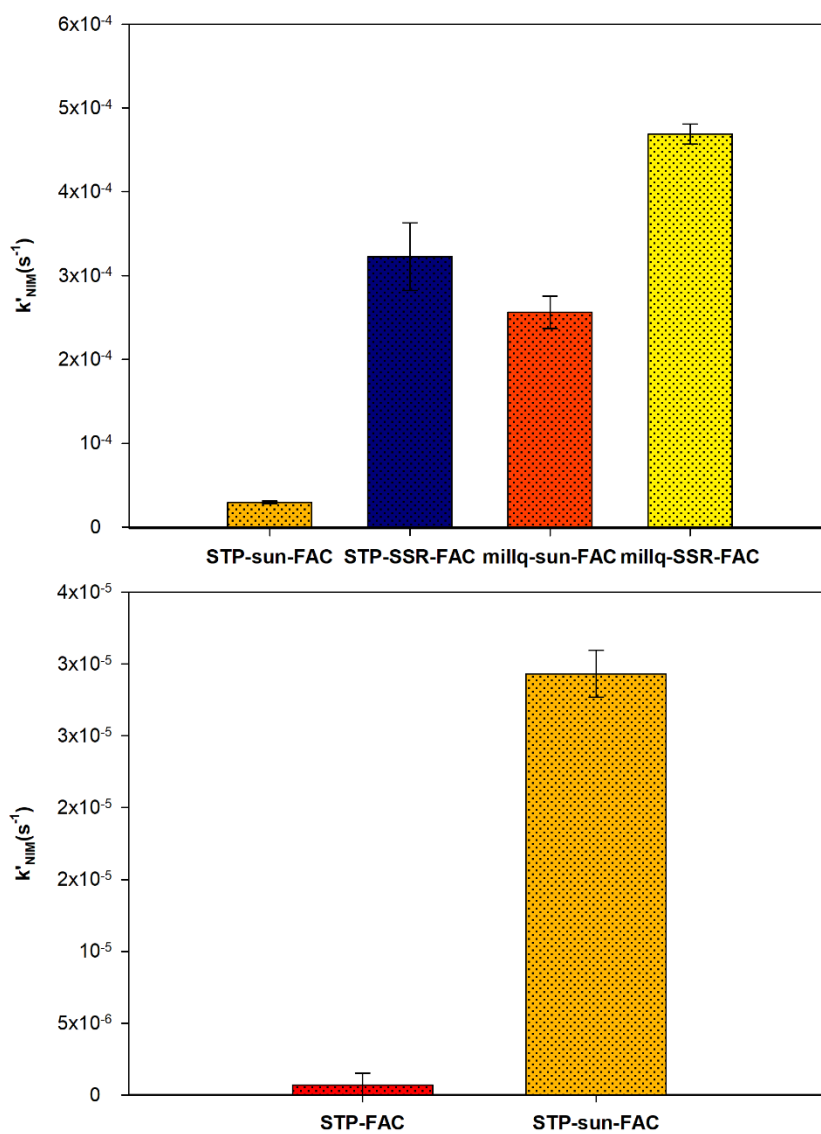


Figure 17 Degradation constant of NIM 10 μ M at pH 7.5 in STPW and Milli-Q water under UVA and solar irradiation.

3.1.4 Conclusions

The documented high toxicity of by-products generated during wastewater disinfection with free active chlorine, as evidenced by previous studies, underscores the significance of exploring alternative oxidation processes. While it is commonly observed that oxidation processes using hypochlorite result in the formation of by-products, this study presents a novel perspective.

Contrary to expectations, our investigation reveals that the hypochlorite/light process, despite producing a higher number of chlorinated by-products, demonstrates a more advanced state of oxidation, closely resembling mineralization compared to the initial material. This assertion is substantiated by the findings of Total Organic Carbon tests, emphasizing the pivotal role of UV light in achieving effective mineralization.

The combination of FAC and SSR has proven to be an excellent method of oxidising emerging pollutants, particularly Nimesulide. The generation of highly oxidising radicals succeeds in decreasing the organic carbon present, demonstrating a higher mineralisation of the compounds that is completely absent in the process with FAC alone. Furthermore, the method is relatively easy to implement as chlorine is currently used in most wastewater treatment processes, and therefore only requires the implementation of an illumination system with UVA light during disinfection processes with FAC.

This study contributes to the understanding of wastewater treatment dynamics by highlighting the efficacy of oxidation in the presence of both simulated and real solar light. This light approach emerges as a superior strategy for degrading persistent pharmaceuticals when compared to treatment solely employing hypochlorite. The consequential heightened mineralization of the pollutants underscores its potential in mitigating the environmental impact posed by the emerging contaminants.

In conclusion, our research offers a promising alternative that combines hypochlorite with solar light for enhanced wastewater treatment. This innovative approach not only

facilitates superior degradation of persistent pharmaceuticals but also contributes significantly to the overall reduction of environmental pollutants, marking a notable advancement in the field of wastewater treatment.

Chapter 4 Sartans: Disinfection By-Products from Different Oxidation Treatments

4.1 Introduction

Sartans are chemical compounds with antagonistic action toward the AT1 receptors of angiotensin II, a multifunctional peptide with vasoconstrictor action and one of the main peptides responsible for regulating blood pressure. Losartan (progenitor of the group), Valsartan, Irbesartan, Candesartan, Eprosartan, Telmisartan and a few other compounds belong to this class. The name of this family comes from the fact that all these molecules possess the suffix “sartan” in their name.

Sartans, represent a cornerstone in modern therapy of hypertension and related cardiovascular diseases. These drugs act by selectively inhibiting the binding of angiotensin II to its type 1 receptor (AT1R), thereby reducing vasoconstriction, aldosterone secretion and other processes that contribute to elevated blood pressure (Timmermans, 1999), these substances block the activation of these receptors inducing a decrease in blood pressure (Schramm et al., 2012). First introduced in the 1990s, sartans such as losartan, valsartan, and irbesartan have rapidly spread in clinical practice due to their therapeutic efficacy and more favorable safety profile than angiotensin-converting enzyme inhibitors (ACE inhibitors), which are often associated with adverse effects such as persistent cough and angioedema (Schramm et al., 2012). The therapeutic importance of sartans goes far beyond the treatment of hypertension, extending to the management of diabetic nephropathy and heart failure. Losartan, introduced in 1995 as the first member of this class, has served as a model for the development of other molecules including valsartan, irbesartan and telmisartan (Bhardwaj, 2006). In Europe, sartans dominate prescribing trends; in Germany, for example, valsartan achieved annual sales of more than 39 tons in 2012, while irbesartan totalled 21 tons (Bergmann et al., 2011). This prevalence is fuelled not only

by their pharmacological efficacy, but also by advantageous pharmacokinetic properties, such as prolonged half-life, variable bioavailability, and lower incidence of metabolic activation than ACE inhibitors. Chemically, sartans share a basic biphenenyl structure with a tetrazole ring, which can improve receptor affinity and oral bioavailability. More specific structural differences, such as the presence of imidazole rings in Losartan and Valsartan, result in variations in potency and pharmacokinetics (Adams & Trudeau, 2000). However, the poor metabolic degradability of these molecules, coupled with their massive diffusion, has led to sartans being classified as emerging micropollutants, characterized by significant persistence in the environment (Ladhari et al., 2021).

Sartans, including valsartan, losartan, and irbesartan, are frequently detected in surface waters and wastewater effluents. For instance, valsartan concentrations reached up to 5.8 µg/L, and irbesartan up to 1.3 µg/L in the Llobregat River, Spain (Gracia-Lor et al., 2012; Huerta-Fontela et al., 2011). Losartan was identified at concentrations as high as 620 ng/L in Spanish rivers and exceeded 2.0 mg/L in industrial areas of India (Bayer et al., 2014; Fent et al., 2006). Effluents from Swedish WWTPs contained irbesartan and e Prosartan at 450 ng/L and 260 ng/L, respectively (IVL Swedish Environmental Research Institute, 2011). Sartans are detected in treated drinking water in Spain at concentrations between 260 and 685 ng/L. Removal rates after chlorination vary significantly: 75% for irbesartan, 62% for losartan, and only 9% for valsartan (Huerta-Fontela et al., 2011). Valsartanic acid, a transformation product of different sartans, has been found in different WWTPs and it is currently used for detecting the sartan pollution of the waters. Near pharmaceutical manufacturing sites in India, sartans have been found at concentrations exceeding 2 mg/L, reflecting direct industrial discharge into the environment (Bayer et al., 2014). In 2007, Larsson et al. found Losartan concentrations in real plant samples from wastewater treatment that ranged from 2400 to 2500 µg/L in Patancheru, near Hyderabad, India (Larsson et al., 2007). Losartan was found in the waters of the Llobregat River (Spain), reaching a maximum concentration of 620 ng/L (Huerta-

Fontela et al., 2011). In 2018, Cortez et al. found Losartan in all analyzed samples, with maximum concentrations of up to 8.7 ng/L in marine samples taken from Santos Bay, Sao Paulo, Brazil. They demonstrated that even concentrations that were equivalent to environmental levels led to cyto-genotoxic effects in the gills and hemolymphs of the *Perna perna* species, showing that Losartan pollution, even at low concentrations, poses increasingly significant issues (Cortez et al., 2018). Sartans such as Irbesartan and Losartan tend to decrease in concentration following water disinfection treatments, resulting in a decrease of up to 75% for Irbesartan and 62% for Losartan (Ladhari et al., 2021), leading to the formation of many disinfection by-products. These by-products have been the subject of study in recent years, with some studies considering valsartan acid, produced by the oxidation of different sartans, as an indicator of sartan pollution (Nödler et al., 2013).

During chlorination, sartans are converted into disinfection by-products (DBPs). Valsartanic acid is one such DBP, formed consistently in WWTPs and showing high persistence (Kern et al., 2010; Romanucci et al., 2020). An additional critical aspect concerns the transformations that sartans undergo during disinfection processes, such as chlorination. These reactions generate numerous disinfection by-products, sometimes more persistent and toxic than the starting compounds. In the case of irbesartan, some studies in the literature have already shown the formation of disinfection by-products formed during chlorination simulating the condition of WWTPs (Mutha et al., 2018), in this chapter new molecules formed precisely during these oxidations, whose presence and possible environmental effect was unknown, will be extensively studied and highlighted.

Similarly, losartan and valsartan undergo oxidative degradations that lead to the formation of by-products that can maintain the pharmacophore while acquiring new toxicological properties (Carpinteiro et al., 2018).

Valsartan by-products, such as valsartanic acid, show reduced degradation rates and increased resistance to treatment processes such as ozonation and filtration on

granular activated carbon (Kern et al., 2010). Such persistence raises ecological concerns, as valsartanic acid has been associated with bioaccumulation in aquatic organisms (Berkner & Thierbac, 2014).

Ecotoxicological implications are particularly relevant. Toxicity tests conducted on model organisms (e.g., algae, rotifers, and crustaceans) have shown that sartans and their by-products can alter fundamental biological functions, such as reproduction and mobility. Chronic exposure studies suggest that although some DBPs may be less acutely toxic than the original compounds, their long-term effects remain significant (Romanucci et al., 2019). Addressing the critical environmental issues related to sartans requires the use of advanced wastewater treatment technologies. Processes such as the use of membrane bioreactors or advanced oxidation with peroxymonosulfate have shown promising results in reducing concentrations of these compounds. However, these methods also produce new DBPs, requiring further studies to assess their ecotoxicological impact and identify more effective mitigation strategies.

This chapter aims to provide an overview of sartans, from their clinical relevance to their environmental behavior and possible approaches to mitigate their ecological impact. Different oxidation processes will be highlighted, both classical such as oxidation with hypochlorite and advanced oxidation processes for removing and decreasing the toxicity of different sartans studied as model emerging pollutants.

4.2 Disinfection By-Products of Irbesartan after Hypochlorite Treatment, and Associated Ecotoxic Risk

This paragraph presents a study aimed at understanding the environmental fate of irbesartan when subjected to treatment with activated sodium hypochlorite, with a focus on the formation and toxicity of Irbesartan disinfection by-products (IDBPs). In a previous study (Romanucci et al., 2020), the behavior of irbesartan during the chlorination disinfection process under conditions normally applied in wastewater

treatment plants was examined (Chusaksri et al., 2012). This investigation led to the isolation and characterization, by mass spectrometry and nuclear magnetic resonance, of six new Irbesartan disinfection by-products (IDBPs) (Figure 18).

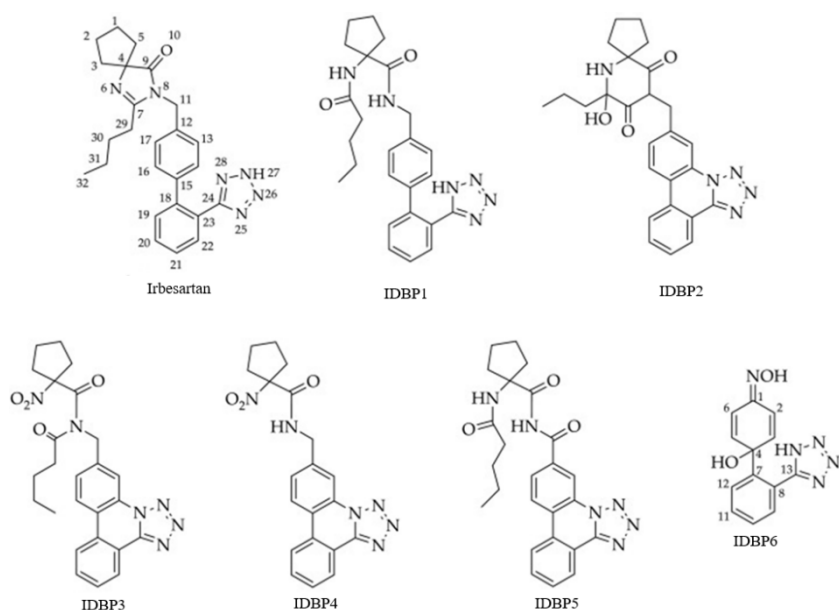


Figure 18 Irbesartan disinfection by-products Romanucci et al., 2020

The results showed that more than 75% of irbesartan was completely mineralized, 15 percent remained unchanged, and 6% converted to these IDBPs. In parallel, the toxicological effects of the chlorinated derivatives of irbesartan were evaluated, showing increased toxicity. In addition, a mechanism of formation of the detected by-products was proposed, assuming a series of reactive intermediates.

In this present study, the isolation and structural determination of twelve new IDBPs has been reported. Finally, to assess the reactivity and biological impact of irbesartan during chlorination processes, toxicity studies were conducted on *D. magna*, *A. fischeri* and *R. subcapitata*, three model organisms belonging to different trophic levels (algae, bacteria and crustaceans). This integrated approach provided a more

comprehensive view of the ecotoxicological risks associated with the presence of DBPs from irbesartan chlorination.

4.2.1 Results and Discussion

4.2.1.I Chlorination experiments

A 1.0×10^{-5} M solution of Irbesartan was used to monitor by HPLC the main disinfection by-products formed during a 30 min reaction in a 10% hypochlorite solution at room temperature (Romanucci et al., 2019; Zhang et al., 2019), in conditions close to those of a typical wastewater treatment plant. The disinfection by-products were identified by comparing their retention time with those obtained by preparative experiments. The concentration of the by-products IDBP7-IDBP18 reached the maximum after 2h with percentages of 0.1, 0.3, 0.2, 1.0, 1.6, 0.4, 0.8, 0.5, 0.3, 0.3, 0.2, 0.3%w/w.

4.2.1.II Structure elucidation of IDBP7

The MS-TOF analysis showed a molecular ion peak at m/z 249.11 $[M + H]^+$ corresponding to the molecular formula $C_{14}H_8N_4O$. The ^1H NMR spectrum did not show the presence of the *n*-butyl side chain, the A ring, or proton H-11, just as the ^{13}C NMR spectrum did not show the corresponding carbons nor the signals of carbons C-7 and C-9 (B ring). In the ^1H NMR spectrum, only aromatic signals were present, particularly, only seven protons at 9.19, 8.73, 8.28, 8.61, 7.98, 7.92, and 8.86 ppm, as well as the signal of an aldehyde proton at 10.30 ppm. The HSQC spectrum allows for correlating the aforementioned signals to the carbons at 127.19, 126.57, 125.41, 120.18, 125.09, 130.95, 124.03, and 190.01 ppm, respectively. Based on the ^1H - ^1H COSY and the HMBC spectra, the first three signals were attributed to protons H-13, H-16, and H-17 of 1,2,4-trisubstituted benzene (C ring). The next four were attributed to protons H-19, H-20, H-21, and H-22 of 1,2-disubstituted benzene (D ring), while the last one was attributed to the aldehyde proton H-11. Evidently, the starting product has undergone hydrolysis of the bounds C-7/N-8 and N-8/C-9 (Carpinteiro et al., 2019; Mutha et al., 2018), along with the partial oxidation of carbon C-11 and the

concomitant closure of a new six-term ring. The latter could result from an aromatic nucleophilic substitution reaction on the C-14 carbon of the previously chlorinated aromatic B-ring by the adjacent N-28 nitrogen of the tetrazole ring (Shah et al., 2010). The plausible mechanism of IDBP7 formation from irbesartan is shown in Figure 20-21.

4.2.1.III. Structure elucidation of IDBP8

In the ^1H NMR spectrum, the compound IDBP8, like IDBP7, showed the presence of only seven aromatic protons, indicating the formation of a new six-term ring. The 1D and 2D NMR data also show the presence of the A ring and protons H-11. In the HMBC spectrum, these (at 5.16 and 4.94 ppm) were correlated to the two carbons at 186.43 and 159.43 ppm, identified as carbonyls C-9 and C-7, respectively. In fact, if the C-9 was correlated to protons H-3/H-5 at 1.91 ppm, carbon C-7 was correlated to the protons at 4.34 and 1.27 ppm. These last ones were identified as H-29 and H-30, respectively, indicating the presence of the *n*-butyl side chain, which, however, was oxidized to carbon C-29, in alpha to carbonyl C-7.

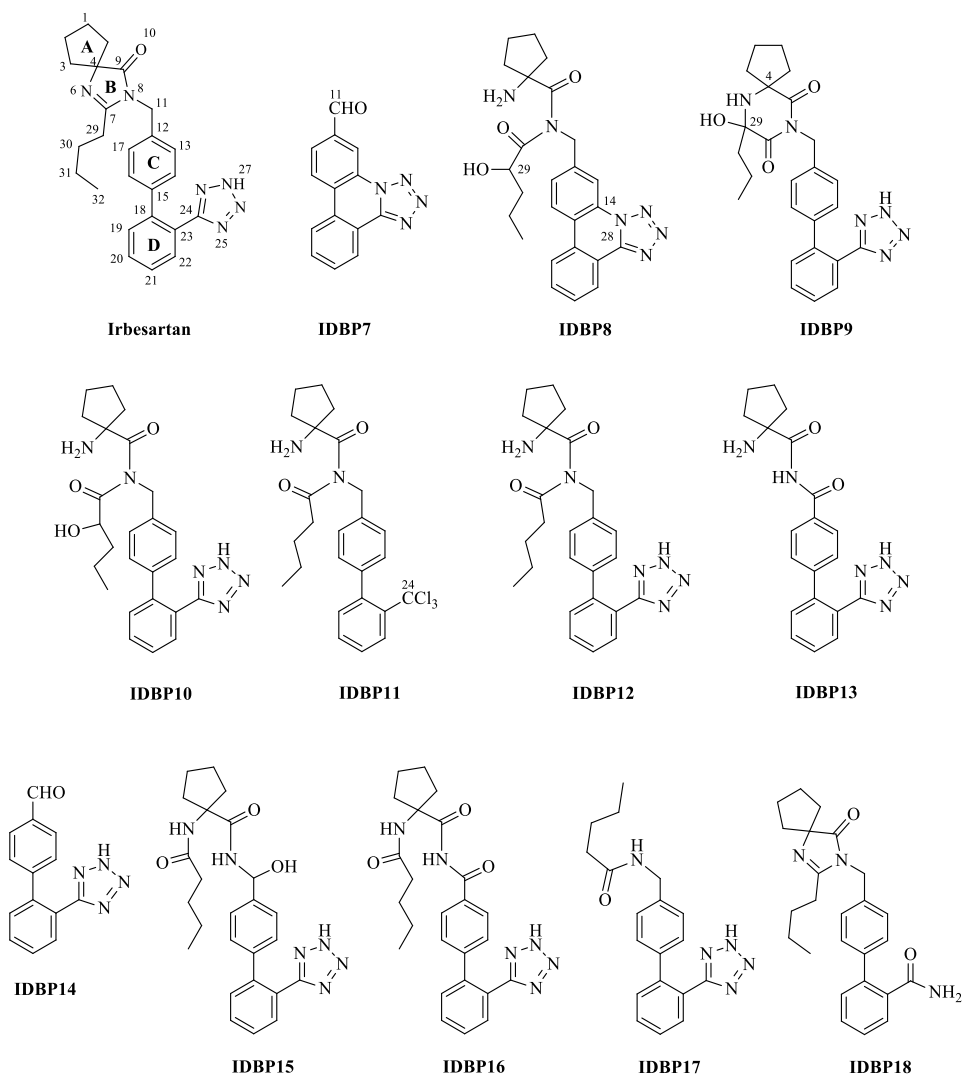


Figure 19 Chemical structures of irbesartan and its disinfection by-products IDBP7–IDBP18.

4.2.1.IV Structure elucidation of IDBP9

The NMR data of the compound IDBP9 show the presence of the A, C, and D rings, the protons H-11 (at 5.25 and 46.84 ppm) and carbons identified as C-9 and C-7 at 187.48 and 158.02 ppm, respectively. In particular, in the HMBC spectrum, both carbonyls were correlated to protons H-11, but only the first one was correlated to protons H-3/H-5, and only the second one to the protons at 2.67 ppm, identified as

proton H-30. The 1D and 2D NMR data show the *n*-butyl side chain, but with a quaternary carbon at position C-29. In fact, the ^{13}C NMR spectrum showed the presence of a carbon at 83.09 ppm, which was identified as an oxygenated quaternary carbon engaged in the closure of a six-term cycle with the amino function at carbon C-4. Compound IDBP9 was, therefore, very similar to the compound IDBP2 reported in the previous article on the degradation products of irbesartan (Figure 18), without the formation of a new bond between the position N-28 and C-14.

4.2.1.V. Structure elucidation of IDBP10

Compound IDBP10 was very similar to compound IDBP8 but without the bond between the positions N-28 and C-14. Indeed, the ^1H NMR spectrum showed the presence of eight aromatic protons, attributed to two 1,4-disubstituted rings.

4.2.1.VI. Structure elucidation of IDBP11

The 1D and 2D NMR data indicate the opening of the B ring by hydrolysis of the bond C-7/N-6. Indeed, in the HMBC spectrum, carbonyl C-7 at 176.01 ppm was correlated with the signals at 2.49 and 1.54 ppm, which were identified as protons H-29 and H-30 of the *n*-butyl side chain, while carbonyl C-9 at 180.05 ppm was correlated with the signals at 2.12 and 1.96 ppm, which were identified as protons H-3/H-5 of the cyclopentane group. The MS-TOF analysis showed a molecular ion peak at m/z 495.88 $[\text{M} + \text{H}]^+$ in addition to the signals at m/z 494.13, 496.15, and 498.13, corresponding to the molecular formula $\text{C}_{25}\text{H}_{29}\text{Cl}_3\text{N}_2\text{O}_2$ and to the presence of three chlorine atoms. Indeed, in the HMBC spectrum, proton H-22 at 8.24 ppm was correlated to the carbons at 134.75 and 141.89 ppm, identified as carbons C-20 and C-18, respectively, and to carbon C-24 at 97.40 ppm, evidently obtained from the degradation of the tetrazole ring.

4.2.1.VI Structure elucidation of IDBP12

The nuclear magnetic resonance spectra were in accordance with those reported in the literature (Luongo et al., 2020).

4.2.1.VII Structure elucidation of IDBP13

The MALDI-TOF analysis of IDBP13 showed a molecular ion peak at m/z 377.18 $[M + H]^+$ corresponding to the molecular formula $C_{20}H_{20}N_6O_2$. The NMR and mass data are like those of IDBP12 but without the C_5 chain bound to the nitrogen N-8 and with carbon C-11 oxidized at the carbonyl group. This last one at 166.70 ppm was correlated in the HMBC spectrum to protons identified as H-13/H-17 at 7.84 ppm.

4.2.1.VIII Structure elucidation of IDBP14

The NMR data of compound IDBP14 are very similar to those of compound IDBP7 but without the bond between the positions N-28 and C-14. Evidently, the starting product was only hydrolyzed to the bonds C-7/N-8 and N-8/C-9 and partially oxidized at carbon C-11 (Cousaert et al., 2005).

4.2.1.IX Structure elucidation of IDBP15

The MS-TOF analysis showed a molecular ion peak at m/z 463.26 $[M + H]^+$ corresponding to the molecular formula $C_{25}H_{30}N_6O_3$. The NMR data of IDBP15 are very similar to those of the IDBP1 reported in Romanucci et al. (2020). The difference is due to carbon C-11, present as methylene in compound IDBP1 and as partially oxidized methine in the IDBP15. In fact, the ^1H NMR spectrum shows a singlet signal of a proton at 6.66 ppm, bound in the HSQC spectrum to the carbon at 83.04 ppm and correlated in the HMBC spectrum with carbonyl carbon C-9, as well as with carbons C-12 and C-13/C-17 at 169.00, 135.15, and 127.53 ppm, respectively.

4.2.1.X Structure elucidation of IDBP16

Compound IDBP16 was the corresponding oxidized product of IDBP15. In fact, in the ^{13}C NMR spectrum the signals of three carbonyl carbons appeared at 176.73, 168.48, and 168.42 ppm. In the HMBC spectrum the third one, identified as carbon C-11, was correlated to protons H-13/H-17 of the 1,4-disubstituted aromatic C ring, at 7.60 ppm. The IDBP16 compound also corresponded to the IDBP5 reported by

Romanucci et al. (2020), without the bond between the positions N-28 and C-14.

4.2.1.XI. Structure elucidation of IDBP17

The nuclear magnetic resonance spectra were in accordance with those reported in the literature (Luongo et al., 2020).

4.2.1.XII. Structure elucidation of IDBP18

The IDBP18 compound was very similar to irbesartan but with the tetrazole ring partially degraded to amide function. In fact, proton H-22 at 7.78 ppm, bound in the HSQC spectrum to the carbon at 130.73 ppm, was correlated in the HMBC spectrum to a carbonyl at 170.51 ppm, which was identified as carbon C-24. The MS-TOF analysis showed a molecular ion peak at m/z 404.54 $[M + H]^+$ corresponding to the molecular formula $C_{25}H_{29}N_3O_2$ (Rao et al., 2007).

It is possible to propose a mechanism that explains the obtaining of all 18 compounds isolated to date from the chlorination of irbesartan, including compounds IDBP2–IDBP5 and IDBP7–IDBP8 with a tetracyclic aromatic nucleus (Figures 20-21).

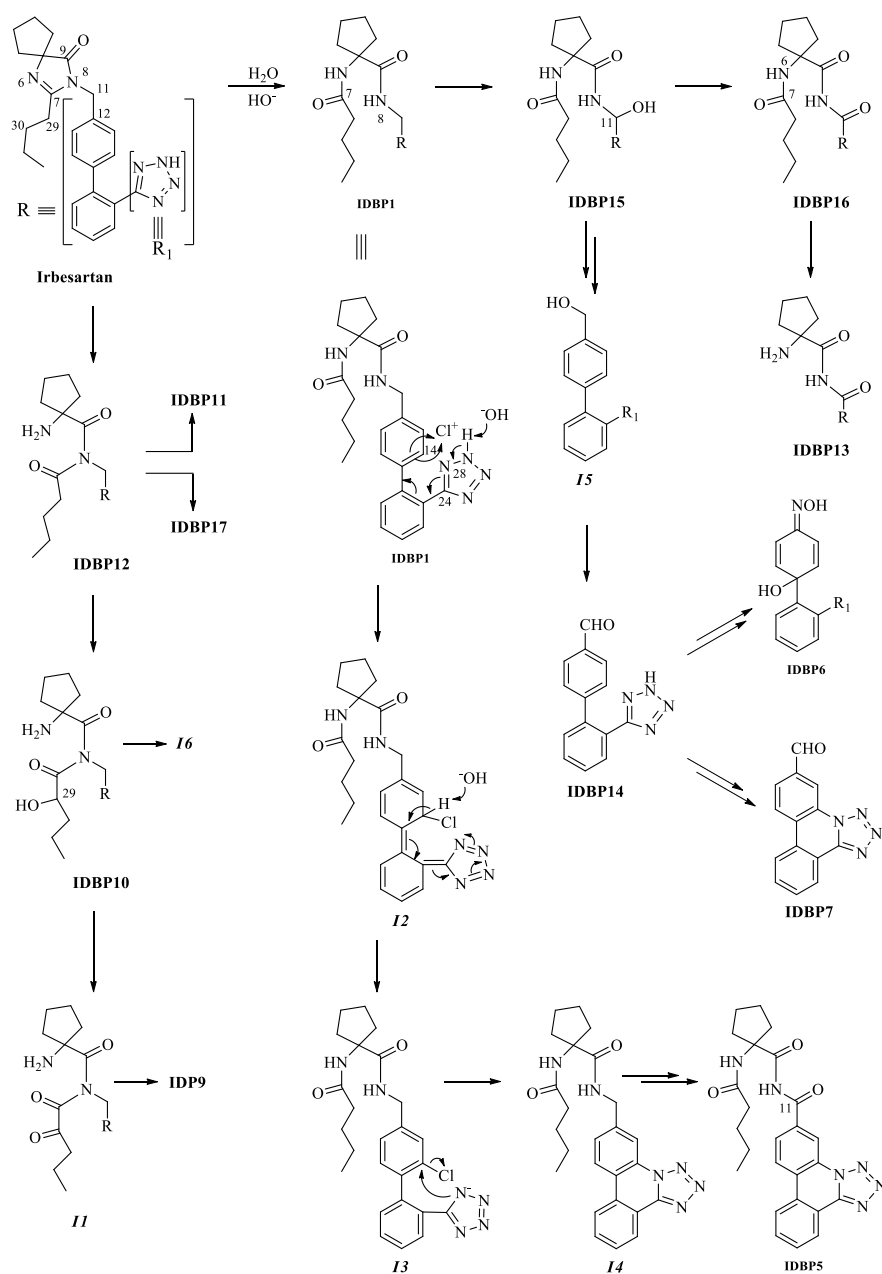
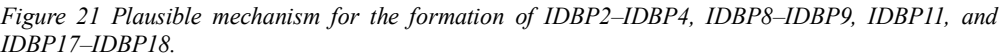


Figure 204 Plausible mechanism for the formation of IDBP1, IDBP5–IDBP7, IDBP10, and IDBP12–IDBP16.



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corresponding carbinol IDBP15, and then to the corresponding oxidized compound IDBP16. The hydrolysis of the N-6/C-7 bond of IDBP16 led to compound IDBP13. From compound IDBP15, through intermediate *I5*, product IDBP14 was obtained, and from this, the IDBP6 oxime and compound IDBP7.

It could be hypothesized that, in the used strongly oxidizing reaction conditions, a neutral intermediate *I2* can be formed by the addition of chlorine on the aromatic C ring of compound IDBP1 and the consequential loss of a proton from the tetrazole ring. The loss of the proton geminal to the chlorine atom would allow the aromaticity of the ring to be restored and would yield intermediate *I3* with a negative charge on the nitrogen of the tetrazole ring. This intermediate would evolve to intermediate *I4* by an intramolecular nucleophilic attack, and from the latter, IDBP5 would then be obtained by the oxidation of the benzyl carbon C-11.

Conversely, the hydrolysis of the C-7/N-6 bond of irbesartan leads to the formation of the by-product IDBP12, and from this, it is possible to obtain by-product IDBP10 by the oxidation of the *n*-butyl side chain at the C-29 carbon. The subsequent oxidation of carbon C-29 of the by-product IDBP10 would lead to intermediate *I1* and from this, with an intramolecular nucleophilic attack, by-product IDBP9 would be obtained.

The by-product IDBP12 could provide IDBP17 by hydrolysis of the N-8/C-9 bond. In turn, the by-product IDBP17 would lead to the by-product IDBP3, through the *I7-I9* intermediates, and to the by-product IDBP2, through the *I7-I12* intermediates. Finally, it could be hypothesized that the by-product IDBP18 could be obtained by the oxidation of the tetrazole ring of the starting irbesartan. the by-product IDBP4 could be obtained from the corresponding IDBP3 for the hydrolysis of the C-7/N-8 bond, and the by-product IDBP8 starting from IDBPB10, through the *I6* and *I13* intermediates.

4.2.1.XIII Ecotoxicity Data

Irbesartan compound is proven to be relatively non-toxic with the *D. magna*, *A. fischeri*, and *R. subcapitata* screening tests with $EC_{50} > 100$ mg /L (Romanucci et al., 2020). This research shows that the chlorination of irbesartan involves more complex IDBPs. The ecotoxicological tests displayed dynamic and variable toxicity of irbesartan and its chlorinated by-products (Figure 22). The lowest toxicity occurred at around -32% (indicating stimulation) of IDBP7 in the algal test, while the highest toxicity was at 84% of the mixture in the bacteria test.

Among the tested organisms, *A. fischeri* was the most sensitive to the IDBPs, followed by *R. subcapitata*, and finally, *D. magna*, showing significant species-specific toxicity. The sensitivity to IDBP effects increases with decreasing trophic levels, according to a previous study (Cui et al., 2021). In contrast to species-specific toxicity differences, only IDBP10 showed the same range of toxicity in all the tested organisms, but no unique physicochemical characteristics have been associated with contributing to the observed effects.

This sensitivity, and consequently toxicity changes, could be attributed to the new smaller molecules after cleavage of functional groups that make them reactive against non-target organism (Cui et al., 2021; Prasse et al., 2020). This also agrees with previous studies that stated the introduction of a chlorine atoms into a molecule, or the formation of chlorinated compound led to an increase in acute toxicity (Grbović et al., 2015).

In addition, this study found that 64% of IDBPs exhibited stimulation (hormesis) to *R. subcapitata*. From environmental perspective, considering the occurrence of IDBPs and algal blooms in water bodies receiving sewage, the observed hormetic effect by IDBPs on algal population needs further evaluation based on a realistic contribution of IDBPs to phytoplankton blooms (Lv et al., 2020).

It should be noted that, the co-existence of many other IDBPs in the environment their high toxicity and the potential interactions among them (Laskowski et al., 2010) suggest that the chlorine product could induce high environmental risks.

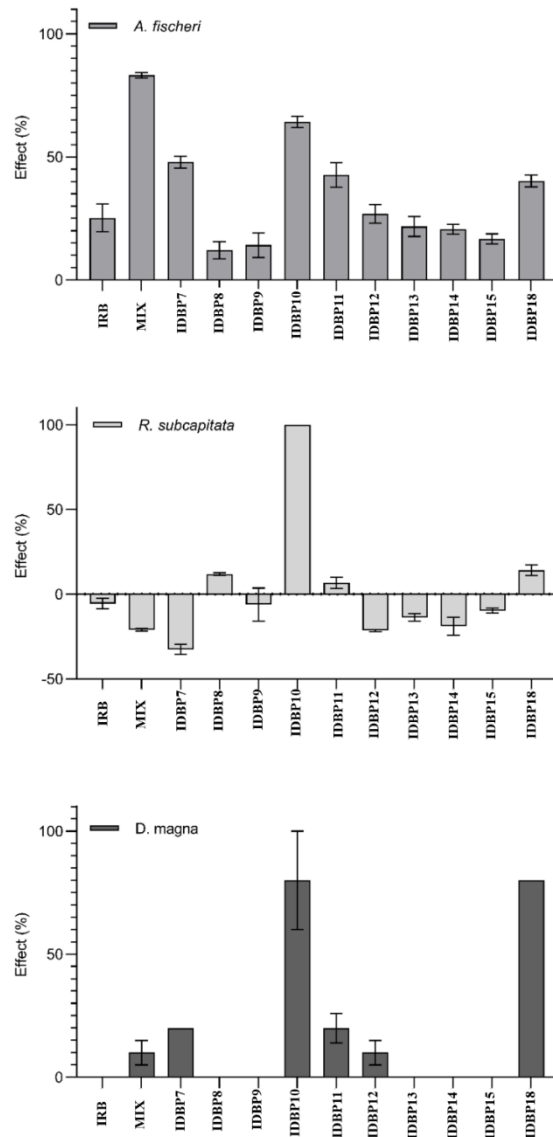


Figure 22 Toxicity results after exposure to Irbesartan and its disinfection by-products.

4.3 Percarbonate Mediated Advanced Oxidation of Irbesartan

This paragraph presents a study aimed at understanding the environmental fate of irbesartan when subjected to treatment with activated sodium percarbonate, with a focus on the formation and toxicity of Irbesartan disinfection by-products (IDBPs). The objective is to compare the results obtained with those already known about the use of sodium hypochlorite, previously characterized in similar studies. The proposed treatment showed the formation of at least eleven DBPs, eight of which were identified for the first time, isolated by different chromatographic techniques and characterized by Nuclear Magnetic Resonance and Mass Spectrometry, including the Time-of-Flight technique. Based on the data collected, a mechanism for the formation of these compounds was also proposed, which can be attributed to the oxidation of the n-butyl chain, the pentane cycle or one of the two aromatic rings.

To evaluate the biological impact of irbesartan subjected to SPC treatment, a toxicity study on DBPs was conducted using three model organisms, *D. magna*, *A. fischeri* and *R. subcapitata*. Ecotoxicity was further analyzed through the ecological structure-activity relationship program (ECOSAR), and the results obtained were compared with experimental data. Compared with sodium hypochlorite treatment, SPC treatment showed a lower mineralization rate (about -43%) and at least twice the amount of DBPs. In addition, toxicity assessment showed that DBPs formed during treatment with activated sodium percarbonate were more toxic than those from chlorination. ECOSAR predictions were in line with experimental data. Finally, DBPs exhibit varying levels of toxicity, mainly attributable to the presence of aromatic and hydroxyl groups in their chemical structure. These evidences indicate that SPC treatment is not suitable for the purification of irbesartan-polluted waters. This study investigated the environmental fate of irbesartan subjected to treatment with activated percarbonate, with a focus on the formation of disinfection by-products (IDBPs) and their toxicity, comparing the results with those obtained by sodium hypochlorite.

4.3.1 Results and Discussion

Eleven disinfection by-products IDBP19- IDBP29 (Figure 23) were obtained from the irbesartan oxidation reaction, isolated by column chromatography and HPLC and fully characterized by employing NMR and MS analyses. Their concentrations were at the maximum in the preparative experiments after ~1h, at percentages of 0.26, 0.49, 0.26, 0.90, 0.52, 0.65, 1.16, 0.52, 10.6, 0.27, and 4.00, respectively. IDBP19, IDBP21- IDBP25, IDBP27 and IDBP28 were isolated for the first time, IDBP20 is a known product isolated from the urine of patients taking irbesartan, evidently a product of their metabolism, and finally, IDBP26 and IDBP29 were identified by comparing their retention times with those of the standard compounds obtained from previous chlorination reactions.

It is curious and interesting to note that none of IDBPs exhibit hydrolysis of the N-6/C-7 or C-7/N-8 bond, a common reaction in the case of treatment with sodium hypochlorite. Only one product (IDBP27) features the closure of a new six-membered ring with the formation of a bond between carbon C-14 and nitrogen N-28. The isolated products are characterized by the presence of a hydroxyl group, which confirms the oxidation of the side chain (at positions C-32 and C-31 for IDBP19 and IDBP20, respectively), of the cyclopentane ring (at position C-3, forming the two diastereoisomers IDBP21 and IDBP22, respectively), of the aromatic ring A (at position C-14, resulting in IDBP23), or of ring B (at positions C-20 and C-21, resulting in IDBP24 and IDBP25, respectively). The other three products are the valsartanic acid IDBP26, well-known and common in the degradation of various sartans, the product lacking the aromatic nucleus IDBP28, and pentanoic acid IDBP29.

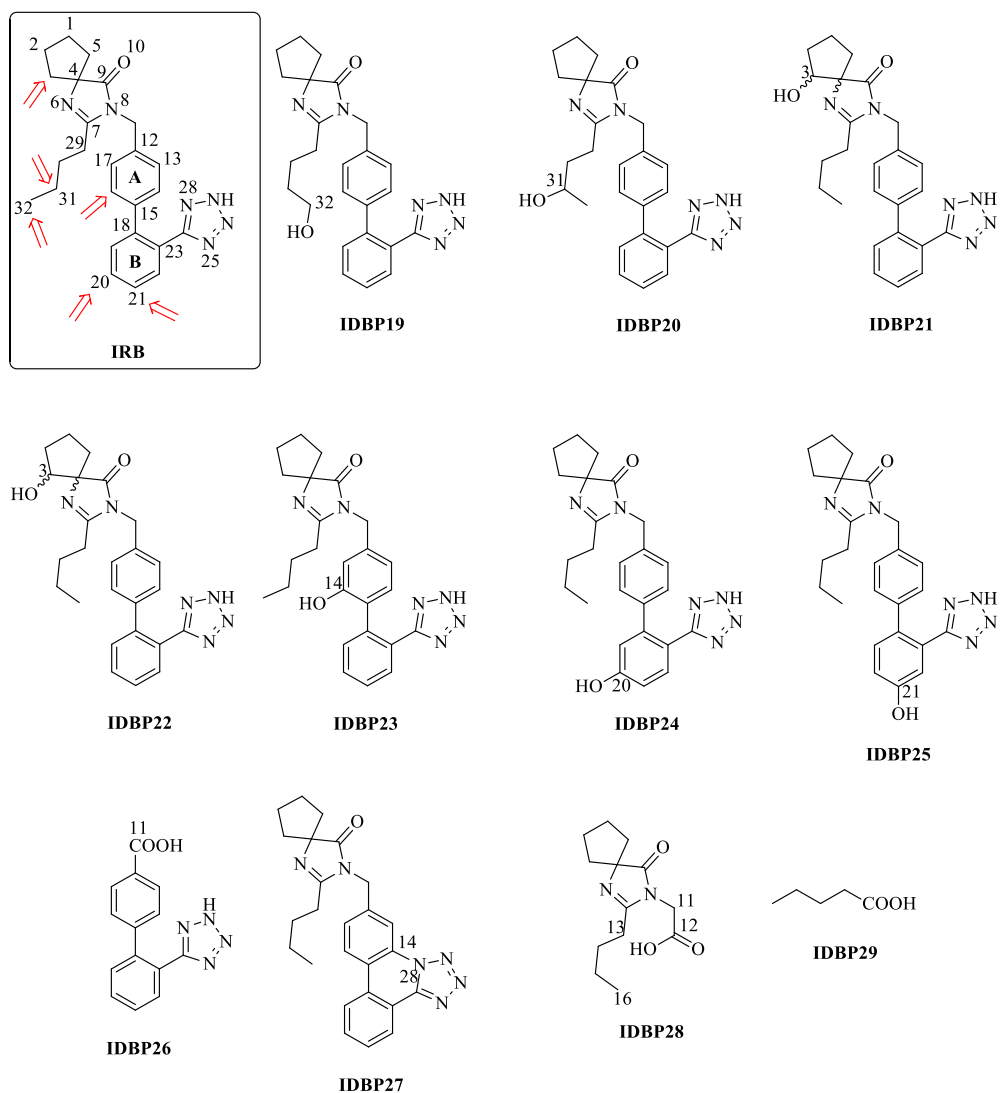


Figure 23 Chemical structures of disinfection by-products IDBP19–IDBP29 obtained by oxidation with sodium percarbonate.

4.3.1.1. Structure Elucidation of IDBP19

The NMR data (Table SMd12) of IDBP19 clearly indicated the presence of the 1,4-disubstituted aromatic ring A and the 1,2-disubstituted ring B. Furthermore, the imidazole-like ring and the cyclopentane ring were present without any traces of

substitutions. The signals related to the *n*-butyl side chain C-29/C-32 bound to carbon C-7 showed differences. In fact, the ^1H NMR spectrum exhibited a triplet signal at 3.51, identified as corresponding to the protons H-32. These protons were found in the HSQC (Heteronuclear Single Quantum Coherence Spectroscopy) spectrum to be attached to a carbon at ppm 60.87 and in the ^1H - ^1H COSY (CORrelated SpectroscopY) spectrum to be correlated with the multiplet signal at ppm 1.52, identified as corresponding to the protons H-31. The MS-TOF analysis confirmed the structural hypothesis, showing a molecular ion peak at m/z 445.23 $[\text{M} + \text{H}]^+$ corresponding to the molecular formula $\text{C}_{25}\text{H}_{28}\text{N}_6\text{O}_2$.

4.3.1.II. Structure Elucidation of IDBP20

Compound IDBP20 was very similar to compound IDBP19. These ones were found to be structural isomers, with IDBP20 being oxidized at carbon C-31 instead of C-32. In fact, the ^1H NMR spectrum (Table SMd13) exhibited a doublet at 1.14, identified as corresponding to the three protons H-32, which were, in the HSQC spectrum, attached to a carbon at ppm 22.18. These protons were correlated, in a ^1H - ^1H COSY spectrum, with the multiplet at ppm 3.74, identified as corresponding to the proton H-31. The MS-TOF analysis confirmed the structural hypothesis with a molecular ion peak at m/z 445.24 $[\text{M} + \text{H}]^+$ corresponding to the molecular formula $\text{C}_{25}\text{H}_{28}\text{N}_6\text{O}_2$.

IDBP20 has been identified in the urine of individuals taking irbesartan and is a product of its metabolism (Chando et al., 1998).

4.3.1.III. Structure Elucidation of IDBP21

The NMR data of IDBP21 (Table SMd14) clearly indicated the presence of the aromatic rings A and B, 1,4-disubstituted and 1,2-disubstituted, respectively, of the imidazole-like ring, and of the *n*-butyl side chain. In this case, the higher polarity of the product compared to that of the starting material was due to the oxidation of the

cyclopentane ring. Indeed, the ^1H NMR spectrum showed a triplet signal at ppm 4.54, identified as corresponding to the proton H-3 and was correlated to the carbonyl carbon C-9 at ppm 185.70 and to the quaternary carbon C-4 at ppm 74.90, as well as to the carbons C-1, C-2, and C-5 at ppm 34.60, 34.60, and 44.60, respectively.

4.3.1.IV Structure Elucidation of IDBP22

The NMR data of IDBP22 (Table SMd15) were very similar, but not identical, to those of the previous compound, under the same solvent conditions, confirming the presence of the aromatic rings A and B, the imidazole-like ring, and the *n*-butyl side chain. In particular, the ^1H NMR spectrum showed a triplet signal at ppm 4.53, identified as corresponding to the proton H-3 and attached, in an HSQC spectrum, to the carbon at ppm 72.70. This proton, in an HMBC spectrum, was correlated to the carbonyl carbon C-9 at ppm 185.75 and to the quaternary carbon C-4 at ppm 74.80, as well as to the carbons C-1, C-2, and C-5 at ppm 34.31, 34.44, and 44.60, respectively. Evidently, the oxidation at the carbon C-3 caused its dissymmetry and that of the adjacent carbon C-4, resulting in a total of four stereoisomers, namely two pairs of enantiomers.

4.3.1.V Structure Elucidation of IDBP23

The NMR data of IDBP23 (Table SMd16) revealed the presence of two aromatic rings, one being the 1,4-disubstituted B ring, and the other being the A ring, which appeared 1,2,4-trisubstituted. Evidently, compared to the starting material, there was a substituent in one of the two equivalent positions, C-13/C-17 or C-14/C-16. In fact, the ^1H NMR data showed a narrow doublet at ppm 6.51, a double doublet at ppm 6.73, and a doublet at ppm 7.17. While the first two signals were correlated in an HMBC spectrum to the benzyl carbon C-11 and the quaternary carbon C-15, the third signal was correlated to the quaternary carbons C-14 and C-18. The data clearly indicated

the presence of a substituent on the carbon C-14, which was identified as a hydroxyl group based on the chemical shift values of the carbon it is attached to and those of the adjacent carbons and hydrogens. Additionally, the MS-TOF analysis confirmed the structural hypothesis with a molecular ion peak at m/z 445.27 $[M + H]^+$ corresponding to the molecular formula $C_{25}H_{28}N_6O_2$.

4.3.1.VI Structure Elucidation of IDBP24

The NMR data of IDBP24 (Table SMd17), unlike those of the previous compound, indeed showed the presence of two aromatic rings, with one being the 1,4-disubstituted A ring and the other being the B ring, which appeared 1,2,4-trisubstituted. Evidently, compared to the starting material, there was a substituent present, but not on the A ring, rather on the B ring, in one of the four different positions, C-19/C-22. The ^1H NMR data revealed a narrow doublet at ppm 6.85, a double doublet at ppm 6.83, and a doublet at ppm 7.35, which were consistent with the presence of a substituent at either position C-21 or C-22, in both cases resulting in a 1,2,4-trisubstituted ring. The HMBC spectrum showed correlation of the doublet at ppm 7.35 with the carbon C-24 of the tetrazole ring and with the quaternary carbon C-18, to which the protons H-14/H-16 of the A ring were also correlated. Conversely, the doublet at ppm 6.85 correlated with the carbon C-15, to which the protons H-13 and H-17 of the A ring were correlated. This confirmed the presence of a substituent at position C-22, identified as a hydroxyl group based on the MS-TOF mass spectrum data.

4.3.1.VI Structure Elucidation of IDBP25

The NMR data of IDBP25 (Table SMd18) clearly showed that this compound was a structural isomer of the previous product. Once again, the B ring appeared 1,2,4-trisubstituted, in this case with the substituent located at position C-21. The ^1H NMR

data revealed a narrow doublet at ppm 7.04, a double doublet at ppm 7.05, and a doublet at ppm 7.34. However, in this case, the HMBC spectrum showed correlation of the narrow doublet at ppm 7.04 with the carbon C-24 of the tetrazole ring and with the quaternary carbon C-18, to which the protons H-14/H-16 of the A ring were also correlated, while the doublet at ppm 7.34 correlated with the carbon C-15, to which the protons H-13 and H-17 of the A ring were correlated. This confirmed the presence of a substituent at position C-21, identified as a hydroxyl group based on the MS-TOF mass spectrum data.

4.3.1.VII Structure Elucidation of IDBP26

The nuclear magnetic resonance spectra were in accordance with those reported in the literature (Kumaraswamy et al., 2020).

4.3.1.VIII. Structure Elucidation of IDBP27

Compound IDBP27 is the only one among those isolated that exhibits the formation of an additional six-membered ring due to the covalent bond formation between carbon C-14 and nitrogen N-28. Indeed, the aromatic ring A is 1,2,4-trisubstituted, as highlighted by the ^1H NMR spectrum (Table SMd19) showing a narrow doublet at ppm 8.47, a broad doublet at ppm 8.76, and a double doublet at ppm 7.66. Of these protons, the first and third were correlated in an HMBC spectrum to both the benzyl carbon C-11 and the quaternary carbon C-15, while the doublet at ppm 8.76 correlated with the quaternary carbons C-12 and C-18. These correlations, consistent with the data from the MS-TOF mass spectrum, which presented an ion peak at m/z 427.23 $[\text{M} + \text{H}]^+$ corresponding to the molecular formula $\text{C}_{25}\text{H}_{26}\text{N}_6\text{O}$, confirmed the formation of a new ring resulting in a tetrazole[1,5-f]phenanthridine nucleus.

4.3.1.IX Structure Elucidation of IDBP28

Compound IDBP28 does not feature the aromatic nucleus but retains the imidazole-like ring, the cyclopentane, and the *n*-butyl side chain (Table SMd20). Evidently, oxidation of the aromatic nucleus occurred, leaving only carbon C-12 of ring A. The MS-TOF mass spectrum presented an ion peak at m/z 253.16 $[M + H]^+$ corresponding to the molecular formula $C_{13}H_{20}N_2O_3$. The ^{13}C NMR spectrum showed a carboxylic carbon at ppm 170.71. This signal, along with the carbonyl carbon C-9 at ppm 185.47, correlated with the signal at ppm 4.21, identified as relative to protons H-11, bound to the carbon at ppm 43.05, as highlighted by the HSQC spectrum.

4.3.1.X Structure Elucidation of IDBP29

The nuclear magnetic resonance spectra were in accordance with those reported in the literature (Osako et al., 2015).

4.3.1.XII Mechanism of Irbesartan Disinfection in Percarbonate-Based Advanced Oxidation Reaction

It is possible to propose a mechanism that explains the obtaining of all 11 compounds isolated to date from the degradation of irbesartan (Figure 24).

In a percarbonate-based advanced oxidation reaction with a pH ranging from 8 to 9, as in our case, the prevalent species is the bicarbonate ion HCO_3^- , which exists in equilibrium with other reactive species such as $CO_3^{\cdot-}$, $HO_2^{\cdot}$, $O_2^{\cdot-}$ and $HO^{\cdot}$. Specifically, this latter species could abstract a hydrogen from carbon C-32 of irbesartan to form intermediate I1, which could then react with $HO_2^{\cdot}$ to yield the hydroalkylperoxide intermediate I2. Decomposition of this intermediate, with removal of OH^- , would lead to the formation of IDBP19. Similarly, if the reaction occurs at carbon C-31 of the side chain, carbon C-3 of the cyclopentane, one of the two equivalent positions C-

14/C-16 of aromatic ring A, or carbon C-21 or C-22 of aromatic ring B, it will result in the formation of products IDBP20- IDBP25, respectively.

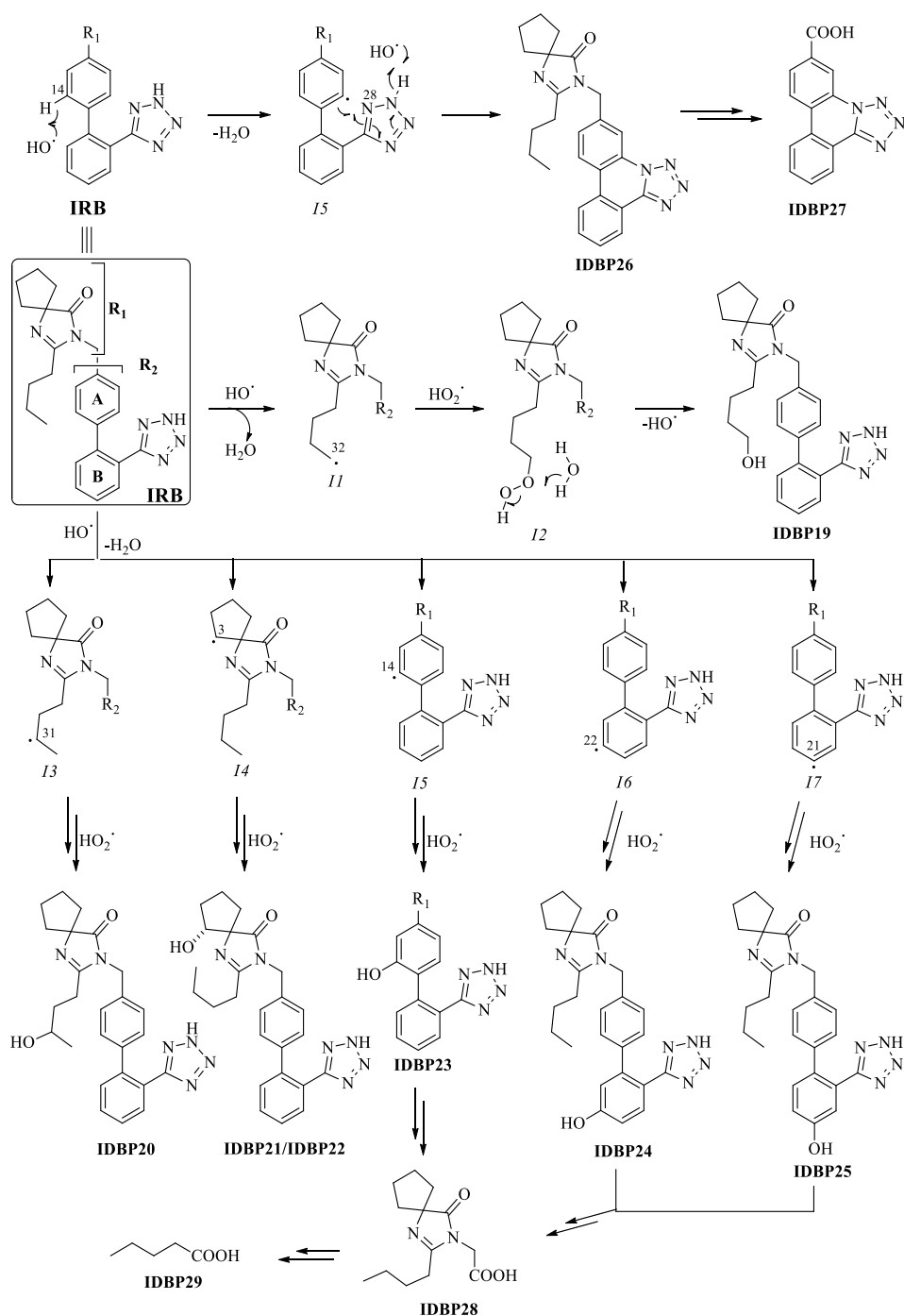


Figure 24 Plausible mechanism for the formation of IDBP19- IDBP29.

Starting from the oxidized products on rings A and/or B, IDBP23- IDBP25, further oxidation reaction could lead to the complete degradation of the biphenyl aromatic nucleus, forming compound IDBP28, which retains only carbon C-12 oxidized to a carboxylic group from biphenyl. Subsequent degradation of this would lead to the formation of compound IDBP29, one of the simplest and final products of the overall oxidation reaction. It is interesting to note that hypothesized intermediate I5, which leads to the formation of IDBP23, could undergo dehydrogenation of the tetrazole ring, resulting in transposition of the two double bonds and the formation of a new six-membered ring due to the formation of a new bond between carbon C-14 and nitrogen N-28. This would explain the obtaining of IDBP26, which, upon further degradation of the imidazole-like ring, would lead to IDBP27.

4.3.1.XIII. Ecotoxicity Data

The evaluation of IRB toxicity across three bioassays revealed distinct response patterns (Figure 25). When exposed to IRB, *D. magna* exhibited only 13.3% immobility recorded at 48h. Additionally, IRB demonstrated a slight stimulatory effect, as indicated by luminescence inhibition with *A. fischeri*, showing a negative percentage (-10.0%) and growth inhibition with *R. subcapitata* experiencing -14.9% of effect upon exposure to IRB. Overall, the results aligned with previous studies (Romanucci et al., 2020), indicating that IRB may not pose significant toxicity.

The toxicity of individual IDBPs revealed distinct response patterns across the tested organisms. In *D. magna*, IDBP20, and IDBP27–IDBP29 exhibited high immobilization rates (87%-100%), while IDBP22–IDBP24, and IDBP26 showed lower toxicity, with rates ranging from 27% to 47%. IDBP19, IDBP21 and IDBP25 demonstrated no toxic effects. *A. fischeri* exhibited varying sensitivity, with effects ranging from stimulatory to inhibitory and percentages ranging from -12.6% (stimulation) to 51% (inhibition). IDBP24 and IDBP29 demonstrated higher toxicity,

while IDBP21 and IDBP27 showed slight toxicity. IDBP19, IDBP20, IDBP23, IDBP25, and IDBP28 exhibited no toxicity, whereas IDBP26 and IDBP22 displayed stimulatory effects.

Regarding *R. subcapitata*, although all IRB-related IDBPs tested showed toxicity, with growth inhibition percentages ranging from 47% to 87%, the narrow range of results did not allow for the detection of variability in sensitivity. However, IDBP19–IDBP23, and IDBP28 induced higher algal growth inhibition with percentages ranging from 70% to 87%, underlining the significance of understanding the toxicity of IDBPs, particularly those that impact aquatic ecosystems.

The toxicity of by-product mixture (MIX) was exclusively observed in *R. subcapitata*, resulting in high algal growth inhibition, with a recorded percentage of 60.8%.

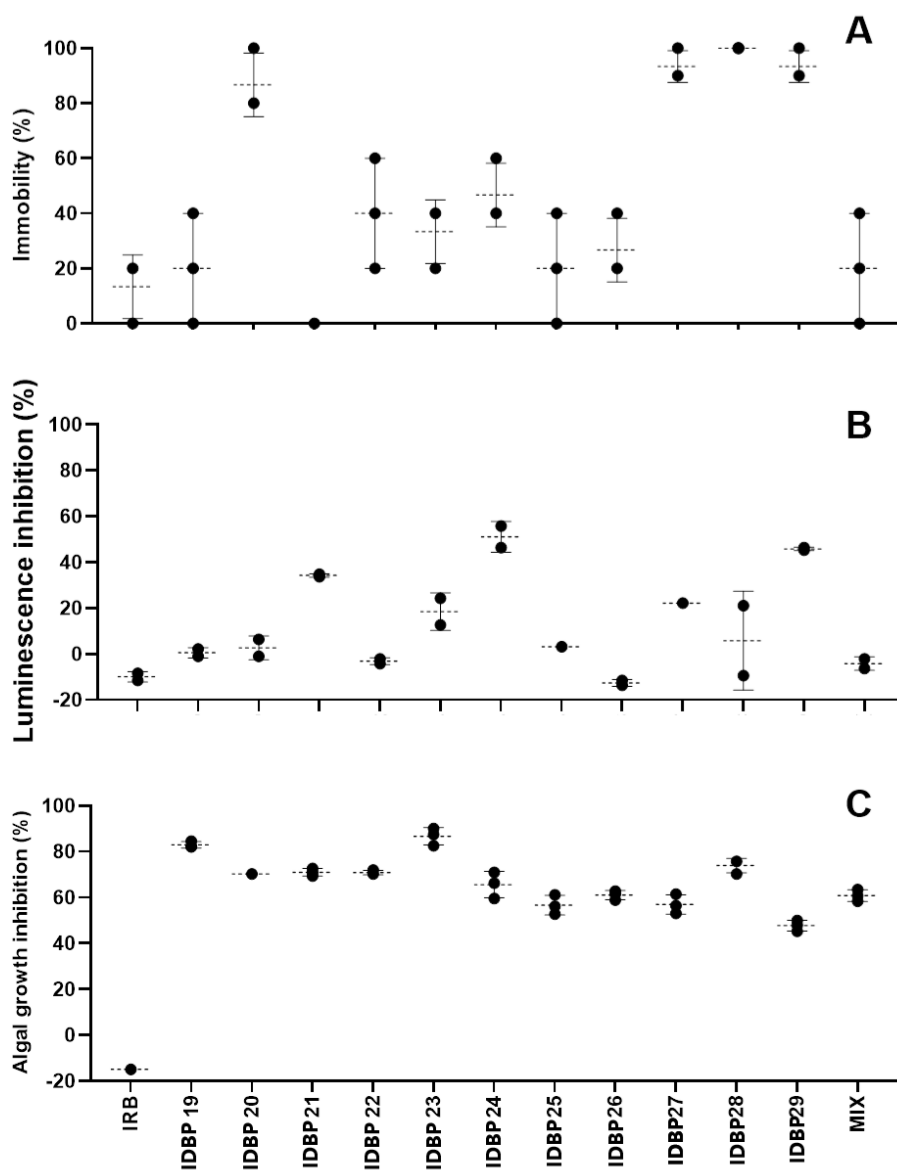


Figure 255 Toxicity results following exposure to irbesartan and its oxidation by-products on (a) *D. magna*, (b) *A. fischeri*, and (c) *R. subcapitata*

4.3.1.XIV Comparative toxicity of IRB- IDBPs

IDBPs from this study, along with those investigated by Romanucci et al. (2020) and those illustrated in chapter 4.1, were categorized into five primary groups based on structural features: presence or absence of a third six-membered ring, position of the OH group, oxidation/hydroxylation of the side chain, reduction/oxidation state, and presence of aromatic or saturated portions.

For instance, IDBPs differing by the presence of a third six-membered ring, such as IRB, showed lower toxicity. The position of OH groups was also significant, as seen with IDBP20, which, hydroxylated at C-31, exhibited moderate toxicity. A clear difference in toxicity was observed between the reduced form (IDBP11) reported as non-toxic in chapter 4.1, and the oxidized form (IDBP26) of the IDBPs. Oxidized compounds, such as IDBP26, demonstrated low toxicity.

Compounds containing aromatic or saturated portions, such as IDBP28, exhibited high toxicity. This finding aligned with previous studies indicating that compounds containing aromatic portions exhibited high toxicity, corroborating the well-established understanding in the field. The presence of aromatic structures in chemical compounds has often been associated with heightened toxicity levels, a phenomenon widely documented across various environmental and pharmaceutical by products studies (Han et al., 2021; Souissi et al., 2013; Xu et al., 2022).

The increase in toxicity from IDBP19 to IDBP29 could be attributed to several factors, including the addition of hydroxyl groups during the oxidation processes. The incorporation of hydroxyl groups leads to structural changes that enhance the compounds' affinity for biological targets or increase their reactivity with cellular components, resulting in heightened toxicity levels (Souissi et al., 2013).

4.2.1.XVI ECOSAR prediction

To further classify the toxicity of IDBPs, the results of the aquatic toxicity tests were compared to the predictions of the ECOSAR model, which were made in the chemical

domain of the neutral organics. The ECOSAR predictions for IRB- IDBPs of previous studies (Romanucci et al., 2020; Siciliano et al., 2023) and the present study are summarized in Table 1: their toxicity range was classified based on LC_{50}/EC_{50} values and the criteria defined by the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) (United Nations, 2023). The GHS classification based on prediction results made by ECOSAR, were as follows: Very Toxic: $LC_{50}/EC_{50}/ChV \leq 1$ mg/L; Toxic: $1 \text{ mg/L} < LC_{50}/EC_{50}/ChV \leq 10$ mg/L; Harmful: $10 \text{ mg/L} < LC_{50}/EC_{50}/ChV \leq 100$ mg/L; Not Harmful: $LC_{50}/EC_{50}/ChV > 100$ mg/L.

The results (Table 7) showed a range of toxicity among the different IDBPs tested. Eight compounds (IDBP7, IDBP8, IDBP14, IDBP23, IDBP24, IDBP25, IDBP27, IRB) exhibited $LC_{50}/EC_{50}/ChV$ values ≤ 1 mg/L, falling them as "Very Toxic" according to GHS criteria. Nine compounds (IDBP4, IDBP6, IDBP13, IDBP16, IDBP17, IDBP19–IDBP22) were classified as "Toxic", with values ranging from 1 to 10 mg/L. Twelve compounds (IDBP1, IDBP3, IDBP5, IDBP9–IDBP12, IDBP15, IDBP18, IDBP28) fell into the "Harmful" category due to their values ranging from 10 to 100 mg/L. Lastly, three compounds (IDBP2, IDBP26, IDBP29) were deemed "Not Harmful" as their values exceeded 100 mg/L.

Results from the aquatic toxicity tests did not entirely follow the predicted ECOSAR toxicity trend. Notably, for select compounds such as IDBP8, IDBP13, IDBP20, IDBP24, and IDBP27, an evident alignment emerges between the experimental and predicted toxicity values. For instance, both sets of data consistently fall within the 'Toxic' classification, indicating a congruent trend. Conversely, compounds like IDBP7, IDBP14, and IDBP26 exhibit notable discrepancies, where the experimental toxicity percentages differ significantly from the predicted values. This discrepancy underscores the challenges in accurately predicting toxicity solely based on chemical properties, as variations in experimental conditions and organism sensitivity can influence outcomes.

The ECOSAR approach generally performs well for congeners that exhibit toxicity through similar mechanisms, particularly those involving membrane disruption (Dom et al., 2010; Moore et al., 2003). However, in the case of IRB-related IDBPs, the applicability of ECOSAR predictions varied.

This study revealed that while ECOSAR predictions generally provided useful insights into the potential toxicity of IRB-related IDBPs, discrepancies between predicted and experimental data were observed. These disparities may be attributed to several factors, including the complexity of IDBP interactions with biological systems and the diverse mechanisms through which these compounds exert toxicity across different organisms.

Moreover, significant interspecies differences in the mode of action of IRB-related IDBPs were evident in our results. When the mode of action varies considerably among species, as observed in our study, the predictive capacity of ECOSAR models may be limited, as they often rely on simplistic relationships between toxicity and physicochemical properties such as log Kow (Dom et al., 2010).

Table 7 Predicted acute and chronic toxicity of IRB and its oxidation by-products, and GHS classification.

Compound	Acute toxicity			Chronic toxicity (ChV)			Toxicity classification	
	(mg/L)			(mg/L)				according to GHS
	Fish	Daphnid	Algae	Fish	Daphnid	Algae		
	(LC ₅₀)	(LC ₅₀)	(EC ₅₀)	(LC ₅₀)	(LC ₅₀)	(EC ₅₀)		
IDBP1	74.565	50.890	21.296	2.800	6.739	11.513	Harmful	
IDBP2	1373.658	895.725	334.936	106.961	91.378	17.639	Not harmful	
IDBP3	55.296	60.053	8.900	5.712	10.041	0.875	Harmful	
IDBP4	0.644	2.868	10.879	0.058	0.279	3.802	Toxic	
IDBP5	1.082	13.356	140.289	0.240	1.522	41.765	Harmful	

IDBP6	0.822	5.515	31.173	0.108	0.569	10.227	Toxic
IDBP7	0.097	0.045	0.017	0.002	0.005	0.013	Very toxic
IDBP8	0.489	0.348	0.458	0.047	0.092	0.458	Very toxic
IDBP9	106.534	69.563	13.806	29.296	6.726	0.930	Harmful
IDBP10	92.059	60.259	27.663	3.754	7.235	12.533	Harmful
IDBP11	135.318	105.637	63.472	18.473	1.746	19.726	Harmful
IDBP12	58.795	101.811	6.282	35.190	8.594	26.190	Harmful
IDBP13	4.453	3.123	4.059	0.436	0.543	2.017	Toxic
IDBP14	0.390	0.622	2.800	0.016	0.059	1.240	Very toxic
IDBP15	90.827	23.743	13.766	3.123	2.210	5.558	Harmful
IDBP16	0.695	3.735	12.537	0.069	0.319	4.328	Toxic
IDBP17	17.236	11.481	8.623	1.109	1.741	4.624	Toxic
IDBP18	62.912	46.057	31.707	7.251	5.010	10.601	Harmful
IDBP19	7.548	5.147	5.015	0.603	0.873	2.857	Toxic
IDBP20	8.756	5.954	5.608	0.683	0.990	3.150	Toxic
IDBP21	8.756	5.954	5.608	0.683	0.990	3.150	Toxic
IDBP22	8.756	5.954	5.608	0.683	0.990	3.150	Toxic
IDBP23	0.714	0.567	0.401	0.029	0.109	0.119	Very toxic
IDBP24	0.717	0.570	0.405	0.029	0.110	0.121	Very toxic
IDBP25	0.717	0.570	0.405	0.029	0.110	0.121	Very toxic
IDBP26	3120.000	1750.000	1220.000	300.000	163.000	308.000	Not harmful
IDBP27	0.126	0.098	0.227	0.017	0.028	0.196	Very toxic
IDBP28	115.175	78.340	110.329	6.063	12.225	29.360	Harmful
IDBP29	2080.0	1140.0	715.0	194.0	98.9	171.0	Not harmful
IRB	0.379	0.292	0.767	0.053	0.067	0.396	Harmful

4.4 Advanced Oxidation Process of Valsartan by Activated Peroxymonosulfate: Chemical Characterization and Ecotoxicological Effects of its By-Products

The possible degradation of Valsartan (VAL) was investigated by means of a Fenton-type reaction with peroxymonosulphate activation in the presence of Fe(II) under neutral conditions. Fourteen degradation products were isolated and fully identified by nuclear magnetic resonance and mass spectrometry; five of them were discovered for the first time. A mechanism for their formation was also proposed. Subsequently, the potential acute and chronic toxicity effects of valsartan and its degradation products in the aquatic environment were evaluated, considering key organisms of the freshwater trophic chain, such as the producer *R. subcapitata* (alga) and the consumer *B. calyciflorus* (rotifer). Acute effects were observed at concentrations in the order of tens to hundreds of mg/L, much higher than those of environmental interest. Regarding chronic effects, algae were not particularly affected by either the main compound or its derivatives, whereas rotifers were less sensitive to derivatives (effective concentrations in the order of units/decades of $\mu\text{g/L}$) than to valsartan (effective concentrations in the order of hundreds of ng/L)

4.4.1 Results and Discussion

4.4.1.1 Degradation experiment

VAL degradation experiments were performed at approximately 10^{-5} M, concentration higher than the one detected in wastewater (Kasprzyk-Hordern et al., 2009). Specifically, drug solutions were treated for 30 min with Fe(II)/OXONE[®] (VAL/Fe(II)/OXONE[®] molar ratio of 1:1:1) (Rizzo et al., 2019), under magnetic stirring and at room temperature. Several oxidation tests and approximately always at the same concentration of VAL provided the same type of degradation. The identification of the by-products obtained occurred by comparing their retention times with those obtained by repeating the experiments at much higher concentrations of

the contaminant ($>10^{-3}$ M), but with a lower ratio of VAL/oxidizing agent (1:25). The reaction, followed at regular time intervals of 15 min, after approximately 2h showed the VDBPs obtained in the highest possible quantities to facilitate their isolation and to proceed with their structural identification. VDBP1–VDBP14 (Figure 26) were isolated at relative percentages of 1.90, 0.95, 1.33, 1.14, 1.14, 2.10, 2.10, 1.33, 1.90, 0.38, 0.19, 4.00, 4.19, and 2.86. The proposed mechanism of their formation from VAL is shown in Figure 27, and VDBP1–VDBP2, VDBP7–VDBP8 and VDBP11 were isolated for the first time.

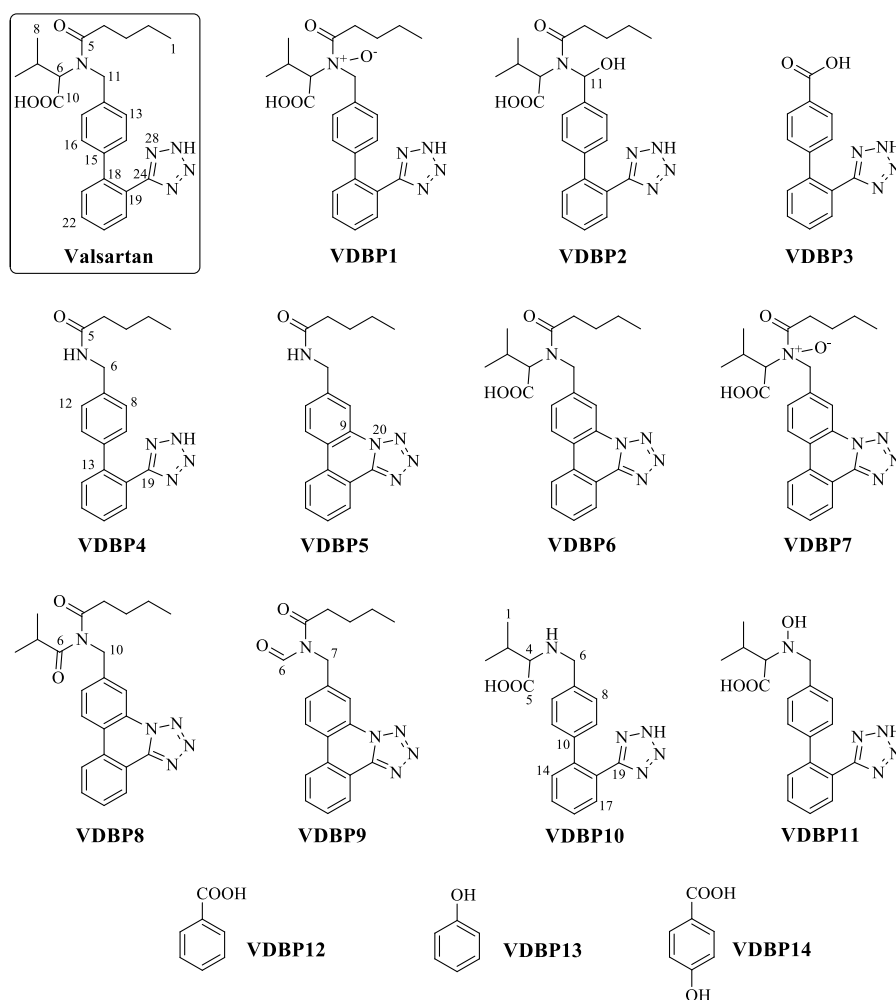


Figure 266 Chemical structures of Valsartan and its degradation by-products VDBP1–VDBP14.

4.4.1.II Proposed Mechanism for the Formation of Degradation By-Products VDBP1–VDBP11

VAL in the presence of hydroxyl radicals, may first form intermediate *I1* and then *I2*, which would lose a proton to yield the corresponding oxidation product VDBP1. From VDBP1, through prototropic tautomerism, the corresponding constitutional isomer hydrogenated at nitrogen N-28 could be obtained instead of nitrogen

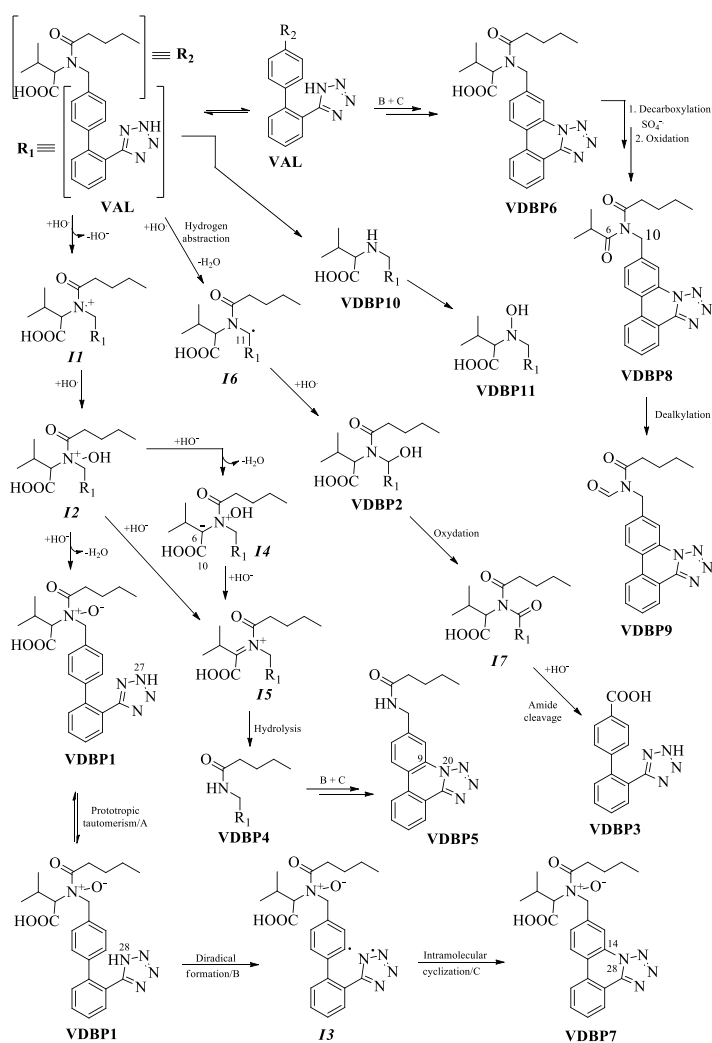


Figure 27 Hypothesized Mechanism for the Formation of VDBP1–VDBP11.

N-27 (Bauer et al., 1998). This offers the possibility of obtaining the diradical *I3*, which could undergo intramolecular cyclization to form VDBP7, closing a new six-membered ring between carbon C-14 and nitrogen N-28.

Intermediate *I2* could evolve into intermediate *I4*, through deprotonation of carbon C-6 in alpha position to carbon C-10 of the carboxylic function, or directly into intermediate *I5*, which, then, for subsequent hydrolysis, would yield VDBP4. VDBP4,

through tetrazole tautomerization (Step A: prototropic tautomerism), diradical formation (Step B), and intramolecular cyclization (Step C), would lead to VDBP5.

On the other hand, if VAL loses a hydrogen from the benzyl carbon C-11, it will result in intermediate *I6*, stabilized by the adjacent aromatic ring, which is a precursor of VDBP2. The subsequent oxidation of VDBP2 would lead to intermediate *I7*, and its amide bond hydrolysis at the alpha carbon C-11 would produce VDBP3, i.e., Valsartan acid. The tautomerization of the starting product and the two subsequent steps B and C could explain the formation of VDBP6, which, after oxidative decarboxylation probably caused by $\text{SO}_4^{\cdot-}$ radical, would yield VDBP8, and further dealkylation would give VDBP9. Finally, it is conceivable that the hydrolysis of the amide bond in the starting molecule would lead to the formation of VDBP10, and its oxidation would produce the corresponding VDBP11 (Figure 27).

4.4.1.III Spectral Data

See Chapter Supplementary Material E SMe

4.4.1.IV Acute toxicity tests

The acute toxicity results obtained by exposing the rotifer *B. calyciflorus* for 24h to the parent compound valsartan, its degradation by-products, and MIX are reported in Table 1 and are expressed as median lethal concentrations ($\text{LC}_{50\text{s}}$).

Table 8 Acute toxicity results expressed as LC_{50} [mg/L] of valsartan (VAL), degradation by-products (VDBPs) and MIX obtained in *B. calyciflorus*, with 95% confidence intervals in brackets.

Sample	LC_{50}	Sample	LC_{50}
VAL	128.00 (92.69–176.80)	VDBP7	33.86 (21.07–54.41)
VDBP1	27.72 (19.70–39.09)	VDBP8	9.30 (7.84–11.03)
VDBP2	36.59 (18.50–72.36)	VDBP9	4.18 (2.88–6.09)
VDBP3	50.69 (20.58–124.90)	VDBP12	8.22 (5.17–13.06)
VDBP4	1.40 (0.88–2.20)	VDBP13	16.13 (11.21–23.20)
VDBP5	8.25 (4.96–13.72)	VDBP14	16.10 (9.76–26.56)

VDBP6	35.16 (17.36–71.20)	MIX	8.60 (5.60–13.17)
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Valsartan caused 50% lethality in exposed organisms at very high concentrations, in the order of hundreds of mg/L. All derivatives were more toxic than the parent compound causing median lethality at tens or units of mg/L. The most toxic by-product was VDBP4 ($LC_{50} = 1.40$ mg/L) followed by VDBP9 ($LC_{50} = 4.18$ mg/L) which, compared to valsartan molecular structure, were characterized by the loss of alkyl groups (VDBP4) and by both the loss of alkyl groups and the increase in the number of oxygen-carbon bonds (VDBP9). A separate remark deserves the MIX which showed a potential synergistic effect among all the residues. No differences between negative control and solvent control were found (Unpaired t-test, data not shown). To our knowledge, acute toxicity data on freshwater organisms on valsartan is scarce in the literature, and the present research addresses the study on rotifers for the first time. However, it is possible to try to compare our results with those obtained in earlier studies in which other primary freshwater consumers were exposed to this sartan molecule. In 2014, Bayer et al., exposed the crustacean *Daphnia magna* to valsartan for 48h obtaining 50% immobilisation at concentrations higher than the maximum tested equal to 200 mg/L, furthermore in 2016, Mínguez and collaborators testing the same molecule in the same crustacean, found an EC_{50} higher than 100 mg/L, confirming results obtained in this study. Considering other sartan molecules, Adams, et al., (2021) tested Losartan in *D. magna* obtaining an acute effect greater than 70% at concentrations in the order of units of mg/L; conversely, Romanucci et al., (2020) testing irbesartan in the same daphnid obtained immobilisation <10% at 100 mg/L. All these studies, together with the results obtained in the present research in which the observed acute toxic effects of valsartan occur at concentrations far higher than those measured in freshwaters (going from tens of ng/L to units of µg/L, Ladhari et al. 2021), highlight the low toxic impact of sartans in freshwaters consumers, with no environmental concern in case of acute exposure.

Also, regarding the acute toxicity of valsartan by-products obtained after treatment with peroxymonosulfate, data are lacking in the literature, and it is possible to consider the toxic effects of other by-products obtained by different treatments of other sartans. In this regard, the two papers mentioned above (Romanucci et al., 2020, Adams et al., 2021) where irbesartan and losartan were exposed to different treatments (hypochlorite, UVC/photolysis and UV/H₂O₂) the by-products obtained were more toxic than the respective parent compounds as found in the present study. In line with the EU Directive 93/67/EEC (1996) where substances are classified into different risk classes according to their EC₅₀ values, valsartan can be classified as “non-toxic to aquatic organisms” after short term exposure, with EC₅₀ higher than 100 mg/L; VDBP1, VDBP2, VDBP3, VDBP6, VDBP7, VDBP13 and VDBP14 can be classified as “harmful to aquatic organisms” with 11 mg/L < EC₅₀ < 100 mg/L, and VDBP4, VDBP5, VDBP8, VDBP9, VDBP12 and MIX can be classified as “toxic to aquatic organisms” with 1 mg/L < EC₅₀ < 10 mg/L. To date, to the best of our knowledge, the mechanism of action of sartans in non-target organisms is not known. Nevertheless, in 2021, Adams et al., reported that the acute toxic effects of losartan and its by-products in *D. magna*, may be caused by cellular stress. Since it is well known that in humans sartans cause the block of the action of angiotensin II and that oxidative stress plays a key role in altering this system (Wen et al., 2012), it is possible to hypothesize similar alterations also in non-target organisms. Hence, as it is known from the literature, some drugs can affect angiotensin system elements in invertebrates and dysregulate angiotensin converting enzyme-like (AnCE) gene, an evolutionarily conserved gene, implicated in osmoregulation, ion balance, growth, memory, and immune processes (Salzet et al. 2001; Russo et al., 2018).

Although acute L(E)C_{50s} generally are obtained at concentrations higher than environmental ones, acute data are useful to enrich databases for the environmental risk assessment, and to estimate the potential toxicity of little studied molecules, especially for byproducts not yet commercially available, by using computational tools as Quantitative Structure–Activity Relationship (QSAR) models (Madden et al.,

2009; Diehle et al., 2019). The toxicity estimations determined using these models cannot replace toxicity testing, which are necessary to verify the real effects induced in aquatic organisms by xenobiotics.

4.4.1.IV Chronic toxicity tests

Considering that aquatic organisms are exposed to valsartan and its by-products in natural ecosystems for their whole lifespan, the effects due to long term exposure should be considered.

Chronic toxicity results obtained exposing the freshwater producer *R. subcapitata* (72 h) and the consumer *B. calyciflorus* (48 h), are reported in Table 9.

Table 9 Chronic toxicity of VAL, VDBPs and MIX in *R. subcapitata* (72 h) and *B. calyciflorus* (48 h).

	<i>Raphidocelis subcapitata</i>	<i>Brachionus calyciflorus</i>
VAL	0.27·10 ⁴ (0.22·10 ⁴ –0.34·10 ⁴)	0.23 (0.14–0.38)
VDBP1	2.39·10 ⁴ (2.04·10 ⁴ –2.78·10 ⁴)	6.21 (3.10–12.40)
VDBP2	4.75·10 ⁴ (3.93·10 ⁴ –5.72·10 ⁴)	6.80 (5.04–9.20)
VDBP3	2.79·10 ⁴ (1.64·10 ⁴ –4.74·10 ⁴)	12.08 (8.33–17.52)
VDBP4	6.03·10 ⁴ (4.62·10 ⁴ –7.88·10 ⁴)	23.54 (16.53–33.53)
VDBP5	5.34·10 ⁴ (2.64·10 ⁴ –10.80·10 ⁴)	42.99 (23.87–77.42)
VDBP6	> 10.00·10 ⁴	42.73 (23.32–78.27)
VDBP7	> 10.00·10 ⁴	29.52 (17.09–50.97)
VDBP8	> 10.00·10 ⁴	87.32 (45.16–168.9)
VDBP9	4.50·10 ⁴ (2.29·10 ⁴ –8.85·10 ⁴)	29.38 (16.28–53.02)
VDBP12	0.62·10 ⁴ (0.37·10 ⁴ –1.02·10 ⁴)	68.82 (43.95–110.90)
VDBP13	0.71·10 ⁴ (0.33·10 ⁴ –1.52·10 ⁴)	23.17 (14.72–36.38)
VDBP14	0.56·10 ⁴ (0.27·10 ⁴ –1.18·10 ⁴)	16.57 (8.58–32.00)

MIX	$1.28 \cdot 10^4$ ($0.67 \cdot 10^4$ - $2.43 \cdot 10^4$)	21.70 (8.50–55.41)
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The consumer was the most sensitive freshwater organism to valsartan with concentrations determining 50% reproductive inhibition (EC_{50}) in the order of tenths of $\mu\text{g/L}$, with environmental concern. The unicellular alga *R. subcapitata* was less sensitive to valsartan showing median inhibition of growth at concentrations in the order of units of mg/L , four orders of magnitude higher than rotifers. For both organisms, no differences between negative control and solvent control were found (*Unpaired t-test*, data not shown). As well as for acute also for chronic toxicity data are scarce, and in freshwater multicellular species are completely lacking. In 2016, Luongo and collaborators exposed *R. subcapitata* to valsartan obtaining EC_{50} higher than 100 mg/L , and in 2014 Bayer and collaborators, in *Desmodesmus subspicatus*, another unicellular green alga, found inhibition of growth at concentrations higher than 85 mg/L . Furthermore, the present study is also in line with Luongo et al. (2021) who observed that unicellular organisms (bacteria and algae) were less sensitive than multicellular species (*D. magna*) to valsartan.

As for parent compound, also for most of by-products and MIX, rotifers were the most sensitive organisms with EC_{50} values in the order of tens of $\mu\text{g/L}$. Only the derivatives VDBP1 and VDBP2 induced inhibition of rotifer reproduction at concentrations in the order of units of $\mu\text{g/L}$, one order of magnitude higher than valsartan. From Figure 28, in addition to the comparison between the two organisms tested, it is easily evident that all by-products were less toxic than the parent compound, differently from acute results. A certain reduction in toxicity from parent compound to by-products can be observed also in a study of Diehle and coauthors (2019) who estimated the effects of valsartan and its ozonation VDBPs by QSAR models in green algae, obtaining for the majority of VDBPs higher estimated EC_{50} values (from tens to thousands of mg/L) compared to that of Valsartan (EC_{50} at units of mg/L), indicating a decreased toxicity.

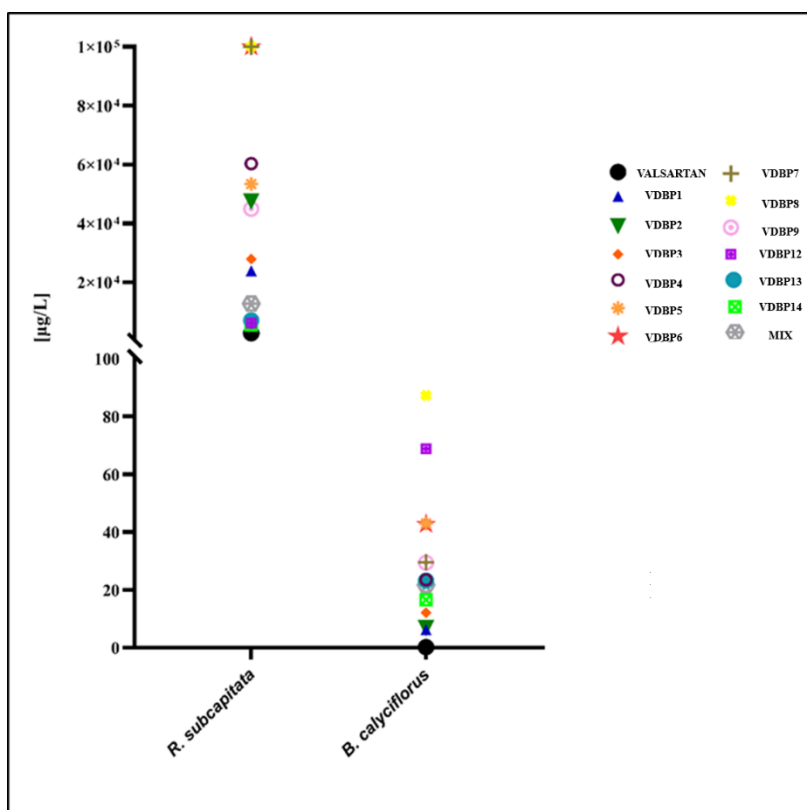


Figure 287 Comparison between the chronic toxicity values in *R. subcapitata* and *B. calyciflorus*. of valsartan, VDBPs and MIX. Results are reported as EC₅₀ [µg/L].

In Figure 29, the concentration/effect curves are reported to compare the whole toxic trend of Valsartan, by-products and MIX. VDBP8, VDBP9, VDBP13 and VDBP14 at lower tested concentrations (units/tenths of µg/L), induce a hormetic effect in *R. subcapitata* appreciable from the curves that reveal a biostimulation especially for VDBP13 and VDBP14. Luongo and collaborators (2021) observed the same trend in *R. subcapitata* testing some by-products of candesartan and olmesartan. The interesting chronic results of the present study, suggest that the advanced oxidation process of valsartan activated by peroxymonosulfate might be applied in wastewater treatment plants as algae are not particularly affected by the parent compound and its

derivatives, although some of them could determine, in some conditions, a potential algal bloom. The rotifers, representative of the second trophic level of the freshwater chain, are less affected by derivatives than valsartan that surely occurs at concentrations higher than those of by-products.

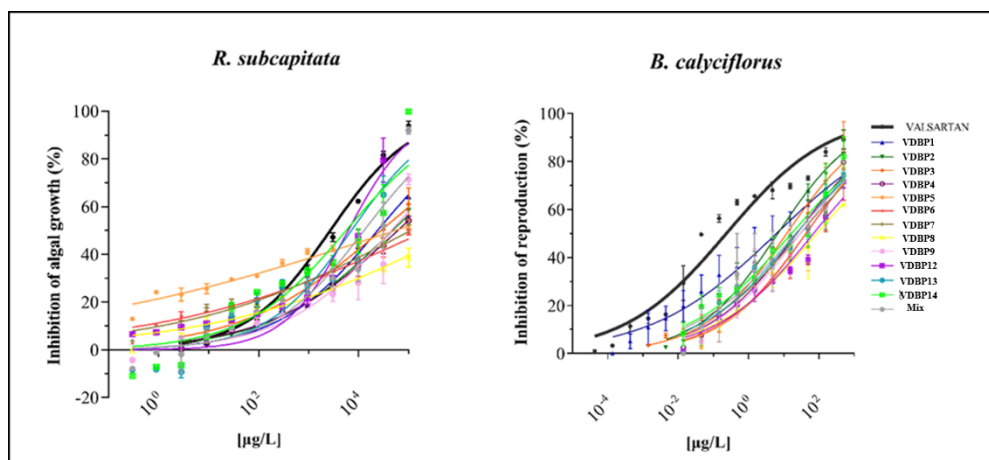


Figure 298 Concentration/effect curves of chronic toxicity of valsartan, VDBPs and MIX in *R. subcapitata* and *B. calyciflorus*. Results are reported as EC_{50} [$\mu\text{g/L}$]. Bars show Standard Deviation (SD, $n = 3$).

4.5 Advanced Oxidation Process of Losartan by Activated Peroxymonosulfate: By-Products Formation and Kinetic Evaluation

Degradation of Losartan was investigated in the presence of activated peroxymonosulfate, leveraging ferrous iron as a catalyst to enhance the oxidation process. Utilizing advanced analytical techniques such as NMR and mass spectrometry, nine distinct by-products were characterized. Notably, seven of these by-products were identified for the first time, providing novel insights into the degradation pathway of Losartan. The study delved into the kinetics of the degradation process, assessing the degradation efficiency attained when employing the catalyst alone versus when using it in combination with PMS. The results revealed that Losartan degradation reached a significant level of 64%, underscoring the efficacy of PMS/Fe(II) AOP techniques as promising strategies for the removal of Losartan from water systems.

4.5.1 Results and Discussion

4.5.1.1 Degradation Experiments

The experimental procedures for the oxidation of LOS were conducted assuming conditions that ensure the complete degradation of LOS, both in analytical and preparative settings. A solution of the drug at a concentration of 10^{-5} M was subjected to treatment for 30 min (LOS:PMS:Fe(II) molar ratio of 1:1:1) at room temperature. Subsequently, experiments were repeated using contaminant concentrations exceeding 10^{-3} M, employing a significantly higher ratio of LOS:PMS:Fe(II) (1:24:24) to ensure effective degradation of the target contaminant and to yield adequate quantities of Losartan degradation products (LDBPs) for subsequent structural elucidation. The resulting LDBPs were purified through column chromatography and HPLC, followed by comprehensive characterization via NMR and MS. (see Chapter Supplementary Materials SMf).

From the oxidation of Losartan, nine degradation products have been isolated (Figure 30), all retaining the basic diphenyl skeleton (rings A and B) and the tetrazole cycle (ring C). Four of the nine LDBPs (LDBP1–LDBP4) have the tetrazole attached only to ring B, through a bond between carbon C-24 of the tetrazole itself and carbon C-23 of the phenyl ring. The last 5 LDBPs (LDBP5–LDBP9) have the tetrazole attached to both phenyl rings A and B, forming a new six-membered cycle (ring E), due to the formation of a new bond between nitrogen N-25 of the tetrazole and carbon C-14 of aromatic ring A. The oxidative processes have affected the imidazole ring D, partially oxidized (in products LDBP1, LDBP6, and LDBP8) or opened (in products LDBP3, LDBP4, and LDBP9), or the tetrazole ring C (cyclized to aromatic ring A in product LDBP5, or cyclized and oxidized at one of the two positions N-26 or N-27 in the two products LDBP2 and LDBP7). Product LDBP1 (product 2a, Figure 31) originates from the oxidation of the side chain of the imidazole ring of Losartan (product 1a), specifically carbon C-6, with the hydroxymethyl function giving rise to the corresponding aldehyde function.

Product LDBP2 (product 3a) can be considered as obtained from the previous one, by oxidation of the tetrazole ring, oxidized to the nitrogen N-26. It should be noted that the ring C can exhibit a particular type of tautomerism—reported in scientific literature as desmotropy—which involves the possible presence of a hydrogen on each of the four different nitrogen atoms of the ring. However, considering the equivalence of the two nitrogen atoms bonded to the carbon C-24 and the free rotation of the tetrazole ring around the bond C-23–C-24, the tautomers in the liquid state are reduced to two, with the possibility that the hydrogen (and therefore the hydroxyl group in the corresponding oxidation product) is bonded to nitrogen N-25/N-28 or N-26/N-27. Generally, the hydrogen is reported to be bonded to nitrogen N-26 because in the solid state, this tautomer, indicated as form B, is the main one.

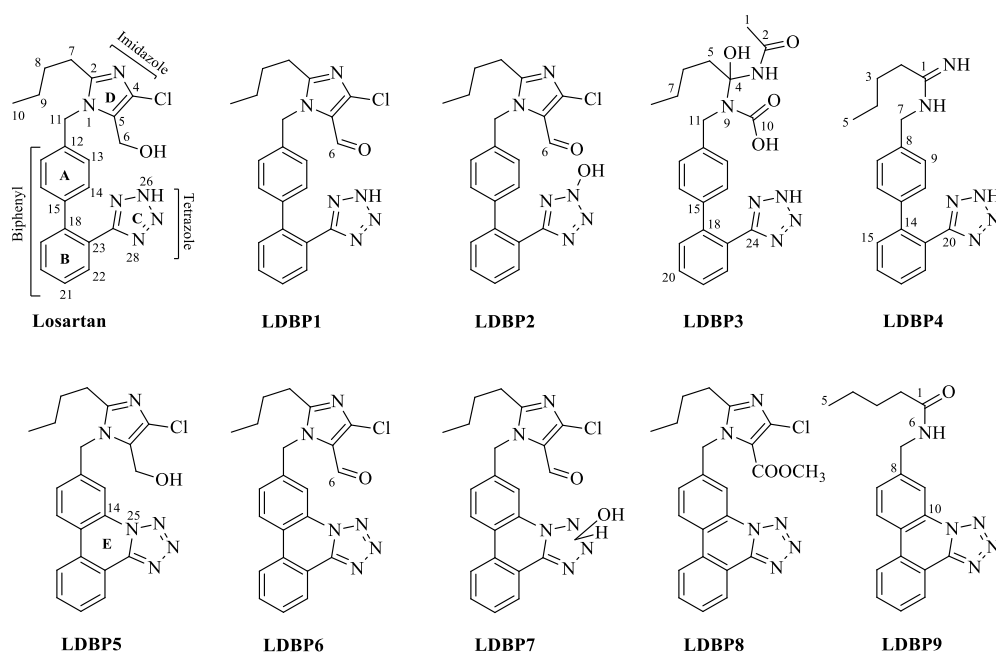


Figure 309 Chemical structures of Losartan and its degradation by-products.

The product LDBP1 (product 2a) could then undergo oxidation at the aldehydic function (C-6) to obtain the corresponding carboxylic acid, product 4a (Figure 31). The latter could undergo a reductive decarboxylation, known as the Kolbe reaction, to obtain product 6a, which could then lead to product 7a, where oxidative dechlorination of the imidazole ring has occurred. The latter could undergo hydroxylation at the carbon C-5 to yield product 8a. The oxidation could then proceed at positions C-4 and C-5 (product 9a) and further to lead to ring opening (product 10a). An initial reductive decarboxylation would yield product 11a, which could then form degradation product LDBP3 (product 14a) or undergo a second decarboxylation to give rise to product LDBP4 (product 12a).

The product LDBP5 (product 1b) is the first of those that exhibit the closure of a six-membered ring between the two aromatic rings and the tetrazole. We hypothesized (Figure 32) that this product could be obtained by the addition of a hydroxyl group to the carbon C-14 of ring A—forming intermediate I°_1 —followed by

the abstraction of a proton from the same position to re-aromatize ring A and obtain a nucleophilic nitrogen on the adjacent tetrazole (intermediate I_2). The final step would involve the closure of the cycle by the nucleophilic attack of the nitrogen N-25 of the tetrazole on the carbon C-14 of the adjacent aromatic ring.

Alternatively, one could hypothesize the oxidation of ring A at carbon C-14, followed by the abstraction of a proton from the nitrogen N-25 of the tetrazole. The intramolecular nucleophilic substitution would then explain the formation of the degradation product LDBP5.

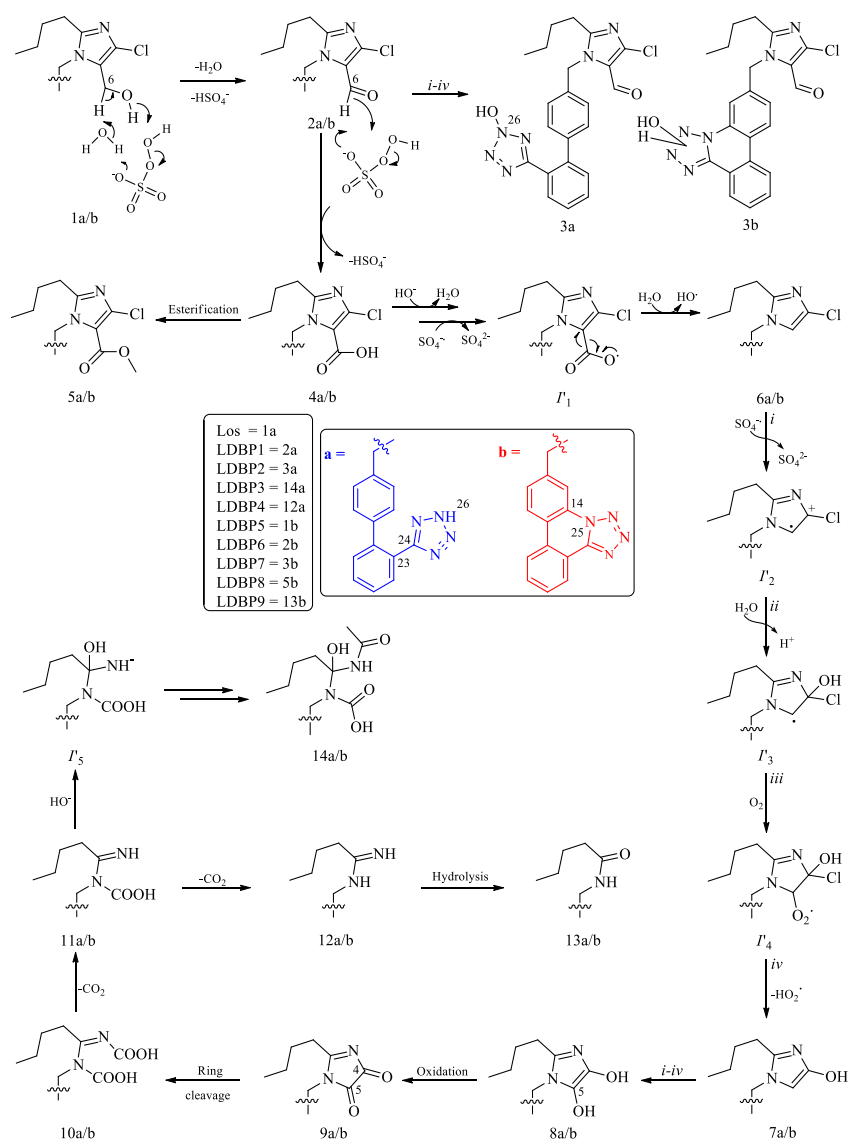


Figure 3110 Plausible mechanism of Losartan oxidation with peroxymonosulfate.

Degradation product LDBP5 (product 1b) can be considered the precursor to the last four degradation products obtained (LDBP6–LDBP9). Specifically, LDBP6 (product 2b) is the corresponding aldehyde of LDBP5, obtained by oxidizing the hydroxymethyl group at C-6. In turn, the oxidation of LDBP6 on the tetrazole ring, with the hydroxyl group oxidation at nitrogen N-26 (or N-27), would explain the

formation of LDBP7 (product 3b), while the oxidation of carbon C-6 in LDBP6 to the corresponding carboxylic acid 4b and its subsequent methylation would yield the corresponding ester LDBP8 (product 5b).

Finally, the reductive decarboxylation of product 4b and the subsequent oxidation and opening of the imidazole ring would account for the formation of product LDBP9 (product 13b), corresponding to the hydrolysis product of LDBP4 (product 12a) with the additional formation of cycle E.

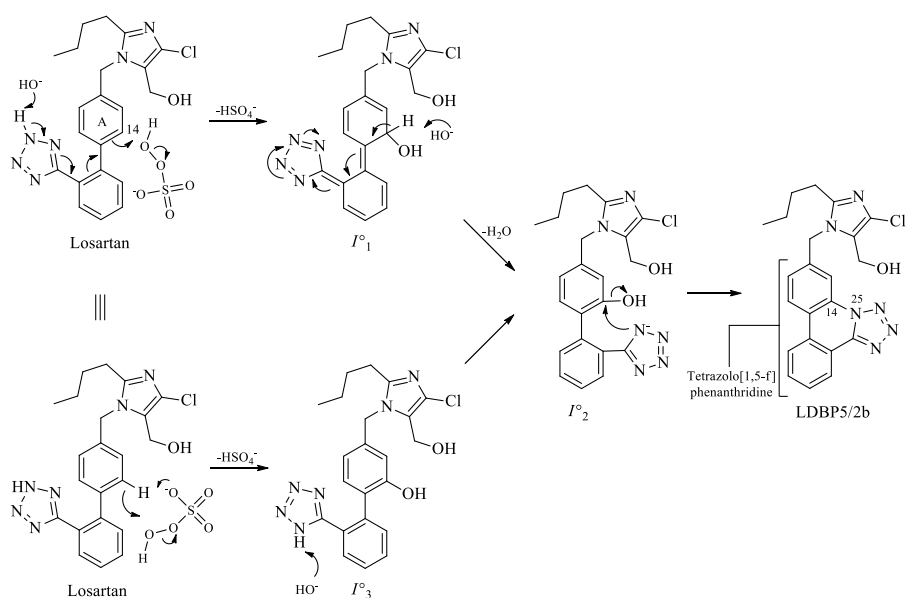


Figure 32 Plausible mechanisms of formation of tetrazolo [1,5-f]phenanthridine.

Observing the nine identified LDBPs resulting from the oxidation reaction with peroxymonosulfate, two main structural modifications are noticeable: a) the oxidation of the hydroxymethyl side chain of the imidazole ring (LDBP1 and LDBP6); b) the formation of an intramolecular bond and the closure of ring E (LDBP5–LDBP9). Thus, degradation product LDBP1 can be considered the precursor for obtaining products LDBP2–LDBP4, while product LDBP5 can be considered corresponding to LDBP1 and the precursor for obtaining products LDBP6–LDBP9. Similarly, it is straightforward to consider LDBP2 and LDBP7 as corresponding, differing only in

the absence or presence of the hydroxylated ring E. Likewise, products LDBP4 and LDBP9 can be considered corresponding, with the latter featuring the ring E and a carbonyl function instead of an imine one. In general, starting from LDBP1 and LDBP5, the generation of other degradation products can be hypothesized by oxidation of carbon C-6 and subsequent degradation of ring D, as summarized in Figure 33. It is conceivable that other products, albeit in lesser quantities, may have formed, not yet identified or degraded more rapidly than others, which could confirm the proposed mechanism.

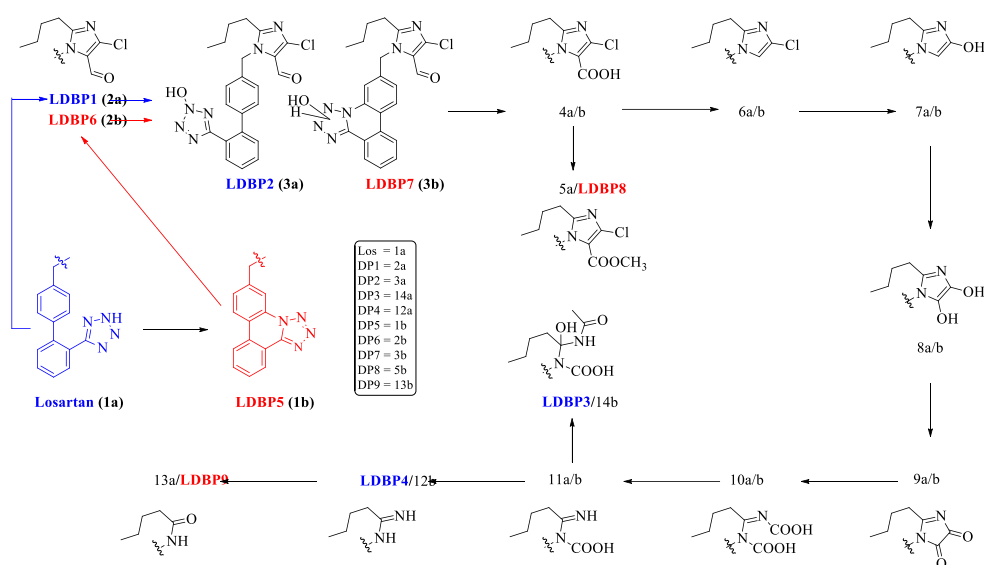


Figure 33 Degradation products isolated and those that could form but have not yet been isolated.

4.5.1.II Kinetics of Losartan degradation in PMS/Fe(II) system

To comprehensively investigate the oxidation process of LOS in the presence of the PMS/Fe(II) system, we initially examined the stability of the pollutant at pH 3 in the presence of Fe(II) sulfate heptahydrate, which resulted in only a 5% removal of the pollutant (Figure 34). Subsequently, another experiment was conducted to assess the degradation directly attributable to the oxidant PMS and was compared with the degradation in the presence of only Fe(II). In this experiment, the degradation halted

at 20%. This highlights the difference between the degradation in the presence of iron and the other solely in the presence of PMS.

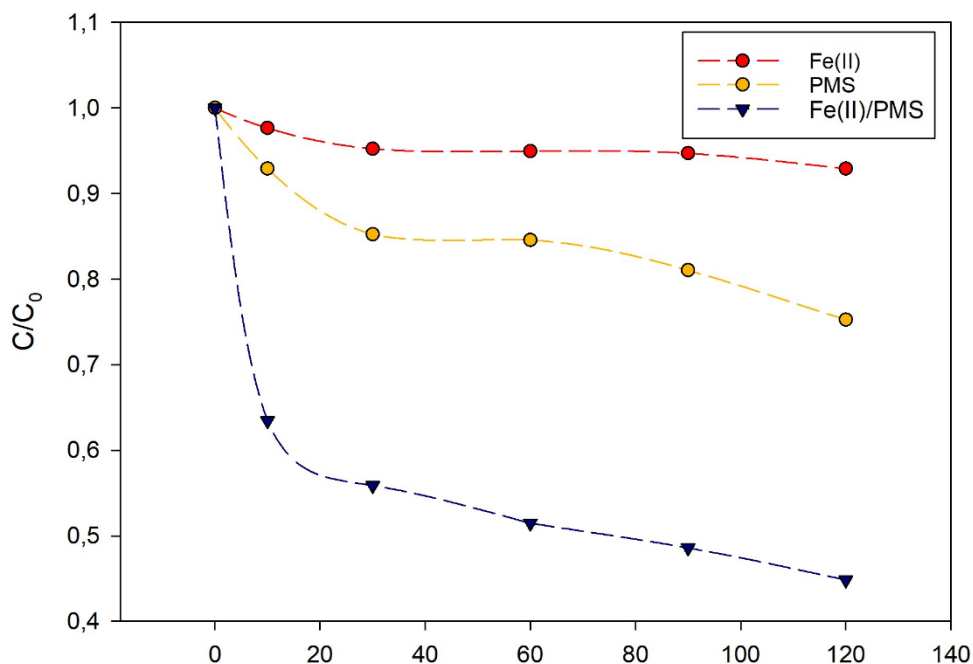


Figure 34 Different $[LOS] = 2.14 \text{ mM}$ removal in presence of $[Fe(II)] = 52 \text{ mM}$ and/or $[PMS] = 52 \text{ mM}$.

As evident from Figure 34, a synergistic effect arises from oxidation in the presence of the Fe(II) catalyst, likely due to the rapid reaction between Fe(II) and PMS, yielding highly reactive radical species such as $SO_4^{\cdot-}$.

Further studies were conducted to evaluate the degradation of Losartan as a function of temperature, conducting trials at 25 °C, 35 °C, and 45 °C (Figure 35). As anticipated, increasing the temperature resulted in a higher rate constant for k'_{LOS} , calculated as pseudo-first order decay using the following equation 3:

$$\ln \frac{[LOS]}{[LOS]_0} = -k'_{LOS} t \quad (1)$$

where $[LOS]_0$ and $[LOS]$ represent the initial and residual concentrations of LOS at time t , respectively, and k'_{LOS} is the pseudo-first-order apparent rate constant in s^{-1} .

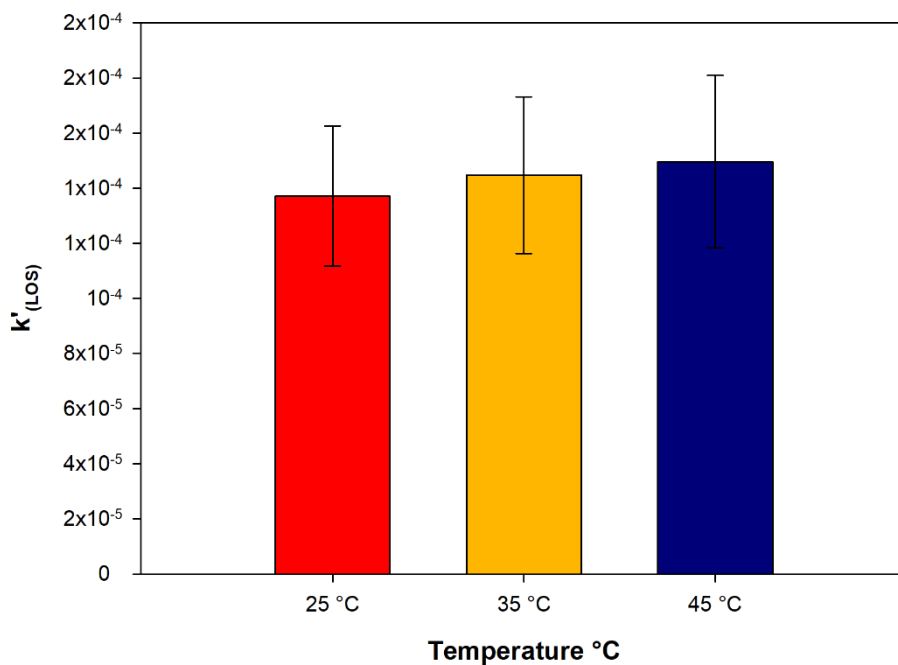


Figure 3511 Pseudo first order rate constant for k_{LOS} , at different temperature.

4.6 Methodology for Oxidation, Isolation, and Toxicological Profiling of Sartans Derivatives

4.6.1 Drugs and Reagents

Irbesartan (99.5%), Valsartan (98%), Losartan (98%) were purchased from Sigma Aldrich (Milan, Italy)., OXONE[®] (triple salt $\text{KHSO}_5 \times 0.5\text{KHSO}_4 \times 0.5\text{K}_2\text{SO}_4$, of which the oxidizing species is KHSO_5 called potassium monopersulfate or potassium peroxymonosulfate/PMS) and iron (II) sulfate heptahydrate ($\text{FeSO}_4 \times 7\text{H}_2\text{O}$) were purchased from Merck (Darmstadt, Germany). All the other chemicals and solvents were purchased from Fluka (Saint-Quentin Fallavier, France), of HPLC grade, and used as received. For the toxicity assessment, the reference toxicant (potassium dichromate) and salts used for the preparation of artificial freshwater ($\text{CaCl}_2 \times 2\text{H}_2\text{O}$, $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, KCl, NaHCO_3) were used. Double distilled water (Microtech) was used to prepare the dilution water and treatments.

4.6.2 Apparatus and Equipment

Normal-phase chromatography (NPC) was performed using silica gel 60 (0.015-0.040 mm) (Merck KGaA, Darmstadt, Germany) for column chromatography. Reversed-phase chromatography was carried out using a HPLC Shimadzu LC-8A system. The NMR spectra were recorded using a Varian Inova 500 MHz and referenced in ppm to the residual solvent signals. The mass spectrometric analyses were performed on a Voyager De Pro MALDI. UV vis spectra were recorded on a JASCO V-750 UV-Visible Spectrophotometer.

4.6.3 Preparative Irbesartan Chlorination Procedure and Product Isolation

The preparative chlorination of irbesartan was achieved by reacting 1.0 g of the active ingredient (2.33×10^{-3} M) with a 10% hypochlorite solution determined by iodometric

titration (Bedner and Mac Crehan, 2006). From the initial pH of 8.0, the solution reached pH 10.5 after 5 minutes and stayed stationary. The reaction was quenched after 2h with sodium sulfite, and the solution immediately extracted with ethyl acetate obtaining a fraction EA of 835 mg (Figure 36), that was chromatographed with the silica gel CC using a gradient of methylene chloride–methanol (100:0 to 30:70, v/v) to yield 17 fractions. The fraction EA7 (138 mg), eluted methylene chloride–methanol (95:5, v/v), was re-chromatographed on the silica gel CC by elution with a gradient of petrol ether–acetone (90:10 to 70:30, v/v) to yield 20 subfractions. The subfraction EA7.3 (30 mg), eluted with petrol ether–acetone (90:10, v/v), was separated in two subtractions using a preparative HPLC column eluting with a gradient of CH₃COONH₄ (pH 3.8; 20 mM) and methanol (30:70 to 0:100, v/v). The subfractions EA7.3.1 contained IDBP7 (1 mg). The fraction EA8 (101 mg), eluted with methylene chloride–methanol (90:10, v/v), was re-chromatographed with the silica gel CC by elution with a gradient of petroleum ether–acetone (90:10 to 70:30, v/v) to yield 9 subfractions.

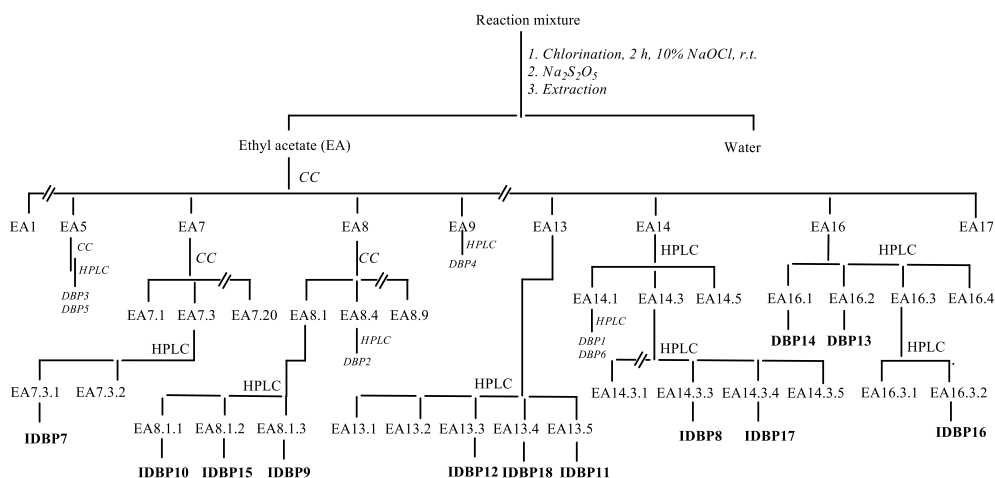


Figure 36 Isolation of 12 Irbesartan identified disinfection by-products.

The subfraction EA8.1 (30 mg), eluted with petrol ether–acetone (90:10, v/v), was purified by HPLC using a reversed phase column and elution with a gradient of $\text{CH}_3\text{COONH}_4$ (pH 4.0; 10 mM) and methanol (25:75 to 0:100, v/v), to yield 3 subfractions containing IDBP10, IDBP15, and IDBP9 (10, 3 and 2 mg, respectively). The fraction EA13 (37 mg), eluted with methylene chloride–methanol (70:30, v/v), was separated in 5 subfractions in the same HPLC conditions as EA7.3 obtaining IDBP12, IDBP18, and IDBP11 (4, 3, and 16 mg, respectively). The fraction EA14 (205 mg), eluted with methylene chloride–methanol (60:40, v/v), was re-chromatographed with the silica gel CC by elution with a gradient of methylene chloride–acetone (100:0 to 65:35, v/v) to yield 5 subfractions. The subfraction EA14.3 (23 mg) and EA14.4 (9 mg), eluted with methylene chloride–acetone (80:20, v/v), contained IDBP8 and IDBP17 (3 and 2 mg, respectively), which were purified by HPLC using a reversed phase column and elution with a gradient of $\text{CH}_3\text{COONH}_4$ (pH 4.0; 10 mM) and methanol (35:65 to 5:95, v/v). The fraction EA16 (37 mg), eluted with methylene chloride–methanol (50:50, v/v), was purified by a preparative HPLC in the same conditions as EA7.3 to yield 4 subfractions, of which the first two contained IDBP14 and the second IDBP13 (5 and 8 mg, respectively). The fraction EA16.3 (15 mg), containing IDBP16 (3 mg), was purified by a preparative HPLC in the same conditions as EA7.3.

4.6.4. Preparative Irbesartan Oxidation Procedure and Product Isolation

A 1.0×10^{-5} M solution of irbesartan was sonicated for 2 min and subsequently treated with $\text{Na}_2\text{CO}_3 \times 1.5\text{H}_2\text{O}_2$ (molar ratio of irbesartan/SPC at a 1:1 concentration) at room temperature. Subsequently, $\text{FeSO}_4 \times 7\text{H}_2\text{O}$ was added to the clear solution (molar ratio of SPC/ FeSO_4 1:1 concentration). The addition of $\text{FeSO}_4 \times 7\text{H}_2\text{O}$ catalyzes the oxidation reaction by SPC, through the formation of a series of radicals including, primarily, the $\text{HO}^\bullet$ species (Li et al., 2023). The progress of the reaction was monitored by HPLC, and the main disinfection by-products IDBP19–IDBP29 (Figure 37) were

identified by comparing their retention times with those obtained in the preparative experiments. In these latest experiments, irbesartan (0.4 g, 0.936 mmol) was dissolved in Milli-Q water (1.0 L) and the resulting suspension was sonicated for 2 min. The clear solution obtained was treated with $\text{Na}_2\text{CO}_3 \times 1.5\text{H}_2\text{O}_2$ (moles of irbesartan/moles of oxidant: 1/10), causing the pH to increase from 6.1 to 9.4. Immediately after, $\text{FeSO}_4 \times 7\text{H}_2\text{O}$ (moles of percarbonate/moles of sulphate: 1/1) was added to the solution. The solution, which immediately turned dark in colour, was left at room temperature with agitation for 30 min. Finally, the solution was quenched by methanol and extracted with ethyl acetate (Figure 37). The organic phase, dried over Na_2SO_4 , filtered, and ultimately evaporated using a rotary evaporator, contained unmodified irbesartan (161 mg). The aqueous phase was treated with 1 N HCl to lower the pH to 3.5 and subsequently extracted with ethyl acetate (EA). The organic phase (EA, 285 mg) was chromatographed on a silica gel column using a gradient of dichloromethane–methanol–acetic acid (94.5:5:0.5 to 74.5:25:0.5, v/v/v) to yield 7 fractions. The fraction EA2 (58 mg), eluted methylene chloride-methanol-acetic acid (94.5:5:0.5, v/v/v), was separated by semipreparative HPLC using a reversed-phase column Phenomenex Kromasil 10 μm 110 Å C18 (250 x 10 mm) eluted with a gradient of methanol and $\text{CH}_3\text{COONH}_4$ (Solution A, pH 4.0; 10 mM) (20:80 to 100:0, v/v) to yield 4 subfractions. The subfractions EA2.1 (17 mg) was chromatographed again by using the same previous column and under the same elution conditions to yield 3 subfractions. The subfractions EA2.1.2 and EA2.1.3 contained IDBP21 (1.1 mg) and IDBP27 (42.4 mg), respectively. The subfraction EA2.4 contained IDBP23 (2.1 mg). The fraction EA3 (31 mg), eluted methylene chloride-methanol-acetic acid (89.5:10:0.5, v/v/v), was separated by semipreparative HPLC using a reversed-phase column Phenomenex Gemini 10 μm 110 Å C18 (250 x 21.20 mm) and elution with a gradient of methanol and Solution A (10:90 to 100:0, v/v) to yield 5 subfractions. The subfraction EA3.2 contained IDBP20 (2.0 mg). The fraction EA5 (85 mg), eluted methylene chloride-methanol-acetic acid (79.5:20:0.5, v/v/v), was separated by semipreparative HPLC using a reversed-phase column Phenomenex Luna 5 μm 110

Å C18 (250 x 10 mm) and eluted with a gradient of methanol and Solution A (10:90 to 100:0, v/v) to yield 7 subfractions. The subfractions EA5.1, EA5.2, EA5.4 and EA5.6 contained IDBP19 (1.1 mg), IDBP22 (3.6 mg), IDBP24 (3.0 mg) and IDBP25 (4.6 mg), respectively.

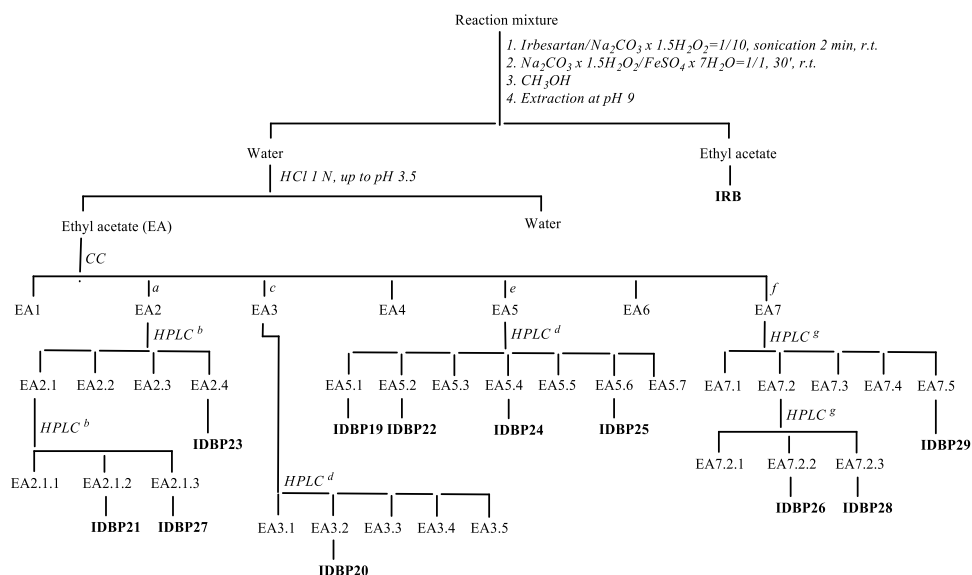


Figure 3712 Isolation of 11 Irbesartan identified disinfection by-products.

In the end, the fraction EA7 (68 mg), eluted methylene chloride-methanol-acetic acid (74.5:25:0.5, v/v/v), was separated by semipreparative HPLC using a reversed-phase column Phenomenex Gemini 10 μm 110 Å C18 (250 x 21.20 mm) and elution with a gradient of methanol and Solution A (60:40 to 100:0, v/v) to yield 5 subfractions. The subfractions EA7.2 (18.1 mg) was purified again by HPLC using the same previous column and under the same elution conditions to yield 3 subfractions. The subfractions EA7.2.2 and EA7.2.3 contained IDBP26 (2.1 mg) and IDBP28 (1.1 mg), respectively. The subfraction EA7.5 contained IDBP29 (16 mg).

4.5.5. Preparative Valsartan Oxidation (PMS) Procedure and Product Isolation

Preparative experiment was performing with a solution of VAL at a concentration of 10^{-3} M, specifically 525 mg (1.20 mmol) of VAL were dissolved in water until a final

volume of 0.5 L. OXONE[®] (9.20 g, 30 mmol, molar ratio VAL/OXONE[®] 1:25) was added under magnetic stirring at room temperature. Then was added iron (II) sulfate heptahydrate (8.30 g, 30 mmol, molar ratio Fe(II)/OXONE[®] 1:1). The pH of the suspension was adjusted to 7.0. Reaction was monitored taking an aliquot of the suspension every 15 min, that was quenched by methanol excess and dried under vacuum. The residue obtained was dissolved in a saturated sodium bicarbonate solution, extracted with ethyl acetate, and analyzed by HPLC. Thus, the reaction was stopped after 2h (so that the degradation of VAL and the formation of its VDBPs are maximum) with an excess of methanol and concentrated by lyophilisation. The residue was dissolved in water and pH-adjusted to 4.0, and this solution was extracted with ethyl acetate. The crude organic fraction (536 mg) was chromatographed on silica gel CC (Figure 38), eluted with a gradient of chloroform:methanol (99:1 to 70:30, v/v) to yield 8 fractions. The fraction Fr. 2 (38 mg), eluted with chloroform:methanol (97:3, v/v), was separated by semipreparative HPLC using a reversed-phase column Phenomenex Kromasil 10 μ m 100 Å C18 (250 $\times$ 10.00 mm) and eluted with a gradient of HCOOH (A, 0.1% in H₂O) and methanol (B), starting with 30% B for 5 min and followed by the installation of a gradient to obtain 100% B over 30 min, at a solvent flow rate of 5 mL/min to yield VDBP1 (10 mg) and VDBP14 (15 mg). The fraction Fr. 4 (51 mg), eluted with chloroform:methanol (95:5, v/v), was separated by semipreparative HPLC using a column Phenomenex Prodigy 10 μ m ODS (250 $\times$ 10.00 mm) and eluted with a gradient of A and B, starting with 40% B for 5 min and followed by the installation of a gradient to obtain 100% B over 30 min, at a solvent flow rate of 4 mL/min to yield VDBP2 (5 mg) and VDBP12 (21 mg). The fraction Fr. 6 (162 mg), eluted with chloroform:methanol (90:10, v/v), was chromatographed on silica gel CC, eluted with a gradient of dichloromethane:ethyl acetate (100:0 to 50:50, v/v) to yield 8 fractions. The fraction Fr. 6.2 (35 mg), eluted with dichloromethane:ethyl acetate (85:15, v/v), was separated by semipreparative HPLC using a reversed-phase column Phenomenex Luna 5 μ m C18 100 Å (250 $\times$ 10.00 mm), and eluted with HCOOH (A, 0.1% in H₂O) and acetonitrile (B), starting with 20% B

for 5 min and followed by the installation of a gradient to obtain 100% B over 30 min, at a solvent flow rate of 5 mL/min to yield VDBP3 (7 mg) and VDBP4 (6 mg). Fraction Fr. 6.4 (19 mg), eluted with dichloromethane:ethyl acetate (75:25, v/v), was separated by semipreparative HPLC using a reversed-phase column HPLC using the same column and the same solvents used for fraction Fr. 6.2, to yield VDBP10 (2 mg) and VDBP11 (1 mg). The fraction Fr. 7 (123 mg), eluted with chloroform:methanol (85:15, v/v), was chromatographed on silica gel CC, eluted with a gradient of dichloromethane:ethyl acetate (100:0 to 70:30, v/v) to yield 7 subfractions.

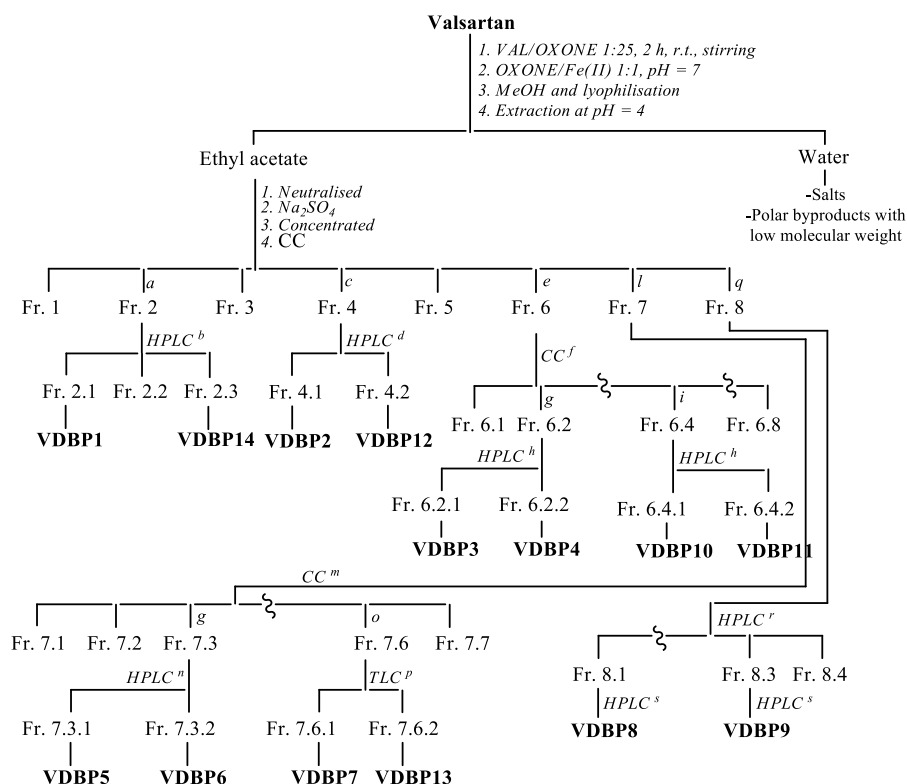


Figure 3813 The isolation of Valsartan different disinfection by-products.

The fraction Fr. 7.3 (61 mg), eluted with dichloromethane:ethyl acetate (85:15, v/v), was separated by semipreparative HPLC using a reversed-phase column Phenomenex Synergi 10 µm 110 Å C18 (250 × 21.20 mm) and eluted with HCOOH (A, 0.1% in

H₂O) and acetonitrile (B), starting with 5% B for 5 min and followed by the installation of a gradient to obtain 100% B over 30 min, at a solvent flow rate of 5 mL/min to yield DBP5 (6 mg) and DBP6 (11 mg). The fraction Fr. 7.6 (40 mg), eluted with dichloromethane:ethyl acetate (70:30, v/v) was chromatographed on TLC, eluted with dichloromethane:ethyl acetate (80:20, v/v) to yield DBP7 (11 mg) and DBP13 (22 mg). The fraction Fr. 8 (61 mg), eluted with chloroform:methanol (80:20, v/v), was separated by semipreparative HPLC using a column Phenomenex Prodigy 10 µm ODS (250 × 10.00 mm) and eluted with a gradient of CH₃COONH₄ (D, pH 4.0; 10 mM) and methanol (B), starting with 30% B for 5 min and followed by the installation of a gradient to obtain 100% B over 30 min, at a solvent flow rate of 4 mL/min to give 4 fractions. The fraction 8.1 was purified by HPLC using a column Phenomenex Synergi 10 µm 110 Å C18 (250 × 21.20 mm) and eluted with HCOOH (A, 0.1% in H₂O) and methanol (B), starting with 5% B for 5 min and followed by the installation of a gradient to obtain 100% B over 30 min, at a solvent flow rate of 5 mL/min to yield give DBP8 (7 mg). Using the same column and gradient from the fraction Fr. 8.3 purification, DBP9 (10 mg) was identified.

4.5.6. Preparative Losartan Oxidation Procedure and Product Isolation

Preparative experiments were conducted using a solution of LOS at a concentration higher than 10⁻³ M, specifically 495 mg (1.07 mmol) of LOS dissolved in water to a final volume of 0.5 L. OXONE[®] (8.20 g, 26 mmol, molar ratio LOS/OXONE[®] 1:24) was added under magnetic stirring at room temperature, followed by the addition of iron (II) sulfate heptahydrate (7.50 g, 26 mmol, molar ratio Fe(II)/OXONE[®] 1:1). The pH of the solution was adjusted to 3.0 with H₂SO₄ 1 M. The reaction progress was monitored by sampling the solution every 15 min, quenching with excess of methanol and drying under vacuum. The resulting residue was dissolved in a saturated sodium bicarbonate solution, extracted with ethyl acetate, and analysed by HPLC. The reaction was terminated after 2h to ensure maximal degradation of LOS and formation of its LDBPs, by quenching with excess methanol and concentrating by lyophilization.

The residue was dissolved in water and pH-adjusted to 7.0 before extraction with ethyl acetate

The organic fraction obtained from the preparative experiment (467 mg) was chromatographed with a gradient of chloroform/methanol (100:0 to 80:20) on silica gel to yield 9 fractions (Figure 39). Fraction 3 (Fr. 3), obtained from elution with chloroform/methanol 98:2, contained by-product LDBP6 (19 mg), which was further purified by HPLC under gradient conditions *f*: HCOOH (0.1% in H₂O) and acetonitrile (70:30 to 0:100) on Phenomenex Luna 5 μ m C18 100 Å (250 $\times$ 10.00 mm) HPLC column, at a solvent flow rate of 5 mL/min. Fraction 4 (Fr. 4), obtained from elution with chloroform/methanol 95:5, was subsequently purified via preparative thin-layer chromatography (TLC) eluted with dichloromethane:ethyl acetate 95:5, resulting in 4 subfractions. By-products LDBP8 (2 mg) and LDBP1 (10 mg) were obtained from subfractions Fr. 4.2 and Fr. 4.4, respectively, and purified using the same gradient *i* in HPLC, namely HCOOH (0.1% in H₂O) and methanol (80:20 to 0:100). Fraction 4.3 (Fr. 4.3) was purified by HPLC under gradient *j*: HCOONH₄ (pH 3.0; 15 mM) and acetonitrile (90:10 to 0:100) on Phenomenex Kromasil 10 μ m 100 Å C18 (250 $\times$ 10.00 mm) HPLC column, at a solvent flow rate of 5 mL/min, resulting in by-products LDBP2 (25 mg) and LDBP7 (12 mg). Fraction 5 (Fr. 5), obtained from elution with chloroform/methanol 90:10, was purified by HPLC under gradient conditions *h*: CH₃COONH₄ (pH 4.0; 10 mM) and methanol (70:30 to 0:100) using a column Phenomenex Prodigy 10 μ m ODS (250 $\times$ 10.00 mm) HPLC column, at a solvent flow rate of 5 mL/min, yielding by-product LDBP5 (18 mg). Fraction 8 (Fr. 8), obtained from elution with chloroform/methanol 85:15, was purified by preparative TLC under conditions *l*: dichloromethane:ethyl acetate (80:20), resulting in 5 subfractions. Subsequently, subfraction 8.1 (Fr. 8.1) was further purified by HPLC under gradient conditions *i*: HCOOH (0.1% in H₂O) and methanol (80:20 to 0:100), using a column Phenomenex Synergi 10 μ m 110 Å C18 (250 $\times$ 10.00 mm), at a solvent flow rate of 5 mL/min, yielding by-product LDBP3 (46 mg). Finally, fraction 9 (Fr. 9), obtained from elution with chloroform/methanol 80:20, was purified by HPLC

under gradient conditions *k*: HCOONH₄, (pH 3.0; 15 mM) and methanol (80:20 to 0:100) using the same column used for Fr. 5, yielding by-products LDBP4 (1 mg) and LDBP9 (1 mg).

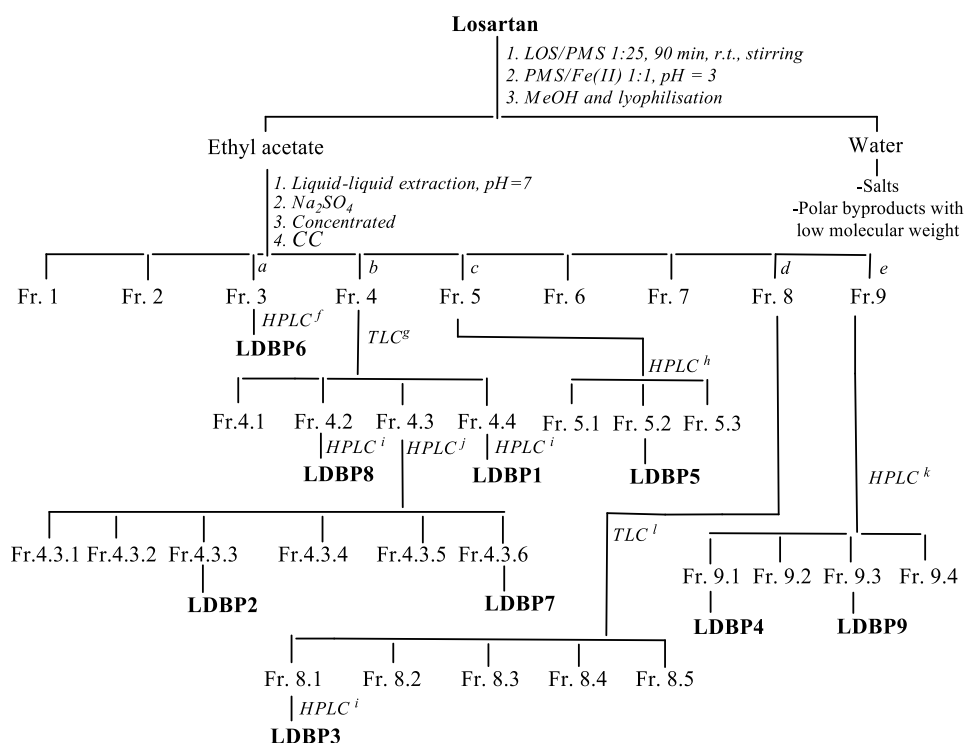


Figure 3914 . Isolation of the by-products LDBP1-LDBP7.

4.6.7. Ecotoxicity Assays Irbesartan

4.6.7.1 *D. magna* assay

The acute immobilization test with *D. magna* was carried out according to UNI EN ISO 6341:2013 (ISO, E., 2012). Daphnids, less than 24h old, were exposed to irbesartan and its ILDBPs, and immobility was assessed after 24h of exposure. This test was conducted in triplicate on 6-well plates, and immobilised organisms were counted under stereomicroscopy (Leica EZ4 HD, Germany). Toxicity was expressed

as the percentage of immobilised organisms relative to the total number of organisms exposed.

4.6.7.II *R. subcapitata* assay

The algal growth inhibition test involving *R. subcapitata* was performed following ISO 8692:2012 (ISO, I.S.O., 2012). Algal cells from a growing pre-culture were exposed to IRB and its IDBPs in triplicate, maintained under continuous illumination (intensity of 70 $\mu\text{mol}/\text{m}^2/\text{s}$), and stirred under orbital shaking at 50 rpm, maintained at a temperature of 23 ± 1 °C, with a 24-hour light cycle averaging an intensity of 70 $\mu\text{mol}/\text{m}^2/\text{s}$. After 72h, the growth rate of the algae was determined, and the inhibition rate of each solution was calculated by comparing it to the growth rate of the control group. The inhibition percentage for each treatment replicate was calculated using the formula: $I(\%) = ((\mu_0 - \mu_T)/\mu_0) * 100$. Here, I (%) represents the percentage of inhibition of the average specific growth rate, μ_0 denotes the mean value for μ in the control (control growth rate), and μ_T represents the value for the growth rate in the treatment replicate. Negative values indicate a stimulation of growth rather than inhibition.

4.6.7.III *A. fischeri* assay

For the acute bioluminescence inhibition assay, the bacterium *A. fischeri* (NRRL-B-11177) was employed, and the test was performed using a Microtox Model 500 analyzer, following international procedure ISO 11348-3:2007 (ISO, 2007). The bacteria were rehydrated with a reconstitution solution and exposed to solutions of IRB and its IDBPs at 15 °C. The bioluminescence intensity was measured after 30 min. This test was conducted in triplicate, incorporating both negative and positive controls. The pharmaceutical and toxic effects were expressed as the ratio of the decrease in bacterial light production to the remaining light.

4.6.7.IV Predicted toxicity data through ECOSAR analysis

ECOSAR program (version 1.11) developed by the USEPA was employed to predict the ecotoxicity of irbesartan (IRB) and its by-products generated by sodium percarbonate. This program enabled us to estimate their hazard indices concerning green algae, daphnia, and fish. The acute toxicity of IRB and its by-products is presented in terms of LC₅₀ values, indicating the concentration at which half of the fish and daphnia populations are lethally affected after 96h and 48h of exposure, respectively. Furthermore, EC₅₀ values were determined signifying the concentration at which the growth of 50% of green algae is inhibited after 96h of exposure. This study also encompassed the prediction of chronic toxicity values (ChV) of IRB and its oxidation by-products on green algae, daphnia, and fish using this same program.

4.6.8. Ecotoxicity Tests Valsartan

For toxicity tests, valsartan (VAL), its derivative products (DBPs, from DBP1 to DBP9) and the mixture of all residues (MIX) were dissolved in 100% DMSO, sonicated for 30 min to obtained stock solutions and stored in the dark at 4 °C. DBP10 and DBP11 were not tested for their toxicity because of their low yield. Each test sample was obtained by mixing the proper volume of the stock solution and specific synthetic media for the considered organisms.

4.6.8.I Acute toxicity tests

B. calyciflorus was used for acute toxicity assay (ASTM E 1440–91, 2004) hatching organisms from cysts, purchased by MicroBioTest Inc. (Nazareth, Belgium), in a synthetic freshwater (hardness: 80-100 mg/L CaCO₃; pH 7.5±0.3) at 25±1 °C, under continuous light at 3000–4000 lux for 16-18h. The tested concentrations of compounds went from 0.03 to 200 mg/L using a geometric progression equal to 3.2. Then, test compounds were put in 36-well plate (0.3 mL/well) and five rotifers were transferred to each test well, in six replicates for each concentration. Both negative control (NC, only the synthetic freshwater) and solvent control (DMSO 0.9% v/v related to the maximum concentration of compounds tested equal to 200 mg/L) were

performed. Plates were incubated for 24h at 25 °C in the dark. The mortality was considered as endpoint and calculated by comparing the number of rotifers in the test dilutions of each compound with those of negative control to determine the effective percentages.

4.6.8.II Chronic toxicity tests

Chronic toxicity assays were performed on the green alga *R. subcapitata* as well as on the rotifer *B. calyciflorus*.

Algal growth inhibition test was performed according to the OECD 201 (2011) and Nugnes et al. (2023). The algae were obtained from laboratory mass culture starting from algal beads obtained by MicroBioTest Inc. (Nazareth, Belgium). 104 cells/mL algal suspension were incubated with the test compound solutions (from 0.30 µg/L to 100,000 µg/L, with a dilution factor of 3.2, in six replicates for each concentration tested) in 96-well plates, at 6,000 lux and at 25±1 °C on a microplate shaker (450 rpm). The absorbance was read at 450 nm using a microplate reader (Synergy H1, Biotek, Winooski, USA) at time zero and after 24, 48 and 72 h. Both NC (the synthetic medium) and solvent control (DMSO 0.4% v/v related to the maximum concentration of compounds tested equal to 100 mg/L) were performed.

Rotifer offspring reduction test was carried out in line with ISO 20666 (2008) on organisms hatched from cysts and less than 2h old. One rotifer per well was transferred in 48-well plates containing 0.9 mL/well of test compounds solutions (from $4.2 \cdot 10^{-5}$ –500 µg/L, using a dilution factor of 3.2, eight replicates for each concentration tested). The rotifers were fed with 100 µL of 107 cells/mL of *R. subcapitata* and then incubated in the dark at 25 °C for 48 h. Both negative control (the synthetic freshwater) and solvent control (DMSO 0.002% v/v related to the maximum concentration of compounds tested equal to 500 µg/L) were performed.

4.6.8.III Data analysis

For both acute and chronic toxicity assays, three experiments were performed, and the effect percentages were calculated by comparing the results of compounds to each specific negative control. Each effect percentage was pooled to estimate the concentrations determining X% effect (L(E)Cx) by non-linear regression (log agonist vs. normalized response-variable slope) using Prism9 software (GraphPad Inc., CA, USA).

The median lethal concentration (LC₅₀) was the endpoint of rotifer acute toxicity, while EC₅₀, EC₂₀, and EC₁₀ were the concentrations giving respectively 50%, 20% and 10% inhibition of reproduction of rotifers or the inhibition of growth in algae. *Unpaired t-test* was performed using Prism9 software to estimate the statistical difference among solvent and negative controls.

4.7 Conclusions

In conclusion, the presence and persistence of sartans in surface and wastewater continue to cause considerable concern, rightfully placing them among the so-called “emerging micropollutants.” The research conducted, starting from the observation of widespread environmental contamination, has highlighted the urgency of developing treatment strategies capable not only of removing the original compound but also of carefully assessing the formation of any degradation products.

In the case of Losartan, the use of advanced oxidation processes such as Fe(II)-activated peroxymonosulfate enabled significant degradation of the contaminant, isolating and characterizing nine degradation by-products by NMR and mass analysis. These by-products, obtained in overall percentages of 27% relative to the starting product and with a total mineralization of 64%, allowed for a better understanding of the transformation mechanisms of Losartan and structurally related molecules,

indicating that these processes do not simply lead to total mineralization, but also to the formation of intermediates with possible environmental implications.

A similar result was found in the case of Valsartan, whose oxidation with peroxymonosulfate-activated Fe(II) produced fourteen by-products, five of which had never been reported before. In this scenario, about 34% of the compound remained unchanged, while 26% were transformed into identifiable degradation products. Toxicity analyses have shown that although the original molecule and its derivatives are not particularly impactful to algae, the estimated chronic concentration for rotifers, comparable to that found in European waters, raises concerns. In some cases, a hormetic response has been observed, indicating that, at low levels of exposure, the compound or its derivatives may even stimulate biological functions rather than inhibit them.

The treatment of Irbesartan with chlorination, adopted in many sewage treatment plants, showed an even more complex picture. 12 new disinfection by-products were isolated and determined, 9 of them for the first time, and formation mechanisms were hypothesized. Some of these DBPs were found to be more toxic than the parent compound. Although the mineralization achieved was considerable (75%), the presence of by-products with toxicity higher than the original contaminant warns of the potential impact of such treatments on the ecological balance.

The use of sodium percarbonate as an alternative oxidant drastically changed the environmental fate of Irbesartan. Compared with chlorination, more of the unaltered compound was recovered (41% vs. 13%) and eleven DBPs were isolated (8 for the first time), but with lower mineralization (40% vs. 75%). Although the total number of by-products was reduced compared with chlorination, their percentage amount compared with starting Irbesartan was higher. Toxicity assays showed variable effects, confirming that biological responses cannot be easily predicted based on chemical and physical properties alone.

This set of studies demonstrates that different oxidation processes (from chlorination to peroxymonosulfate-activated or sodium percarbonate treatments) significantly influence both the degree of sartan removal and the spectrum and toxicity of degradation products generated. As a result of this research, it is now possible to outline more sustainable and informed treatment strategies that can not only reduce the concentration of these compounds, but also minimize the formation of harmful by-products, thus contributing to the protection of water quality and human and ecosystem health.

Chapter 5 Ecotoxicological Impact Assessment and Remediation Techniques for Olive Mill Wastewater

5.1 Introduction

The olive oil industry is one of the oldest and most traditional sectors in the Mediterranean basin, with a history that dates back millennia (Langgut et al., 2019). This industry is not only of great economic importance but also holds significant cultural and social value for many communities (Uduji & Okolo-Obasi, 2019). According to the International Olive Council (IOC, 2022), olive oil production for the 2020/2021 season was around 3.1 million tons, with 63% of this amount produced by European Union countries. Spain is the world's largest producer, accounting just under 15% of the global share on average (Mili & Bouhaddane, 2021). Italy follows as the second-largest producer with an average share of 8.8%, then Greece and Tunisia both at 7.4%, Turkey at 6.6%, and Portugal at 4.2%. Other significant non-Mediterranean producers include Argentina, Australia, California, Chile, Peru, and South Africa.

However, olive oil production generates significant quantities of wastewater, known as olive mill wastewater (OMWW) or vegetation water (Zahi et al., 2022). In fact, the continuous two- and three-phase systems, which have replaced the traditional discontinuous process for olive oil extraction, generate two primary wastes: brownish-black water (OMWW) and a solid waste called pomace (Asses et al., 2009; Obied et al., 2005). Every year, the Mediterranean area alone produces between 7 and 30 million cubic meters of wastewater (Kavvadias et al., 2010).

The indiscriminate release of untreated wastewater poses a significant threat to natural ecosystems, negatively affecting microorganisms, plants, and animals (Aggelis et al., 2003; Roig et al., 2006). Despite seasonal production and lower quantities compared to other sectors, these effluents represent a significant environmental challenge due to their complex composition and difficult management (Al-Bsoul et al., 2020).

Vegetation waters are characterized by a high organic load (including long-chain fatty acids, lipids, simple and complex sugars, proteins; in addition, high levels of polyphenols and tannins), a significant content of suspended solids, inorganic potassium salts, calcium, sodium, magnesium, and iron (Albuquerque et al., 2004; Crognale et al., 2006), and notable acidity. All factors that contribute to its high ecotoxicity.

In particular, the high organic content is mainly responsible for the recalcitrant nature of wastewater (Tsioulpas et al., 2002). Consider also that phenolic compounds, including hydroxytyrosol, tyrosol, vanillic acid, and others, vary widely depending on factors such as olive cultivar, maturity, climatic conditions, storage time, and extraction process (Allouche et al., 2003; Di Meo et al., 2022; Kallel et al., 2009; Roig et al., 2006). On the other hand, it has been experimentally proven that some bioactive molecules extracted from OMWW can be used to produce functional feed and thus represent a possible source of circular economy from an environmental sustainability perspective. Some research indicates that diet supplemented with phenolic compounds from OMWW influences plasma fatty acid composition in lactating Holstein-Friesian cows. Indeed, a close relationship has been demonstrated between the fatty acid composition of the diet and the plasma lipid content of cows with a significant impact on polyunsaturated fatty acids, with an increase in Omega-6 compared to Omega-3, improving the metabolic performance of cows to support high levels of milk production (Di Meo et al., 2023). Likewise, OMWW represent a valuable source of phytotoxic compounds, that could be used as potential bioherbicides or antioxidant and which may have food applications Ladhari et al., 2022).

The fact remains that treating wastewater before releasing it into the environment is a critical concern for a long time (Domingues et al., 2021; Jamrah et al., 2023; Paraskeva & Diamadopoulos, 2006).

Therefore, despite the health benefits of olive oil consumption, such as the reduction of risk factors for coronary heart disease, the prevention of various cancers, and the modulation of immune and inflammatory responses (Bendini et al., 2007; Tuck & Hayball, 2002; Visioli & Galli, 2002; Zafra et al., 2006), its production process presents serious environmental challenges.

Standard ecotoxicological tests, mainly using *A. fischeri* and *D. magna*, show high sensitivity to OMWW, with severe inhibition of luminescence and growth even at significant dilutions (Mekki et al., 2008; Paixao et al., 1999). Furthermore, the use of vegetation waters as a fertilizer can impact soil microbial communities and plant growth, causing changes in microbial populations and reduced enzymatic activity (Mekki et al., 2006; Saadi et al., 2007). These effects highlight the need for effective treatment strategies to mitigate the environmental impact of OMWW.

To address the environmental impact of wastewater, this study explores various treatment methods and their effectiveness in reducing wastewater toxicity. Simple physical processes such as pH treatment, flocculation, combined pH treatment and flocculation, adsorption, oxidation, denaturation and polymerization of the aromatic fraction have been used to treat wastewater. After the treatment, the study evaluated the reduction in toxicity levels, the efficiency of contaminant removal, and the overall improvement in water quality. The ecotoxicological evaluation on the 17 aqueous solutions/suspensions isolated from raw OMWW made it possible to measure the degree of toxicity expressed in terms of EC₅₀ through bioluminescence and root elongation inhibition assays using *A. fischeri* and *Lepidium sativum*, respectively.

5.2 Results

In line with sustainable wastewater resource management, developing efficient treatment techniques is crucial for reducing wastewater pollution loads and considering their reuse. In this study different physicochemical separation techniques have been utilized to treat raw OMWW, including A) pH treatment, B) flocculation,

C) pH treatment and flocculation, D) adsorption, E) Oxidation, F) Denaturation, G) Polymerization of the aromatic fraction (Table 10).

Table 10 Different treatments on olive mill wastewater.

Entry	Treatment	Conditions
1	Olive Mill Wastewater	
A	pH treatment	
2	HCl	99:1 (v/v)
3	H ₂ SO ₄	99:1 (v/v)
4	KOH	25:1 (v/v)
B	Flocculation	
5	Polyaluminium chloride (PAC, 10%)	125:1 (v/v)
6	FeSO ₄	125:1 (v/w)
7	FeSO ₄	250:1 (v/w)
8	FeSO ₄ + Ca(OH) ₂ (37%)	250:1:6 (v/w/v)
C	pH Treatment and flocculation	
9	Ca(OH) ₂ (37%) up to pH 7 + PAC (10%); pH 4	125:3:1 (v/v/v)
D	Adsorption	
10	Zeolite	50:1 (v/w)
11	Vermiculite	50:1 (v/w)
12	Spent Coffee Grounds (SCG)	50:1 (v/w)
13	Wood ash	50:1 (v/w)
E	Oxidation	
14	NaClO 6%	25:2 (v/v)
15	Na ₂ S ₂ O ₈	100:1 (v/w)
F	Denaturation	
16	NaCl	40:1 (v/w)
17	Acetone	15:1 (v/v)
G	Polymerization of the aromatic fraction	
18	H ₂ CO 37%	50:1 (v/v)

The OMWW was characterized by the following parameters: chemical oxygen demand (COD), biological oxygen demand (BOD₅) and total suspended solids (TSS) (Table 11). Other parameters investigated in this study, essential for characterizing OMWW collected after the treatments reported in Table 10, include measurements of oil and grease, phenols (using the Folin-Ciocalteu method), and chlorides. OMWW is characterized by the predominance of polyphenolic compounds, responsible for the typical dark color of the solutions used in this study, which constitute the main cause of toxicity.

The pollutant load expressed in terms of chemical oxygen demand ranged from 20.4 up to 72.3, highest value recorded for Entry 17. The main characteristics of the raw OMWW, identified as Entry 1, were: 68.0 g /L of COD, 1.81 g /L of BOD₅, and 2.71 g /L of TSS. Remaining concentrations of oil and grease, phenols, and chlorides are 1.05 g /L, 7.2 g GAE /L, and 310 mg /L, respectively. The treatment using calcium hydroxide (Ca(OH)₂, 37%) and polyaluminum chloride (PAC, 10%) as flocculant (Entry 9), following the lowering of pH from 7 to 4, led to important reductions in organic matter (70% COD, 50.3% BOD₅, 70.1% TSS, 78.1% oil and grease and 70.8% phenolic compounds), when compared to all other physicochemical separation techniques. The treatment involving flocculation using iron (II) sulfate (FeSO₄) and Ca(OH)₂ (37%) also shows an important reduction of organic matter, evidenced by values close to those recorded for the treatment that couples' pH and flocculation both (Entry 8). In this case the most significant values refer to COD (30.6 g /L), BOD₅ (0.81 g /L), TSS (1.22 g /L), and phenolic compounds (3.8 g GAE /L). The content of oils and grease is the lowest recorded among the 18 samples with a value of 0.13 g /L. Finally, as regards chlorides, the high values refer to entries 2, 13, 14, and 16 with concentrations of 725, 2437, 1632, and 5233 mg /L respectively.

Table 11 COD, BOD and TSS measurements for aqueous solutions/suspensions obtained from different OMWW treatments.

Entry	Chemical Oxidation	Biological Oxidation	Total Suspended
	Demand (COD)	Demand (BOD)	Solids (TSS)
	g /L	g /L	g /L
1	68.0	1.81	2.71
2	57.1	1.82	1.03
3	56.7	1.87	1.56
4	68.0	1.82	2.53
5	42.1	1.12	1.68
6	40.8	1.09	1.63
7	40.4	1.05	1.43
8	30.6	0.81	1.22
9	20.4	0.90	0.81
10	60.5	1.75	2.53
11	60.4	1.81	2.65
12	65.0	1.85	2.30
13	52.0	1.49	2.48
14	55.0	1.53	2.43
15	55.1	1.72	2.70
16	67.5	1.82	2.71
17	72.3	1.50	2.41
18	69.0	1.43	2.63

Table 12 Measurements of Oil and Grease, Folin Ciocalteu, and Chlorides for the aqueous solutions/suspensions obtained from the different treatments of OMWW.

Entry	Oil and Grease	Folin Ciocalteu	Chlorides
	g /L	g GAE /L	mg /L
1	1.05	7.2	310
2	0.85	5.6	725
3	0.97	5.5	310

4	0.91	7.0	313
5	0.65	4.4	310
6	0.63	4.3	310
7	5.43	4.2	310
8	0.13	3.8	310
9	0.23	2.1	310
10	0.96	5.3	310
11	0.97	6.5	310
12	0.98	7.5	310
13	0.71	3.8	2437
14	0.82	4.2	1632
15	1.02	6.8	312
16	1.06	7.2	5233
17	1.02	6.5	310
18	1.04	6.3	310

The olive mill wastewater, just collected from the mill, were allowed to ferment in an open container at room temperature and under a slight flow of air. After 14 days, a copious, dark precipitate with an oily and nauseating smell was observed. The overlying aqueous phase was considered as the starting solution (Table 10, Entry 1).

The change in pH of a wastewater can be used as a pretreatment procedure for the removal of suspended or colloidal matter from wastewater. This is done by adjusting the pH to values of 2-4 through the addition of acids (e.g. HCl, H₂SO₄, HNO₃), or increasing it to 11 through the addition of caustic substances such as KOH, NaOH or Ca(OH)₂. The reduction of pH as a potential pretreatment for wastewater has received little attention so far (Mitrakas et al., 1996). However, by increasing the concentration of hydrogen ions, the negative surface charge of suspended hydrophilic colloids is reduced, leading to their neutralization and destabilization. Indeed, treatment with acids (Entries 2 and 3), followed by gravity settling for at least 48h, resulted in the formation of a precipitate consisting of flakes and sludge, with an aqueous upper part

containing about 1% olive oil. It is worth noting that the pH reduction also contributes to the acid hydrolysis of oils into fatty acids, which could be easily separated from the effluents. On the other hand, increasing the pH as a pretreatment step before evaporation of wastewater helps to retain a higher amount of volatile organic compounds in the solid fraction during evaporation. In our case, treatment with KOH (Entry 4) only caused a slight precipitate.

Flocculation is the technique by which an agent is added to water (wastewater) to induce precipitation, transforming dissolved chemicals into a solid, insoluble form that precipitates. It is known that during storage, olive mill wastewater undergoes natural self-purification through spontaneous flocculation/denaturation of proteins. The suspended material already present in the wastewater interacts with the help of long-chain proteins that act as flocculating agents. Individual particles come together to form flocks, which become denser than the surrounding medium and settle. The first flocculant used is Polyaluminum chloride (PAC). It is a newly conceived flocculant, and its specific properties derive from the action of its basic active component, polyaluminum chloride. It is a polynuclear complex of polymerized hydroaluminum ions with the following formula: $[Al_2(OH)_nCl_{6-n}]_x$. PAC is supplied in the form of an aqueous solution or powder, facilitating transportation, storage operations, and reagent dosing. It has been used alone (Entry 5) or after treatment with $Ca(OH)_2$ (Entry 9) and subsequent pH lowering to around 4.

Ferrous sulphate ($FeSO_4$), the most economical among coagulants, was then used at two different w/v ratios (Entries 6 and 7). Considering that it is suitable for water with a high pH and a sufficient O_2 content, necessary for the oxidation of ferrous hydrate into ferric, which is insoluble, the pH of the treated water was raised by adding $Ca(OH)_2$ (Entry 8).

Adsorption is a physicochemical process in which compounds dissolved in polluted waters are adsorbed onto the surface of a solid substance (adsorbent) and within its pores. In general, the adsorption process has proven impractical due to the high costs

of the commonly used adsorbent, such as activated carbon, the impossibility of reusing it, and the low quantity of absorbed products. In our case, we simulated a fluidized bed process where the suspension obtained after adding the adsorbent to olive mill wastewater is placed in slow but constant agitation. Specifically, the adsorbents used are zeolites (Entry 10), vermiculite (Entry 11), spent coffee grounds (Entry 12), and wood ash (Entry 13). Treatments with zeolite and wood ash are characterized by very low sedimentation rates due to their fine particulate nature. Vermiculite, after treatment, is neither reusable nor combustible but could be recycled as inert material for concrete. Otherwise, it must be disposed of in specific landfills for construction material waste. Instead, zeolites, spent coffee grounds, or ash could be dispersed in the environment, considering their nearly neutral composition that allows their reuse as natural fertilizers. They constitute a valuable source of mineral salts for plants and polyphenolic organic material that could counteract the settlement and growth of fungi, insects, mites, nematodes, etc. In fact, the organic compounds (adsorbates) that can be removed from wastewater are primarily colouring substances (mainly tannic acids), difficult or non-biodegradable pollutants, bactericidal or inhibitory compounds. The use of sodium hypochlorite as a disinfectant for wastewater treatment has always been effective and widely adopted over the years, mainly due to economic reasons. However, it can become problematic if, for any reason, it meets a concentration of organic matter that is too high. This is precisely the case with olive mill wastewater, which contains large amounts of sludge and suspended solids that drastically reduce the disinfection effectiveness of sodium hypochlorite (Entry 14), as those involved in wastewater reclamation are aware. At the same time, disinfection by-products such as chloramines develop due to the reaction between ammonia in the wastewater and the chlorine released from sodium hypochlorite. While chloramines are potential disinfecting compounds, they tend to persist in water longer, making them toxic to aquatic fauna (Parveen et al., 2022). The use of potassium persulfate (PS) (Entry 15) as an oxidizing agent for wastewater treatment offers a valid alternative to conventional solutions such as sodium

hypochlorite. PS has one of the highest standard reduction potentials in the peroxide family, with a potential equal to 2.01 V. PS is widely used, even in industrial settings, for polymerization reactions, pool clarification, and hair decolorization (Block et al., 2004). One of the distinctive features of PS is its ability to minimize the formation of undesired by-products during the disinfection process. Compared to chloramines generated by the reaction between ammonia and chlorine, the oxidation products of PS are characterized by greater stability and lower persistence in the treated water. Its effectiveness, combined with the production of more manageable by-products, makes it a valuable ally for those involved in the management and reclamation of wastewater.

A high salt concentration can induce the precipitation of proteins (salting out) from an aqueous solution. One disadvantage of this protein purification technique is that, in addition to precipitating the specific protein of interest, any impurities and contaminants are also precipitated, which, in these cases, have no weight (Entry 16). Proteins are macroions, and their attractive force is inversely proportional to the dielectric constant of the dispersing medium. Those who work with proteins know that organic solvents lower the dielectric constant of water, reducing protein solubility. Thus, solvents such as methanol, ethanol, and especially acetone can be used for the almost complete precipitation of protein material (Entry 17).

Different is the treatment with formaldehyde (Entry 18). The reactivity of formaldehyde with polyphenolic molecules is known in the literature (Vera et al., 2021; Garro et al., 1996), and thanks to these reactions it is possible to form molecules that can also be used as adhesives. The formation of these higher molecular weight molecules can lead to a decrease in their solubility in water and consequent precipitation from solution. Since vegetation waters are strongly rich in aromatic compounds, the aim was to verify whether their polymerization and subsequent precipitation were achieved.

5.2.1 Ecotoxicological Assessment

The short-term toxicity of raw OMWW (Entry 1) and OMWW after treatment (Entries 2-18) was assessed using the marine bacteria *A. fischeri* and seed *L. sativum*. Table 4 presents the toxicity results (EC_{50}), obtained when exposing *A. fischeri* to olive mill wastewater samples. Extremely low EC_{50} values were recorded, ranging from 0.014% to 0.095%. The highest toxicity reduction was observed in Entries 8 and 18 with EC_{50} values of 0.812% and 0.183%, respectively. As reported by (Babić et al., 2019), the estimated EC_{50} for *A. fischeri* on raw OMWW (Entry 1) is 0.24%, therefore OMWW samples of Entry 16 show a more marked inhibition of bioluminescence, in terms of EC_{50} , compared to this threshold value (Figure 40a). Only in the case of Entry 8 there is no toxicity ($EC_{50} = 0.812\%$), while Entry 18 reflects the data provided by Babic et al. Therefore, our experiments have highlighted that treatment with $FeSO_4$ and $Ca(OH)_2$ (37%) (Entry 8), is the most effective in reducing OMWW toxicity towards marine bacteria such as *A. fischeri*.

Table 13 EC values and corresponding 95% confidence intervals for 50% inhibition levels. EC values are expressed in %.

Entry	<i>A. fischeri</i> EC_{50} (% of dilution)	CI 95% (inf.)	CI 95% (sup.)
1	0.045	0.023	0.089
2	0.034	0.028	0.041
3	0.031	0.012	0.082
4	0.033	0.027	0.040
5	0.068	0.031	0.148
6	0.065	0.036	0.118
7	0.063	0.016	0.237
8	0.811	0.111	5.905
9	0.025	0.014	0.048

10	0.037	0.026	0.054
11	0.089	0.081	0.099
12	0.031	0.023	0.041
13	0.014	0.005	0.040
14	0.095	0.065	0.140
15	0.060	0.043	0.084
16	0.025	0.010	0.058
17	0.075	0.026	0.215
18	0.183	0.107	0.311

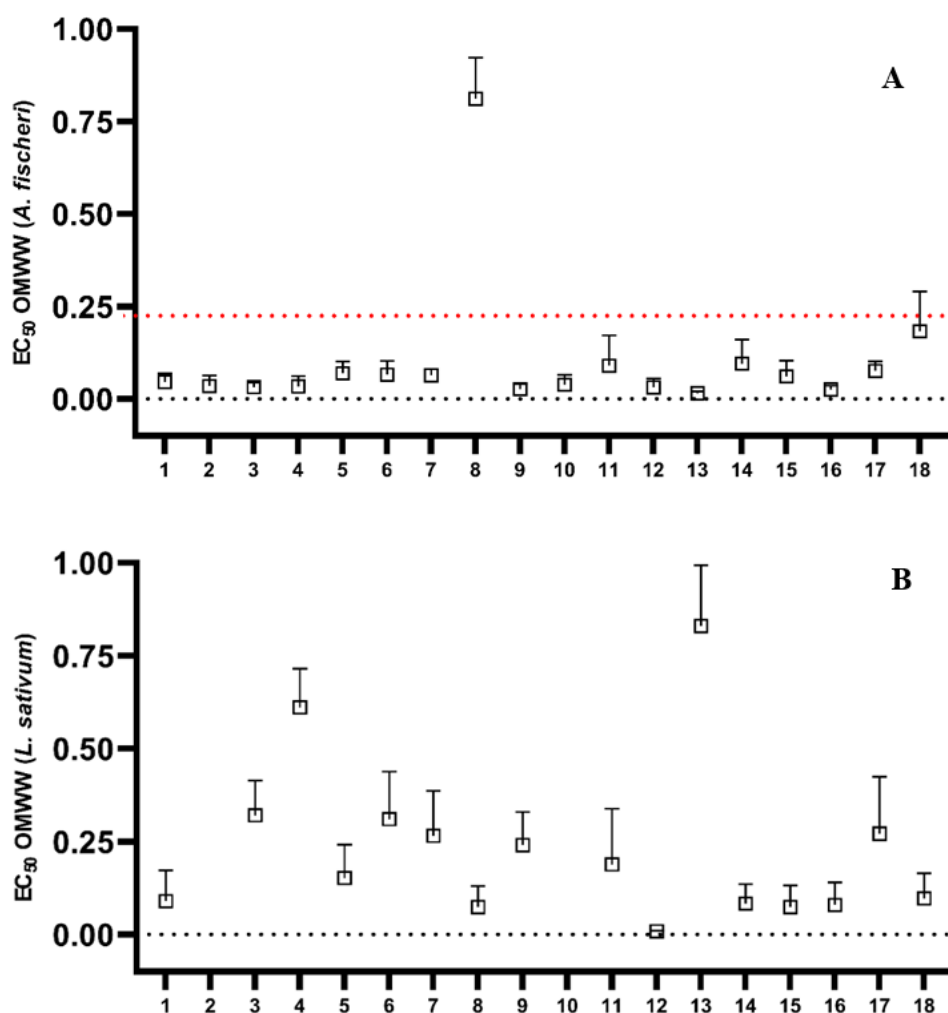


Figure 4015 Boxplot showing a) ecotoxicity, represented as EC_{50} of inhibition of bioluminescence on *A. fischeri* and b) phytotoxicity, represented as EC_{50} of root elongation inhibition on *L. sativum* (Entries 2 and 10 are not indicated in the graph).

To better focus on the possible toxicity effects of OMWW samples on garden cress seed germination, the primary root length was measured at the end of the treatment (72 h). Phytotoxicity was expressed as root elongation endpoint that appeared to be a better indicator to determine toxicity of raw and treated OMWW samples. Subsequently the ecotoxicity values were expressed as EC_{50} (Figure 40b). The test

carried out with raw OMWW supernatant (Entry 1) showed a strong root elongation inhibition given that the EC_{50} is equal to 0.09%. The OMWW treated with physicochemical methods, specifically Entries 3, from 5 to 8, 9, 11 and from 14 to 18, can be considered equally phytotoxic as our experiments demonstrated that the EC_{50} values were approximately 4 orders of magnitude higher to Entry 1 and do not exceed 0.50%. Unlike the tests conducted with *A. fischeri*, in this case the reduction in toxicity can be identified with Entry 4, which use potassium hydroxide (KOH), by exploiting variations in pH, and with Entry 13 which consider the absorption on wood ash. In this chemical and ecotoxicological data case, the phytotoxicity highlighted with *L. sativum*, expressed in terms of EC_{50} , was sensibly reduced, going from 0.09% for the raw OMWW to 0.61% and 0.83% for Entries 4 and 13 at the end of the respective treatments.

A biplot summarizing PCA results concerning is shown in Figure 41. As can be observed from the graph below, the variables referring to the EC_{50} of the two bioindicators used to measure the toxicity of the olive mill wastewaters are not correlated with the chemical variables obtained from the various treatments. However, some of the chemical parameters investigated in this study show a correlation between them, in particular chemical oxygen demand is strictly associated with the presence of phenolic compounds and total suspended solids.

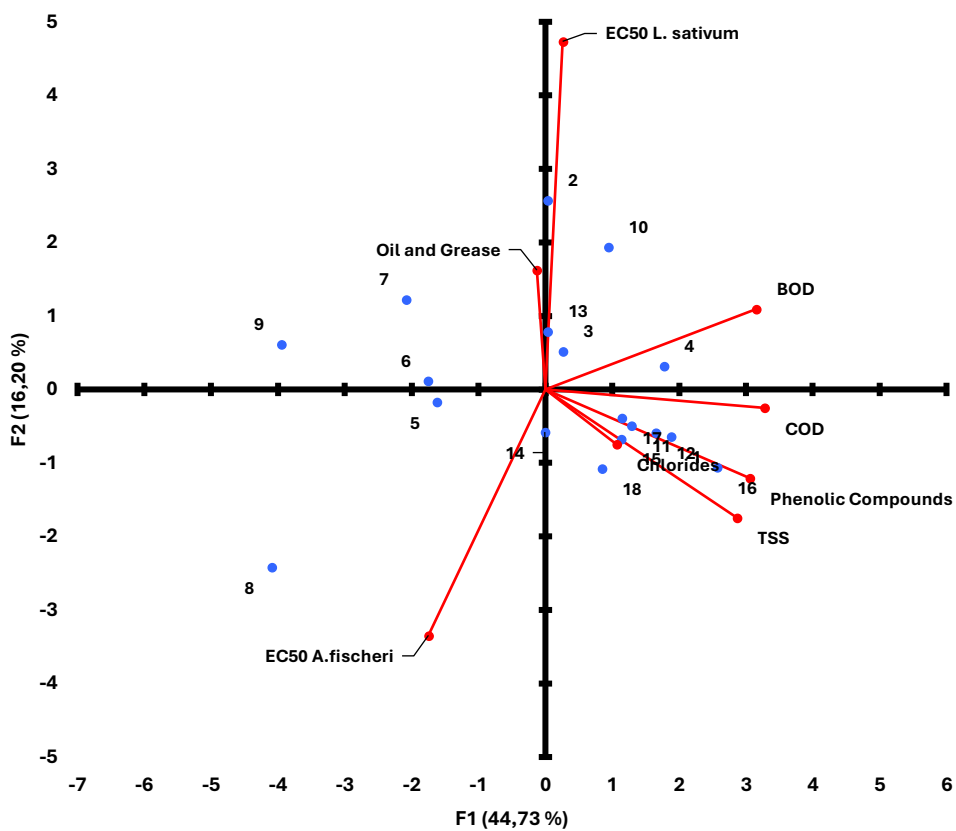


Figure 4116 Principal component analysis (PCA) on chemical and ecotoxicological parameters of OMWW samples from Entries 1–18.

5.3 Materials and Methods

5.3.1 OMWW Samples

Olive mill wastewater samples (25 L) were collected in October 2022 in Melizzano (Campania, Italy) and allowed to ferment in an open container, at room temperature, and under a slight flow of air. After 14 days, there was an abundant precipitate (consisting mainly of cellulose, high molecular weight polyphenols, and pectins), dark, with an oily and nauseating odour, separated by centrifugation. The supernatant aqueous phase is the starting solution/suspension for the considered treatments.

5.3.2 Drug and Reagents

HCl, H₂SO₄, KOH, FeSO₄, Ca(OH)₂, Zeolite, Vermiculite 2-3 mm, NaClO 6%, K₂S₂O₈, NaCl, and Folin-Ciocalteu reagent were purchased from Sigma Aldrich (Milan, Italy) and used without further purification. Polyaluminium chloride 10% w/w was purchased from Biosynth Ltd (London, United Kingdom). Acetone, formaldehyde, and other solvents were purchased from Merck KGaA (Darmstadt, Germania). Wood ashes were obtained through the controlled combustion of hardwood in locally available kilns, sieved to attain a uniform particle size distribution. Spent coffee grounds were sourced from local coffeehouse, collected post the brewing process. To maintain consistency in composition, SCG from a singular coffee blend was exclusively utilized in this study.

Lyophilized bacteria were activated by adding a reconstitution solution (Aqua Science BioLight Recon Solution). A 2% NaCl solution was used for bacterial regeneration and sample dilution and as a control. The osmotic control was made using an osmotic adjusting solution (OAS) of 22% NaCl.

5.3.3 Analytical Measurements

Chemical Oxygen Demand was analysed by CNR-IRSA method 5135, with hatch cuvette by the Reactor Digestion Method (HACH LANGE S.r.l.); Biochemical

Oxygen Demand analysis via O₂ pressure difference (APHA 5210B): Measure initial/final O₂ after inoculation to assess organic matter decomposition by BOD EVO Sensor. Total Suspended Solids TSS measurement was analysed by APHA 2540 D. Grease and oil measurement was analysed by APHA 5520 B. Chloride measurement (APHA 4500-Cl- E) with hatch cuvette by thiocyanate method.

5.3.4 Quantification of the Polyphenolic Fraction Using the Folin Ciocalteu (FC) Method

Total phenolic content was analysed on filtered samples using the Folin-Ciocalteu spectrophotometric method (Box, 1983). Precisely, 0.25 mL of Folin-Ciocalteu reagent and 0.75 mL of a saturated sodium carbonate solution (220 g /L, 2.08 M) were introduced to 5 mL of the wastewater sample. The mixture was allowed to react for 60 minutes in darkness at 20 °C. Subsequently, the UV–Vis spectrophotometer measured the absorbance of the resulting blue solution at a wavelength of 750 nm. The total phenolic content was expressed as gallic acid equivalents (g GAE /L). Ultraviolet-visible (UV-Vis) spectra were recorded using a JASCO V-750 UV–Visible Spectrophotometer.

5.3.5 Microtox Assay with *A. fischeri*

The ecotoxicity of olive mill wastewater samples was assessed following luminescent bacteria tests according to the UNI-EN ISO 11348-3 (ISO, 2007). The endpoint measured in the Microtox assay was the decrease in the intensity of light emitted by the luminescent marine bacteria, *A. fischeri* (NRRL-B-11177), after 30 min of exposure to several dilutions of olive mill wastewater samples (10^{-1} – 10^{-6}). For the final analysis, only the data from the 30 min exposure were reported due to the negligible difference in toxicity between results from different exposure durations. Tests were conducted in duplicate. The averages of bacteria luminescence were calculated and then used to determine the effective concentration that inhibits 50% of bacteria luminescence (EC₅₀). The toxic effect values reflect the ratio of the decrease in bacterial light production to the remaining light.

5.3.6 Germination Test and Root Elongation Evaluation

Seeds of garden cress (*L. sativum*), purchased from Microbiotests (Gent, Belgium), were used for evaluating the effects of OMWW samples on seed germination and root elongation (Co-operation & Development, 2006).

Seeds were sown in 90 mm diameter Petri dishes that contained one sheet of Whatman No. 1 filter paper as support and 3 mL of test solutions. Ten garden cress seeds were placed in each Petri dish. After 72h of incubation in a growth chamber in darkness at 25 ± 1 °C, the biometric indices (number of germinated seeds, length of total plantlet and length of root) were obtained through imaging and data processing by an imaging analysis software (ImageJ 1.54 g). The Germination Index (GI) was calculated as shown in equation (1) by multiplying the germinated seed number (G) and length of roots (L) and expressed as percentage (GI%) with respect to the control as follows:

$$GI (\%) = (L_s \times \%G_s) / (L_c \times \%G_c) * 100 \quad (1)$$

where: L_s : length of roots (mm) in seeds treated with compost extract sample; G_s : number of seeds treated with compost extract sample that germinated; L_c : length of roots (mm) in seeds treated with water (control); G_c : number of seeds treated with water (control) that germinated.

5.3.7 Statistical Analyses

The trace element dataset was analysed using principal component analysis (PCA) (Jolliffe, 2002). The primary goal of PCA is to reduce the dimensionality of a dataset consisting of many interrelated variables while preserving as much variation as possible. This is achieved by transforming the variables into a new set of variables, known as principal components (PCs), which are orthogonal to each other and ordered in such a way that the amount of variation from the original variables decreases as you move down the order of components. A PCA, based on the Pearson correlation matrix, was used to investigate the relationships between the independent variables (chemical and ecotoxicological data) in the 18 samples of OMWW. All statistical

analyses were performed using XLSTAT V. 2016.02.27444 and GraphPad Prism V.8.4.2 (679).

5.4 Conclusions

The results of this study demonstrated the effectiveness of various physicochemical and oxidative approaches in the treatment of olive mill wastewater (OMWW). Treatments employing the combined use of coagulants/flocculants, particularly calcium hydroxide and polyaluminium chloride (Entry 9), as well as iron sulfate with calcium hydroxide (Entry 8), proved to be the most efficient in reducing COD, BOD₅, total suspended solids (TSS), and fats and oils. These treatments achieved a significant reduction in the organic and toxicological load of OMWW.

Oxidative treatments, while less effective in reducing the organic fraction, showed interesting potential for toxicity reduction, particularly with sodium persulfate (Na₂S₂O₈) and formaldehyde. Formaldehyde treatment exhibited a high capacity for stabilizing aromatic and phenolic compounds, resulting in a substantial reduction in ecotoxicological impact.

From an ecotoxicological perspective, the treatments that achieved the most significant reductions in organic compounds also led to a marked decrease in toxicity, as demonstrated by tests on *A. fischeri* and *L. sativum*. This underscores the importance of addressing both the reduction of key chemical parameters and the decrease in ecological toxicity to ensure sustainable wastewater treatment.

In conclusion, the tested treatments offer promising solutions for the sustainable management of OMWW, with potential applications for reuse in agricultural settings or for discharge into natural environments, in line with current regulations. However, further studies are needed to optimize treatment effectiveness under different operational conditions and to assess the long-term potential of these treatments at a real-scale application.

Chapter 6 Pollutant Reduction Through Non-Destructive On-Line Inspection of Products from the Tanning Industry

6.1 Introduction

The industrial innovation is a key factor in the sustainable and competitive development of manufacturing companies. In the current landscape of digital and environmental transition, industry faces complex challenges—not only in adopting new technologies but also in evolving toward a more regenerative, circular, and sustainable model. Here, automation and advanced technologies are not solely directed at production efficiency but are also focused on enhancing social and environmental well-being (Directorate-General for Research and Innovation et al., 2022).

From this perspective, the implementation of non-destructive, online-controllable techniques for monitoring production processes could reduce waste—not only in the final product but also in the overall environmental impact of the industry, including reductions in electricity and water waste. This would lead to optimized processes across the board.

6.2 Leather Tanning Industry

In the leather tanning industry, tanning is a critical stage in the transformation of animal hides, as it imparts durability, resistance to external agents, and rot resistance to the material (Manzo, 1999; Ding et al., 2022). Precise control of tanning agents throughout this process is crucial, not only to ensure the quality of the final product but also to minimize environmental impact. Specifically, the use of non-destructive and inline techniques to monitor tanning agents in drums during the tanning phase

represents an advanced and sustainable technological solution. Such techniques optimize the use of chemicals, reduce waste, and minimize pollutants.

The tanning process alone involves a variety of reagents, including transition metal salts (Li et al., 2003; Wang et al., 2021), aldehydes, and tannins (Hassan et al., 2023; Ammenn et al., 2015), which interact to form various chemical bonds with the collagen in the skin's dermis. These interactions form the basis of different types of leather, each with unique physical and mechanical properties (Covington & Wise, 2019). A key aspect across all tanning types is achieving uniform penetration and fixation of the tanning agent within the tissue. This process must be carefully monitored to avoid material waste and prevent the release of potentially harmful substances into the environment.

The growing emphasis on sustainability and resource optimization in the tanning sector is driving the development of innovative process control methodologies. The adoption of non-destructive techniques enables real-time verification of the distribution and fixation of tanning agents within hides, minimizing excess reagent use and improving waste management. These technological tools not only enhance leather quality but also represent a significant step toward reducing the environmental impact of the tanning industry, making the process more sustainable and safer.

Various tanning types use different reagents, resulting in distinct leather characteristics. Essentially, tanning involves forming cross-links between multiple polypeptide chains; thus, the reagent must have properties that allow it to chemically bond with the collagen fibrils in the dermis. Given the variety of binding sites in collagen, different tanning methods with various tanning agents are possible. Tanning agents can be classified based on the type of bond they form with collagen:

- **Coordination bonds:** chromium, zirconium, iron, titanium, aluminum, zeolites
- **Covalent bonds:** formaldehyde, glutaraldehyde (and other aldehydes)

- **Hydrogen and dipolar bonds:** vegetable and synthetic tannins

It is therefore important to consider that leather stability mainly depends on factors such as the number of cross-links and bond strength (Maina et al., 2019). Regardless of the reagent type, tanning comprises two key phases: tanning agent penetration and its fixation. Fixation tends to hinder diffusion; thus, achieving a uniform distribution of the tanning agent throughout the leather initially requires conditions of minimal fixation.

For example, in coordination bond tanning, the fixation site consists of carboxylic functions on the collagen's side chains. In an acidic environment, these are protonated and exhibit low affinity for the binding metal, allowing deeper penetration into the dermis (Manzo, 1999). Once uniform tanning agent distribution within the hide is achieved, fixation can be completed by adjusting the pH. In mineral salt tanning, for example, increasing the pH deprotonates the carboxylic groups on collagen's side chains, enhancing their affinity for metals between the polypeptide chains.

The high usage of chemicals required in hide transformation processes inevitably produces potentially polluting waste, which must be carefully monitored to ensure environmental compliance and sustainability within the sector (Ahmed et al., 2021). In this context, the application of advanced analytical techniques proves essential for controlling and characterizing wastewater and tanning processes. Key attributes that make Near-Infrared (NIR) Spectroscopy particularly suitable for these applications include its rapid execution, non-destructive nature, capacity for online monitoring using fiber optics and portable sensors, and sustainability, as it does not require chemical solvents or reagents.

6.2 Near Infrared Spectroscopy (NIR)

The so-called optical radiation covers a broad range of wavelengths from 100 nm to 1 mm. This range is divided into different regions: ultraviolet (UV) radiation spans from 100 to 380 nm, visible radiation extends up to 780 nm, and above that lies

infrared (IR) radiation. Infrared radiation itself is subdivided into regions: Near Infrared (NIR) from 780 nm to 2.5 μm , Mid Infrared (MIR) from 2.5 μm to 25 μm , and Far Infrared (FIR) up to 1000 μm . The signals observed in NIR spectra are closely related to bands that mainly correspond to overtones and combinations of fundamental vibrations (Blanco & Villarroya, 2002).

In light of the increasing demand for industrial improvements, particularly within the European community, Near Infrared (NIR) Spectroscopy is well-suited to support these process enhancement goals in industry. NIR spectroscopy is an emerging analytical technique, with highly effective qualitative and quantitative applications that are rapidly gaining popularity, especially in production control. Its appeal lies in its low cost and the fact that it requires no sample preparation. Currently, NIR spectroscopy is widely used for both inline and laboratory quality control in the agro-food (Hetta et al., 2017; Vincent & Dardenne, 2020) and pharmaceutical industries (Amigo et al., 2008; Swarbrick, 2014), and even for waste sorting, as it can distinguish different types of plastics through chemometric databases, facilitating their separation (Zheng et al., 2017).

For instance, NIR spectroscopy can assess moisture content in agricultural products (Lin et al., 2019) and monitor blood oxygenation levels (H. Liu et al., 1995), as the lower-frequency wavelengths used in NIR can penetrate deeper into the skin (Marin & Moore, 2011). In recent studies, coupling NIR with hyperspectral cameras and chemometric methods has even enabled non-destructive, quantitative assessment of collagen content in archaeological artifacts. This allows for strategic sampling points for radiocarbon analysis (Naito et al., 2020). In other cases, NIR spectroscopy has been shown to predict quality parameters in cattle leather (Braz et al., 2018).

6.3 Chemometrics and Multivariate Methods

The information contained in NIR spectra often requires the use of multivariate analysis techniques to correlate spectroscopic features with macroscopic factors, such

as concentration. This approach enables both qualitative and quantitative assessment of samples. NIR spectral information typically appears in broad, frequently overlapping bands, which complicates direct interpretation. In such cases, chemometric techniques facilitate simpler and, in some cases, nearly autonomous interpretation of spectra, providing an optimal sample classification. One example of commonly used chemometric techniques is Principal Component Analysis (PCA). PCA is one of the most widely applied statistical methods across scientific disciplines. It reduces the number of parameters in a sample, such as those within a spectrum, by condensing them into one or more principal components. These components retain the main information, or variance, of the samples, allowing faster and more straightforward interpretation via two- or three-dimensional graphs (Abdi & Williams, 2010; Bro & Smilde, 2014). Specifically, PCA transforms samples into a new set of orthogonal variables, or principal components, that reveal similarities among samples as points on a plane (Greenacre et al., 2023). Thus, PCA efficiently extracts the most relevant information, compressing and simplifying it for easier analysis of data structures.

A practical example is the differentiation of wine samples using PCA or other multivariate methods based on a simple IR/UV analysis (Ranaweera et al., 2021; Yu et al., 2017). This technique is applicable to various datasets, including NMR data (Stoyanova & Brown, 2001), metal concentration data (De Andrade Passos et al., 2010; Loska & Wiechuła, 2003), and reaction parameters (Isaac et al., 2014; Biddau et al., 2024).

Partial Least Squares Regression (PLSR) is a powerful multivariate regression method used to develop predictive (quantitative) models that, for example, relate spectra generated by spectrometers to response data previously obtained through other analytical techniques. PLS regression constructs models that link multiple data groups, such as X and Y (Wold et al., 2001). Here, the X group represents a set of variables describing a system (e.g., an NIR spectrum), while Y represents the

characteristics or properties of the system to be predicted. PLS models create predictive systems even with significant background noise, by generating “latent variables” from the X group, which best predict the Y group, thereby reducing data quantity and complexity (i.e., for incomplete or highly correlated data).

Numerous studies in the literature demonstrate PLSR's applications for quantifying a variety of sample types, from the concentration of illicit drugs in unknown samples (Groberio et al., 2014), to acetone (Ramírez-Sánchez et al., 2024), and mapping soil salinity (Farifteh et al., 2007). PLSR applications to NIR hyperspectral cameras have also enabled the quantitative spatial mapping of collagen on archaeological artifacts (Malegori et al., 2023), chemical parameters in fish muscle (Cheng & Sun, 2016), as well as protein content and hardness in wheat (Mahesh et al., 2014).

6.4 Spectral Pretreatment

In NIR (Near Infrared) spectra, various data pretreatment techniques are applied prior to multivariate analysis to enhance signal quality and reduce noise or unwanted variation. These pretreatments make the models more robust and accurate:

- Normalization: This process scales all data to a common range, typically between 0 and 1, to minimize potential differences in NIR signal amplitudes caused by systematic influences.
- Derivatives (First and Second): Derivatives highlight rapid signal changes and suppress background noise, thereby improving the resolution of absorption bands.
- Standard Normal Variate (SNV): SNV normalizes each spectrum individually by subtracting the mean of the spectral points. This reduces variability not directly related to composition but rather due to scaling and offset effects from physical variations in the spectrum.

6.5 Statistical Validation of Models

The validation of a chemometric method is essential to evaluate its robustness. Commonly used techniques for model validation include cross-validation, with internal validation methods such as Cross-Validation (CV), primarily through the Leave-One-Out (LOO) method (Riedl et al., 2015), also referred to as leave-n-out depending on the number of samples used to form a validation subset. In this approach, samples are separated into subsets. Specifically, in the LOO method, *n* subsets are created for *n* samples. Each subset is used to assess the predictive capability of the chemometric model on the sample excluded from the training set, with all other points serving to evaluate the excluded sample. The leave-one-out strategy is thus a form of cross-validation that involves the rotational exclusion of each individual observation.

6.6 Performance Indicators

To evaluate PLSR-based chemometric models, several key statistical indicators are used to assess the model's suitability and correlation parameters, including the coefficient of determination (R^2) and root-mean-square error metrics, such as those estimated through calibration (RMSEC) and cross-validation (RMSECV) (Cheng & Sun, 2016; Riedl et al., 2015). The R^2 value, or goodness of fit, is calculated as the squared correlation coefficient between observed and modeled values. An R^2 value above 0.91 signifies an excellent model. Conversely, lower RMSE values indicate that the model has minimal error relative to actual values, suggesting better predictive capability (Cheng & Sun, 2016).

The formulas for these indicators are as follows:

$$R^2 = 1 - \frac{\sum_{i=1}^n (y_i - \hat{y}_i)^2}{\sum_{i=1}^n (y_i - \bar{y})^2}$$

$$Rcv^2 = 1 - \frac{\sum_{i=1}^n (y_i - \hat{y}_{cv_i})^2}{\sum_{i=1}^n (y_i - \bar{y})^2}$$

$$RMSEC = \sqrt{\frac{\sum_{i=1}^n (y_i - \hat{y}_i)^2}{n}}$$

$$RMSEC = \sqrt{\frac{\sum_{i=1}^n (y_i - \hat{y}_{cv_i})^2}{n}}$$

where:

- y_i is the observed value,
- $\hat{y}_i$ is the predicted value from the model,
- $\bar{y}$ is the mean of the observed values,
- n is the number of observations, and
- $\hat{y}_{cv_i}$ represents values predicted during cross-validation.

6.7 Materials and Methods

The tanning agents were sourced directly from tanneries and used as-is for analysis. Each sample was homogenized by stirring for a few minutes before measurement, and samples were prepared by weighing and mixing in 3% formic acid solutions to simulate the actual concentrations and pH conditions in the tanning drum (Manzo, 1999). Each sample was then placed in a Suprasil© cuvette with a 2 mm optical path, and measurements were taken with an OnSite-W microNIR sensor, which includes a dedicated accessory for liquid sample acquisition.

The spectra were processed and manipulated using the VIAVI MicroNIR™ Pro Software Suite v3.2, which includes an integrated chemometric system for spectral

manipulation and the construction of analytical methods and PLSR models. Spectra were pretreated using SNV and second derivative transformations via the Savitzky-Golay filter to enhance interpretability for PLSR modeling.

The tanning agents analyzed included chromium(III) hydroxide sulfate, condensed natural tannin from mimosa, synthetic naphthalenesulfonic tannin, zeolite tanning agents (70% Type A zeolite and 30% aluminum sulfate mix), and a 15% glutaraldehyde solution in methanol. Concentrations were expressed in g/L to standardize and compare data against tanning drum concentrations.

6.8 Results and Discussion

Multivariate methods were applied to assess chemometric control of the concentrations of five tanning agents at concentrations typical of those used in tanning drums (150 g/L) down to the minimum achievable concentrations (10 g/L). Model performance was assessed through performance indicators and internal validation using leave-one-out cross-validation, which calculated RMSEV (root mean squared error of validation), RMSEC (root mean squared error of calibration), R^2 , and Rcv^2 .

The PLSR method was applied across the full instrument wavelength range (908–1676 nm), with performance indicators detailed in Table 14.

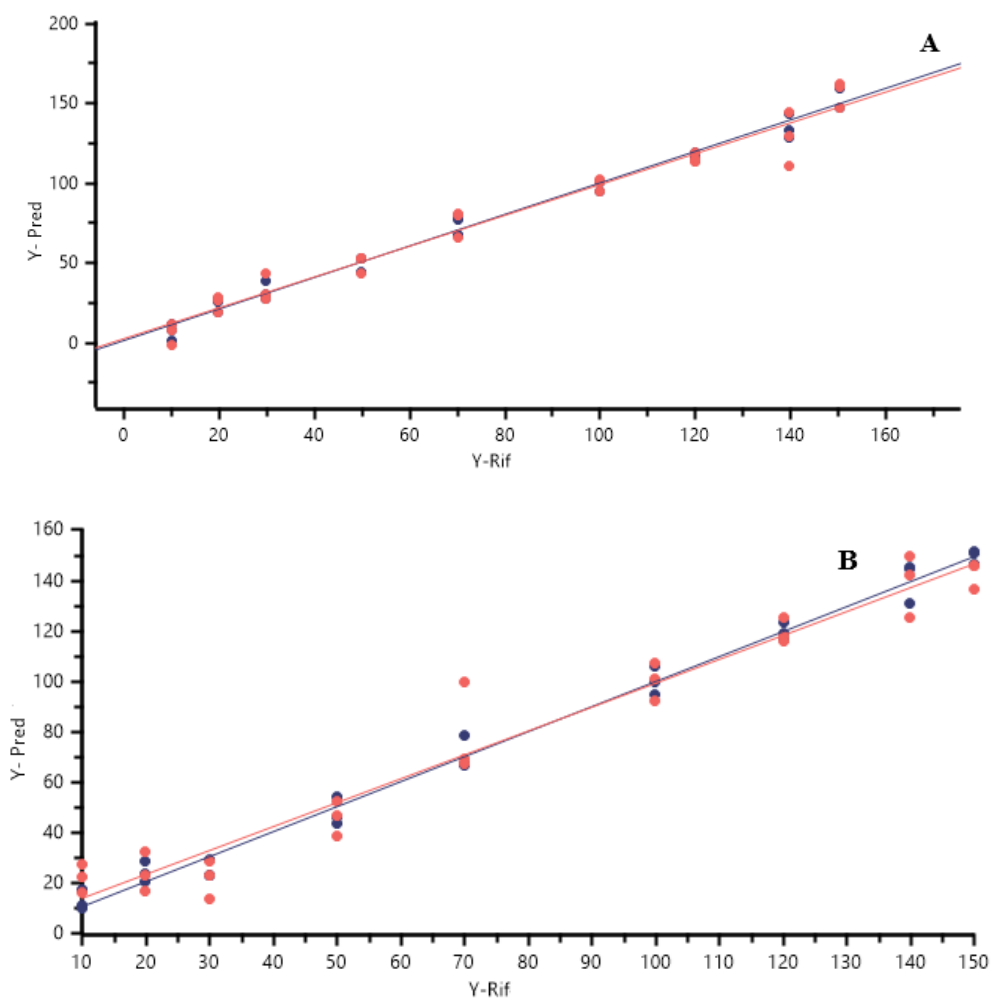
Table 14 Performance Indicators for PLSR method.

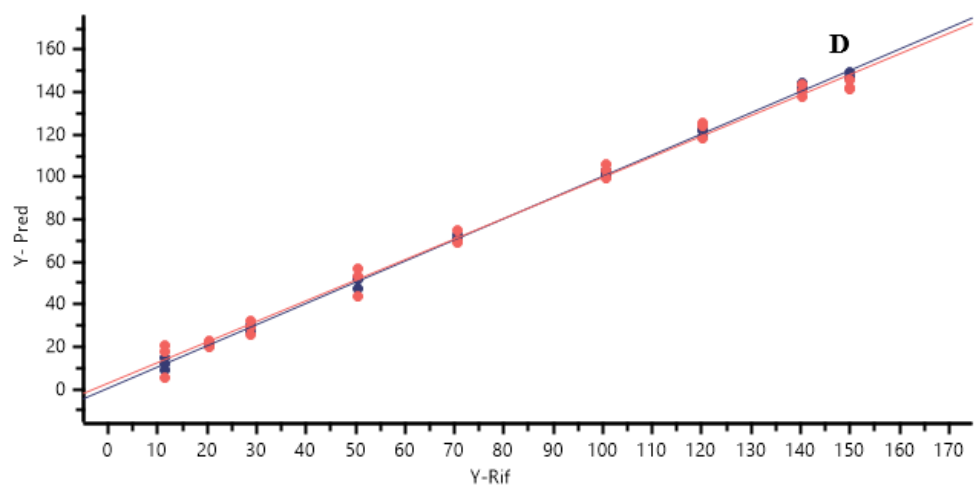
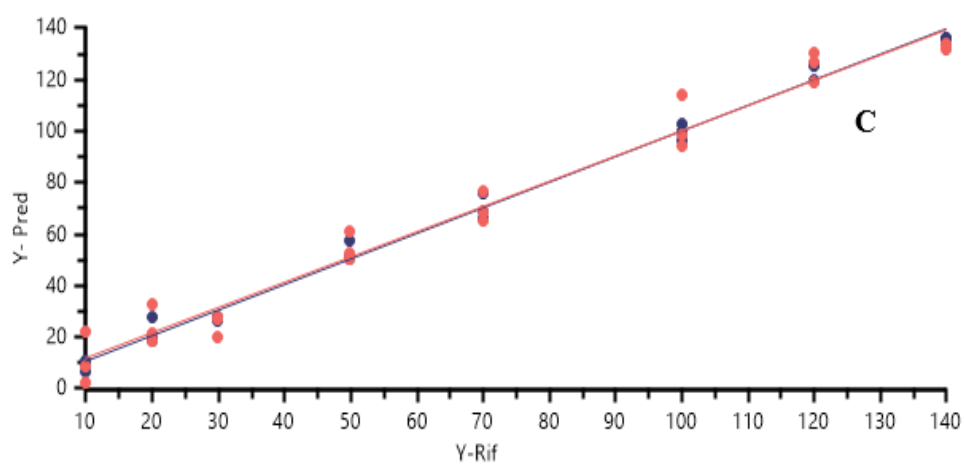
	Chrome	Glutaraldehyde	Mimosa	Syтан	Zeolites
n. samples	29	27	24	27	24
R²	0.987	0.990	0.968	0.996	0.985
Rcv²	0.969	0.959	0.945	0.991	0.934
RMSEC	5.52	4.95	8.03	2.92	5.51
RMSEV	8.55	10.04	10.53	4.98	11.56

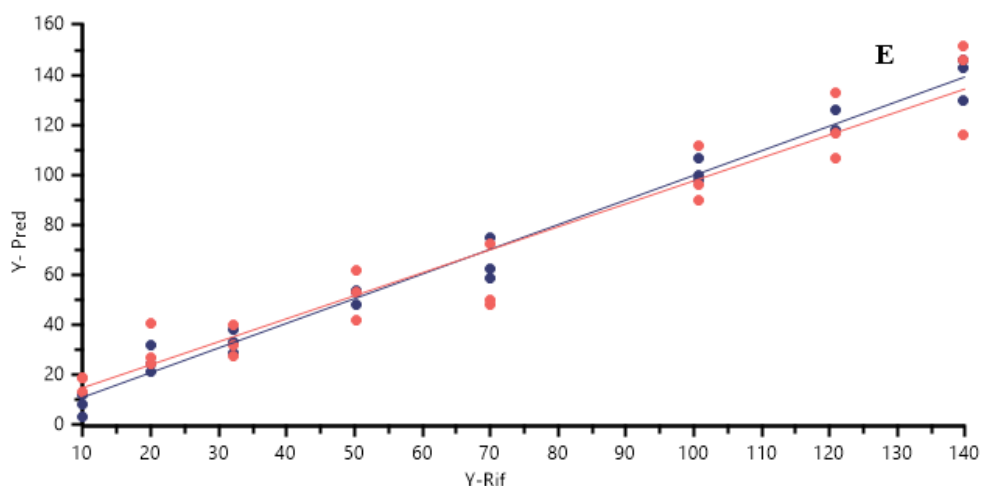
The results, in Table 14, show that PLSR models based on the NIR spectra of synthetic tannin provided the best calibration and validation performance compared to the other tanning agents (Chrome, Glutaraldehyde, Mimosa, and Zeolites). The R² values for both calibration (R² = 0.99656) and validation (Rcv²=0.99128) were the highest, with significantly lower RMSEC (2.92237) and RMSEV (4.98563) values than those obtained for other tanning agents. This robust predictive capacity of the syntan model is likely attributable to specific chemical-physical factors. Synthetic tannins, being synthetic, exhibit regularity in structural composition compared to natural mimosa tannins, which consist of a mix of condensed monomers. Additionally, the synthetic tannins' "purity" may yield higher solution concentrations than other tanning agents, enhancing the NIR spectra with more characteristic and abundant signals. Although not apparent in simple visual analysis, these signals facilitate easier interpretation and interpolation within the PLSR chemometric model.

Figure 42 A-E: Predicted versus reference values obtained through PLSR analysis for five different substances. In each plot, predicted values from cross-validation are shown as orange points, while blue

points represent values read from the calibration line. The substances analysed are: A = Chromium, B = Glutaraldehyde, C = Mimosa, D = Syntan, and E = Zeolites. The linear trend in each figure demonstrates the correlation between predicted and reference values, indicating the predictive accuracy of the model across different chemical compounds.







6.9 Limitations and Remarks

NIR spectroscopy combined with the PLSR technique has been successfully used for sensing and measuring the chemical information in known-concentration solutions of classic and non-classic tanning agents used in tannery operations. The PLSR method, applied to NIR spectra from tanning solutions, has proven to be an effective alternative for developing objective, rapid, nondestructive, noninvasive, and chemical-free techniques for real-time, online detection and control in the tanning industry.

However, the method has inherent limitations as it was studied on a laboratory scale, meaning the developed model may not be directly applicable to tannery drum water samples due to their different matrix compositions. Drum solutions contain additional substances released by hides, various chemicals used during tanning (e.g., formic acid, surfactants), and possibly the combined action of multiple tanning agents. Nonetheless, this study offers a way to evaluate tanning agent concentrations using reliable chemical analyses as reference methods, i.e., direct tanning agent concentrations, measured through known-concentration solutions.

The method appears promising for inline control of tanning agents, and in future applications, it could be coupled with direct analytical analysis of tanning agents in

drum solutions. This would enable inline evaluation of tanning agent concentrations to monitor the process more effectively (e.g., by assessing metal concentrations via IC-MS analysis, or glutaraldehyde and tannin concentrations through HPLC-MS).

6.10 Conclusion

During the tanning process in the drum, NIR technology could allow real-time monitoring of chemical concentrations, optimizing operating conditions, enhancing tanning efficiency, and minimizing the use of chemical agents, thereby contributing to a more sustainable process. To fully leverage NIR spectroscopy's potential, it is essential to integrate chemometric methods with multivariate statistical analysis of the collected data. Tools such as PLSR analysis enable effective data interpretation, helping to identify trends and correlations that can optimize tanning processes and improve water resource management. With adequate model calibration and expert analysis of results, NIR spectroscopy has the potential to become a reference technology for sustainable, non-destructive monitoring in the tanning industry.

Appendix: Ph.D. Course Activity Summary

Publications:

- Lavorgna, M., Medici, A., Russo, C., Orlo, E., Di Fabio, G., Luongo, G., De Nisco, M., Isidori, M., & Zarrelli, A. (2024). Ethylhexyl triazone sunscreen and its disinfection byproducts obtained after chlorine treatment: Ecofriendliness or ecotoxicity? *The Science of the Total Environment*, 955, 177279. <https://doi.org/10.1016/j.scitotenv.2024.177279>
- Medici, A., De Nisco, M., Luongo, G., Di Fabio, G., Brigante, M., & Zarrelli, A. (2024). Degradation Acyclovir using sodium hypochlorite: focus on byproducts analysis, optimal conditions and wastewater application. *Molecules*, 29(16), 3783. <https://doi.org/10.3390/molecules29163783>
- Medici, A., Lavorgna, M., Isidori, M., Russo, C., Orlo, E., Luongo, G., Di Fabio, G., & Zarrelli, A. (2024). Advanced oxidation process of valsartan by activated peroxymonosulphate: Chemical characterization and ecotoxicological effects of its byproducts. *The Science of the Total Environment*, 908, 168337. <https://doi.org/10.1016/j.scitotenv.2023.168337>
- Medici, A., Luongo, G., Di Fabio, G., & Zarrelli, A. (2022). Environmental fate of organic sunscreens during water disinfection processes: The formation of degradation by-products and their toxicological profiles. *Molecules*, 27(14), 4467. <https://doi.org/10.3390/molecules27144467>
- Medici, A., Luongo, G., Pedatella, S., Previtera, L., Di Fabio, G., & Zarrelli, A. (2024). Tackling Losartan contamination: The promise of peroxymonosulfate/Fe(II) advanced oxidation processes. *Molecules*, 29(10), 2237. <https://doi.org/10.3390/molecules29102237>
- Medici, A., Sarakha, M., Di Fabio, G., Brigante, M., & Zarrelli, A. (2024). Efficient Nimesulide degradation via chlorination and sun-simulated radiation: Kinetic insights, reactive species formation, and application to real wastewater. *Journal of Environmental Chemical Engineering*, 12(4), 113247. <https://doi.org/10.1016/j.jece.2024.113247>
- Medici, A., Saviano, L., Siciliano, A., Libralato, G., Guida, M., Previtera, L., Di Fabio, G., & Zarrelli, A. (2022). Octocrylene: From sunscreens to the degradation pathway during chlorination processes: Formation of byproducts and their ecotoxicity assessment. *Molecules*, 27(16), 5286. <https://doi.org/10.3390/molecules27165286>
- Medici, A., Siciliano, A., Libralato, G., Saviano, L., Guida, M., Pedatella, S., Luongo, G., Di Fabio, G., & Zarrelli, A. (2024). Percarbonate mediated advanced oxidation of irbesartan: A suitable alternative to chlorination? *The Science of the Total Environment*, 947, 174730. <https://doi.org/10.1016/j.scitotenv.2024.174730>
- Siciliano, A., Medici, A., Guida, M., Libralato, G., Saviano, L., Previtera, L., Di Fabio, G., & Zarrelli, A. (2023). Newly discovered Irbesartan disinfection byproducts via chlorination: Investigating potential environmental toxicity. *Applied Sciences*, 13(14), 8170. <https://doi.org/10.3390/app13148170>
- Toscanesi, M., Russo, V., Medici, A., Giarra, A., Hmoudah, M., Di Serio, M., & Trifuoggi, M. (2022). Heterogeneous photodegradation for the abatement of recalcitrant COD in synthetic tanning wastewater. *ChemEngineering*, 6(2), 25. <https://doi.org/10.3390/chemengineering6020025>

Communications:

ORAL COMUNICATION: XXIX Conference of the Analytical Chemistry Division, Italian Chemical Society (SCI), 11-15/09/2022 in Castello di Milazzo (Me). Title: "Disinfection byproducts and ecotoxic risk associated with hypochlorite treatment of Irbesartan" in the session 'Environment and Cultural Heritage'. A. Medici, A. Siciliano, M. Guida, G. Libralato, L. Saviano, G. Luongo, L. Previtera, G. Di Fabio, A. Zarrell <https://www.analitica2022.chim.it/index.php/en/information/program>

ORAL COMUNICATION: Department of Institut de Chimie de Clermont- Ferrand (ICCF), Université Clermont Auvergne, Photochemistry Department meeting on June 2nd 2023. Title: "Advanced Oxidation Processes for the Degradation of Emerging Contaminants: A Case Study on Nimesulide Oxidation Using UVA and Solar-Activated Hypochlorite".

ORAL COMUNICATION: XXVIII National Congress of the Italian Chemical Society "Chemistry ELEMENTS OF FUTURE", 26-30 August 2024 in Milano. Title: "Degradation of Nimesulide by Sunlight-Assisted-UVA irradiation: Kinetics and Reactive Species Generation". A. Medici, G. Di Fabio, M. Sarakha, M. Brigante, A. Zarrelli. <https://sci2024.org/>

POSTER COMUNICATION: International School on Mass Spectrometry (IntSMS), Ettore Majorana Foundation and Centre for Scientific Culture, data in Erice (TP). Title: "Octocrylene: From Sunscreens to the Degradation Pathway during Chlorination Processes: Formation of Byproducts and Their Ecotoxicity Assessment". A. Medici, L. Saviano, A. Siciliano, G. Libralato, M. Guida, L. Previtera, G. Di Fabio and A. Zarrelli. www.spettrometriadimassa.it/intsms2022/index.html

POSTER COMUNICATION: Merck Young Chemists Symposium 2023, 13-15 November 2023 in Rimini (Italy). Title: "Efficient Nimesulide degradation by hypochlorite and UVA irradiation: kinetics and reactive species generation". https://www.soc.chim.it/sites/default/files/Program_MYCS2023_12.11.23.pdf

Ph. D. Courses

"Advanced Mass Spectrometry", Prof. A. Amoresano (Unina)

"Smart drug delivery systems", Prof. A. Guaragna (Unina)

"Protein engineering", Prof. A. Duilio (Unina)

"Persistent organic pollutants", Prof. A. Andolfi (Unina)

"Introduction to data analysis", Prof. R. Velotta (Unina)

"First steps in writing and publishing a scientific manuscript", Prof. V. Calabrò (Unina)

"Synthetic glycochemistry", Prof. E. Bedini; A. Iadonisi (Unina)

"Mitigation of the environmental impact of chemical processes for energy production", Prof. F. Montagnaro (Unina)

Schools, Meeting and Seminars:

"1st International School on Mass Spectrometry", Ettore Majorana Foundation and Centre for Scientific Culture, Erice (TP). 25/09/2022

"Prima Scuola Nazionale di Risonanza Magnetica Nucleare per lo Studio di Piccole Molecole Organiche" Università di Napoli Federico II 19-20 February 2024

17 Conference Campania A.I.C.C. Associazione Italiana dei Chimici del Cuoio "Nuove PROSPETTIVE per il Settore Conciario"

“Research & development in a big company” held by Dr. Andrea Di Matteo (STMicronics S.r.l. – Arzano–NA), 21.01.2022

“Chimici in ospedale” held by Dr. Rita Boenzi (Ospedale Monaldi Napoli), 09.03.2022

“La nostra avventura dal dipartimento di Scienze Chimiche a UniConNet” held by Dr. Rosaria Cercola, Dr. Claudio Chesi, Dr. Silvia Marchese e Dr. Fabiana Paragliola (UniConNet), 10.05.2022

“Modulation of type 2 diabetes mellitus by flavonoids through the inhibition of key carbohydrate-hydrolyzing enzymes” held by Prof. Marisa Freitas University of Porto, 25.05.2022

"Food byproducts valorization" held by Prof. Maria Beatriz Oliveira University of Porto, 25.05.2022

“Academic Publishing Workshop:What to consider when publishing your research?” held by Prof. Cristina de Castro, 17/10/2022

“Federico II PLUS–Per L’Uso Sostenibile delle risorse” held by Prof. Fabio Montagnaro, 10.10.2022

“On discovery and sensitivity in (photo)catalysis” held by Prof. Frank Glorius, 05.09.2023

“Introduction to Small Angle X-ray Scattering” held by Dr. Andreas Keilbach, 20/09/2023

“First-principles study of the structural and electronic properties of BN-ring doped graphene”–Dr. Laura Caputo(UC Louvain (UCL), 19/12/2023

“Exploration of Fröhlich polarons from perturbative and variational approaches”–Dr. Bogdan Guster (UC Louvain (UCL) 19/12/2023

"Complessità e il volo degli uccelli" held by Prof. Giorgio Parisi, 29/11/2023

“Protein NMR: two interesting serendipitous results” Prof Gennaro Esposito, 12/01/2024

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Supporting Materials

STA-Supporting text A- Octocrylene

Octocrylene (99%) was purchased from Sigma Aldrich (Milan, Italy). All of the other chemicals and solvents were purchased from Sigma Aldrich (Milan, France) and were of HPLC grade and used as received. All of the chemicals were of analytical grade and supplied by Sigma Aldrich.

The toxicity tests were conducted with two combinations. In the first combination, osmotic adjustment solution (OAS) (22 g /L NaCl) was used as a control for optimal conditions according to the ISO 11348-3 standard. In the second combination, synthetic sea water was used as the control solution according to the ISO 10253 standard. The synthetic sea water used for analytical procedures comprised the following salts: NaCl (22 g /L), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (9.7 g /L), Na_2SO_4 (3.7 g /L), CaCl_2 (1.0 g /L), KCl (0.65 g /L), NaHCO_3 (0.2 g /L) and H_3BO_3 (0.023 g /L).

Column chromatography (CC) was carried out with Kieselgel 60 (230–400 mesh, Merck, Darmstadt, Germany). HPLC was performed on a Shimadzu LC-8A system using a Shimadzu SPD-10A VP UV-VIS detector (Shimadzu, Milan, Italy). Preparative HPLC was performed using an RP Gemini C18-110A preparative column (10 μm particle size, 250 mm x 21.20 mm i.d. Phenomenex, Bologna, Italy) with a flow rate of 8.0 mL/min. The ^1H - and ^{13}C -NMR spectra were recorded with an NMR spectrometer operated at 400 MHz and at 25 °C (Bruker DRX, Bruker Avance) and referenced in ppm to the residual solvent signals (CDCl_3 , at ppm_H 7.27 and ppm_C 77.0; and CD_3OD , at ppm_H 3.30 and ppm_C 49.0). The proton-detected heteronuclear correlations were measured using a gradient heteronuclear single-quantum coherence (HSQC) experiment, optimized for $^1J_\text{HC} = 155$ Hz, and a gradient heteronuclear multiple bond coherence (HMBC) experiment, optimized for $^nJ_\text{HC} = 8$ Hz. The MALDI-TOF mass spectrometric analyses were performed on a Voyager-De Pro MALDI mass-spectrometer (PerSeptive Biosystems, Framingham, MA, USA).

SMA-Supporting Materials A- Octocrylene

Table SMa1. ^1H , ^{13}C and 2D NMR data of Octocrylene in CDCl_3 .

Position	Residue	$^{13}\text{C}^a$	$^1\text{H}^a$, multiplicity	^1H - ^1H COSY	^1H - ^{13}C HMBC
1	C	162.90	-		
2	C	104.10	-		
3	C	168.84	-		7.16
4	C	138.33	-		7.41, 7.16
5/9	CH	128.23	7.16, d	7.41, 7.38	7.41, 7.38
6/8	CH	129.26	7.41, m	7.38, 7.16	7.38, 7.16
7	CH	130.33	7.38, m	7.41, 7.16	7.41, 7.16
10	C	138.65	-		7.41, 7.16
11/15	CH	128.47	7.16, d	7.41, 7.38	7.41, 7.38
12/14	CH	130.21	7.41, m	7.38, 7.16	7.38, 7.16
13	CH	131.55	7.38, m	7.41, 7.16	7.41, 7.16
16	C	116.76	-		

17	CH ₂	68.52	4.02, dd	1.46	1.46, 1.23
18	CH	38.50	1.46, m	4.02, 1.23	4.02, 1.23, 0.83
19	CH ₂	30.05	1.23, m	1.46	4.02, 1.46
20	CH ₂	28.81	1.23, m	1.23	1.46, 1.23
21	CH ₂	22.85	1.23, m	1.23, 0.89	1.23, 0.89
22	CH ₃	14.00	0.89, d	1.23	1.23
23	CH ₂	23.42	1.23, m	1.46, 0.83	4.02, 1.46, 1.23, 0.83
24	CH ₃	10.88	0.83, d	1.23	1.46, 1.23

^aChemical shifts in ppm.

Table SMa2. ¹H, ¹³C and 2D NMR data of DBP2 in CDCl₃.

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
1	C	194.01	-		7.81
2	C	137.98	-		7.81, 7.49
3/7	CH	130.25	7.81, m	7.59, 7.49	7.59, 7.49
4/6	CH	127.95	7.49, m	7.81, 7.59	7.81, 7.59
5	CH	132.60	7.59, m	7.81, 7.49	7.81, 7.49
8	C	137.98	-		7.81, 7.49
9/13	CH	130.25	7.81, m	7.59, 7.49	7.59, 7.49
10/12	CH	127.95	7.49, m	7.81, 7.59	7.81, 7.59
11	CH	132.60	7.59, m	7.81, 7.49	7.81, 7.49

^aChemical shifts in ppm.

Table SMa3. ¹H, ¹³C and 2D NMR data of DBP3 in CDCl₃.

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
1	C	172.63	-		
2	CH	57.39	5.06, s		7.34
3	C	138.79	-		5.06, 7.34
4/8	CH	128.51	7.34, m	7.29	5.06, 7.34, 7.29
5/7	CH	128.65	7.34, m	7.29	7.29
6	CH	127.17	7.29, m	7.34	7.34
9	C	138.79	-		7.34
10/14	CH	128.51	7.34, m m	7.29	7.34, 7.29
11/13	CH	128.65	7.34, m	7.29	7.29
12	CH	127.17	7.29, m	7.34	7.34
15	CH ₂	67.50	4.10, d	1.60	1.60, 1.28
16	CH	38.76	1.60, m	4.10, 1.28	4.10, 1.28
17	CH ₂	30.6	1.28, m	1.60	1.60, 1.28
18	CH ₂	28.84	1.28, m		1.28
19	CH ₂	22.93	1.28, m		1.28, 0.88
20	CH ₃	14.01	0.88, t	1.28	1.28
21	CH ₂	23.76	1.28, m	1.60, 0.88	1.60, 0.88
22	CH ₃	10.95	0.88, t	1.28	1.60, 1.28

^aChemical shifts in ppm.

Table SMa4. ¹H, ¹³C and 2D NMR data of DBP4 in CDCl₃.

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
1	C	170.13	-		
2	C	81.58	-		7.38, 7.33
3	C	147.32	-		7.38, 7.33
4/8	CH	127.85	7.38, m	7.38	7.38
5/7	CH	128.30	7.38, m	7.38, 7.35	7.38, 7.35
6	CH	127.67	7.35, m	7.38	7.38
9	C	147.32	-		7.33
10/14	CH	127.85	7.38, m	7.38	7.40, 7.33
11/13	CH	128.30	7.38, m	7.38, 7.35	7.40, 7.33
12	CH	127.67	7.35, m	7.38	7.33
15	CH ₂	67.08	4.18, d	1.55	1.55, 1.24, 1.22
16	CH	36.68	1.55, m	4.18, 1.24, 1.22	4.18, 1.24, 1.22
17	CH ₂	30.31	1.24, m	1.55, 1.22	1.55, 1.24, 1.22
			1.22, m	1.55, 1.24	
18	CH ₂	28.76	1.24, m	1.22	1.24, 1.22, 0.83
			1.22, m	1.24	
19	CH ₂	22.92	1.24, m	1.22, 0.83	1.24, 1.22, 0.83
			1.22, m	1.24, 0.83	
20	CH ₃	14.02	0.83, t	1.24, 1.22	1.24, 1.22
21	CH ₂	23.75	1.24, m	1.55, 1.22	1.55, 0.81
			1.22, m	1.55, 1.24	
22	CH ₃	10.93	0.81, t	1.24, 1.22	1.55, 1.24, 1.22

^aChemical shifts in ppm.

Table SMa5. ¹H, ¹³C and 2D NMR data of DBP5 in CDCl₃.

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
1	C	161.78	-		
2	C	78.06	-		
3	C	72.96	-		7.55, 7.48
4	C	134.38	-		7.55, 7.43
5/9	CH	127.29	7.55, m	7.44, 7.43	7.44, 7.43
6/8	CH	128.70	7.43, m	7.55, 7.44	7.55, 7.44
7	CH	129.64	7.44, m	7.55, 7.43	7.55, 7.43
10	C	133.69	-		7.48, 7.42
11/15	CH	127.15	7.48, m	7.42, 7.41	7.42
12/14	CH	128.62	7.42, m	7.48, 7.41	7.48, 7.41
13	CH	129.30	7.41, m	7.48, 7.42	7.48, 7.42
16	C	113.78/113.76	-		
17	CH ₂	69.81/69.77	4.01, m	1.43	1.43, 1.25, 1.22
			3.91, m		4.01, 3.91, 1.25,
			1.43, m	4.01, 3.91, 1.25, 1.22	1.22,
18	CH	38.51/38.46	1.22, m		0.83
					4.01, 3.91, 1.43,
19	CH ₂	29.99/29.91		1.43	1.25,
					1.22,
					0.83
20	CH ₂	28.81/28.74	1.22, m	1.25	1.43, 1.25, 1.22,
					0.93

21	CH ₂	22.86/22.85	1.25, m	1.22, 0.93	1.22, 0.93
22	CH ₃	14.03/14.01	0.93, t	1.25	1.25, 1.22
23	CH ₂	23.36/23.32	1.25, m	1.43, 0.83	4.01, 3.91, 1.43, 0.83
24	CH ₃	10.86/10.82	0.83, m	1.25	1.43, 1.22

^aChemical shifts in ppm.

Table SMa6. ¹H, ¹³C and 2D NMR data of DBP7 in CDCl₃.

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
1	C	178.16	-		
2	CH	57.05	5.08		7.36
3	C	138.02	-		5.08, 7.36
4/8	CH	128.66	7.36, m	7.31	5.08, 7.36, 7.31
5/7	CH	128.70	7.36, m	7.31	7.36, 7.31
6	CH	127.47	7.31, m	7.36	7.36
9	C	138.02	-		7.36
10/14	CH	128.66	7.36, m	7.31	7.36, 7.31
11/13	CH	128.70	7.36, m	7.31	7.36, 7.31
12	CH	127.47	7.31, m	7.36	7.36

^aChemical shifts in ppm.

Table SMa7. ¹H, ¹³C and 2D NMR data of DBP8 in CDCl₃.

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
1	C	166.53	-		
2	C	105.17	-		
3	C	163.50	-		7.43, 7.15
4	C	138.48	-		
5/9	CH	129.61	7.43, m	7.50, 7.30	7.50, 7.30
6/8	CH	128.14	7.30, m	7.50, 7.43	7.50, 7.43
7	CH	131.29	7.50, m	7.43, 7.30	7.43, 7.30
10	C	138.75	-		
11/15	CH	130.30	7.15, m	7.39	7.39, 7.38
12/14	CH	128.48	7.38, m	7.39, 7.15	7.39, 7.15
13	CH	130.43	7.39, m	7.38, 7.15	7.38, 7.15
16	C	117.65	-		

^aChemical shifts in ppm.

Table SMa8. ¹H, ¹³C and 2D NMR data of DBP9 in CDCl₃.

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
1	C	170.05	-		
2	C	72.30	-		
3	C	72.69	-		7.54
4	C	138.11	-		7.54, 7.41
5/9	CH	128.61	7.54, m	7.41, 7.33	7.41, 7.33
6/8	CH	129.56	7.41, m	7.54, 7.33	7.54, 7.33
7	CH	129.98	7.33, m	7.54, 7.41	7.54, 7.41
10	C	136.78	-		7.54, 7.35
11/15	CH	128.10	7.54, m	7.35, 7.33	7.35, 7.33
12/14	CH	129.40	7.35, m	7.54, 7.33	7.54, 7.33

13	CH	129.72	7.33, m	7.54, 7.35	7.54, 7.35
16	C	117.85	-		

^aChemical shifts in ppm.

SMB-Supporting Materials B- Ethylhexyl triazone

Ethylhexyl triazone (EHT): *bis(2-Ethylhexyl) 4,4'-((6-(((4-(((2-ethyl-4-methylhexyl)oxy)carbonyl) phenyl)amino)-1,3,5-triazine-2,4-diyl)bis(azanediy))dibenzoate*. White powder. ^1H and ^{13}C NMR: see Table S1; MS-TOF (positive ions): m/z calculated for $\text{C}_{49}\text{H}_{68}\text{N}_6\text{O}_6$ 836.52 $[\text{M}]^+$; found 837.61 $[\text{M} + \text{H}]^+$ (55%), 838.62 (30%).

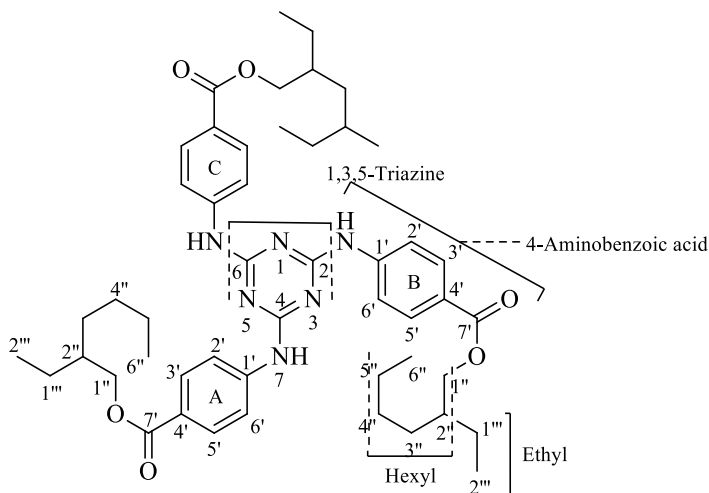


Table SMb1. Data obtained from the mono- and two-dimensional NMR analysis of EHT, in deuterated chloroform (CDCl_3).

Position	Residue	$^{13}\text{C}^a$	$^1\text{H}^a$, multiplicity	^1H - ^1H COSY	^1H - ^{13}C HMBC
2/4/6	C	165.31	-		
NH	-	-	7.51, s		
1'	C	143.84	-		
2'/6'	CH	120.83	7.73, d	8.06	143.84, 126.72, 120.83
3'/5'	CH	132.08	8.06, d	7.73	167.72, 143.84, 132.08
4'	C	126.72	-		
7'	C	167.72	-		
1''	CH_2	68.72	4.28, m	1.77	167.72, 40.35, 32.00
2''	CH	40.35	1.77, m	4.28, 1.51, 1.37	30.41, 12.48
3''	CH_2	32.00	1.51, m	1.77, 1.43	68.72, 40.35, 24.39
4''	CH_2	31.41	1.43, m	1.51, 1.37	40.35, 15.45
5''	CH_2	24.39	1.37, m	1.43, 0.94	32.00, 15.45
6''	CH_3	15.45	0.94, m	1.37	30.41
1'''	CH_2	25.44	1.37, m	1.77, 0.99	68.72, 32.00, 12.48
2'''	CH_3	12.48	0.99, m	1.37	40.35

^aChemical shifts in ppm.

DBP1: *bis(2-ethylhexyl) 4,4'-((6-amino-1,3,5-triazine-2,4-diyl)bis(azanediyl))dibenzoate*. White powder. ^1H and ^{13}C NMR: see Table S2; MS-TOF (positive ions): m/z calculated for $\text{C}_{33}\text{H}_{46}\text{N}_6\text{O}_4$ 590.36 $[\text{M}]^+$; found 591.39 $[\text{M} + \text{H}]^+$ (61%), 592.41 (22%).

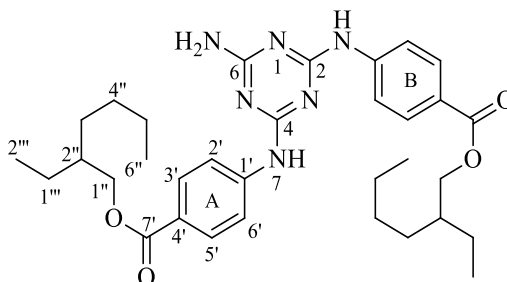


Table SMb2. Data obtained from the mono- and two-dimensional NMR analysis of DBP1, in deuterated chloroform (CDCl_3).

Position	Residue	$^{13}\text{C}^a$	$^1\text{H}^a$, multiplicity	^1H - ^1H COSY	^1H - ^{13}C HMBC
2	C	166.38	-		
4	C	166.55	-		
6	C	167.08			
1'	C	140.12	-		
2'/6'	CH	120.99	7.72, d	8.06	127.04, 120.99
3'/5'	CH	130.55	8.06, d	7.72	165.90, 140.12, 130.55
4'	C	127.04	-		
7'	C	165.90	-		
1''	CH_2	67.60	4.27, m	1.76	165.90, 30.97, 24.,10
2''	CH	38.80	1.76, m	4.27, 1.46, 1.41	30.55, 11.00
3''	CH_2	30.97	1.46, m	1.76, 1.39	67.60, 24.10, 23.00
4''	CH_2	30.55	1.39, m	1.46, 1.26	38.80, 13.90
5''	CH_2	23.00	1.26, m	1.39, 0.91	30.97
6''	CH_3	13.90	0.91, m	1.26	30.55
1'''	CH_2	24.10	1.41, m	1.76, 0.97	67.60, 30.97, 11.00
2'''	CH_3	11.00	0.97, m	1.41	38.80

^aChemical shifts in ppm.

DBP2: *2-ethylhexyl 3-chloro-4-((4-(((2-ethyl-4-methylhexyl)oxy)carbonyl)phenyl)amino)-6-(((2-ethylhexyl)oxy)carbonyl)phenyl)amino)-1,3,5-triazin-2-yl)amino)benzoate*. Gray powder. ^1H and ^{13}C NMR: see Table S3; MS-TOF (positive ions): m/z calculated for $\text{C}_{49}\text{H}_{67}\text{ClN}_6\text{O}_6$ 870.48 $[\text{M}]^+$; found 871.52 $[\text{M} + \text{H}]^+$ (48%), 873.53 $[\text{M} + 2 + \text{H}]^+$ (16%), 872.52 $[\text{M} + 1 + \text{H}]^+$ (22%).

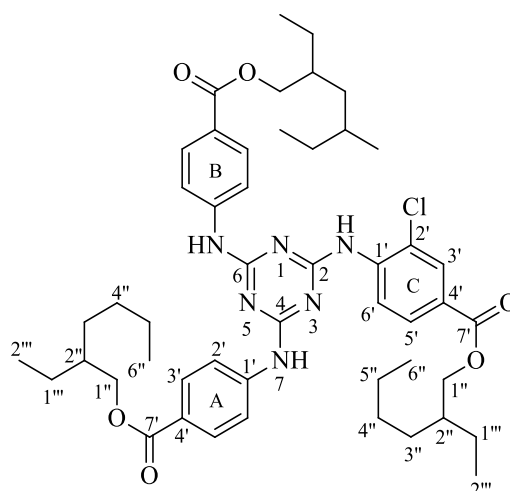


Table SMb3. Data obtained from the mono- and two-dimensional NMR analysis of DBP2, in deuterated chloroform (CDCl₃).

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
2	C	163.56	-		
4/6	C	163.78	-		
1'	C	138.82	-		
2'	C	125.54	-		
3'	CH	130.52	8.07	7.94	165.26, 138.82, 128.78
4'	C	125.81	-		
5'	CH	128.78	7.94	8.59, 8.07	165.26, 138.82, 130.52
6'	CH	120.79	8.59	7.94	125.81, 125.54
7'	C	165.26	-		
1''	CH ₂	67.68	4.24, m	1.73	165.26, 30.53, 23.96
2''	CH	38.87	1.73, m	4.24, 1.46, 1.40	28.98, 11.03
3''	CH ₂	30.53	1.40, m	1.73, 1.34	67.68, 23.96, 22.93
4''	CH ₂	28.98	1.34, m	1.40	38.87, 14.01
5''	CH ₂	22.93	1.34, m	0.91	30.53, 14.01
6''	CH ₃	14.01	0.91, m	1.34	28.98
1'''	CH ₂	23.96	1.46, m	1.73, 0.96	67.68, 30.53
2'''	CH ₃	11.03	0.96, m	1.46	38.87
1'	C	142.11	-		
2'/6'	CH	119.55	7.69, d	8.04	125.74, 119.55
3'/5'	CH	130.71	8.04, d	7.69	166.25, 142.11, 130.71
4'	C	125.74	-		
7'	C	166.25	-		
1''	CH ₂	67.32	4.24, m	1.73	165.26, 30.59, 24.01
2''	CH	38.92	1.73, m	4.24, 1.46, 1.40	28.98, 11.06
3''	CH ₂	30.59	1.40, m	1.73, 1.34	67.32, 24.01, 22.95
4''	CH ₂	28.98	1.34, m	1.40	38.92, 14.01
5''	CH ₂	22.95	1.34, m	0.91	30.59, 14.01
6''	CH ₃	14.01	0.91, m	1.34	28.98
1'''	CH ₂	24.01	1.46, m	1.73, 0.96	67.32, 30.59

2'''	CH ₃	11.06	0.96, m	1.46	38.92
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^aChemical shifts in ppm.

DBP3: 2-ethyl-4-methylhexyl 3-chloro-4-((4-((2-chloro-4-((2-ethylhexyl)oxy)carbonyl)phenyl)amino)-6-((4-((2-ethylhexyl)oxy)carbonyl)phenyl)amino)-1,3,5-triazin-2-yl)amino)benzoate. Gray powder. ¹H and ¹³C NMR: see Table S4; MS-TOF (positive ions): *m/z* calculated for C₄₉H₆₆Cl₂N₆O₆ 904.44 [M]⁺; found 905.53 [M + H]⁺ (39%), 907.54 [M + 2 + H]⁺ (14%), 906.52 [M + 1 + H]⁺ (17%).

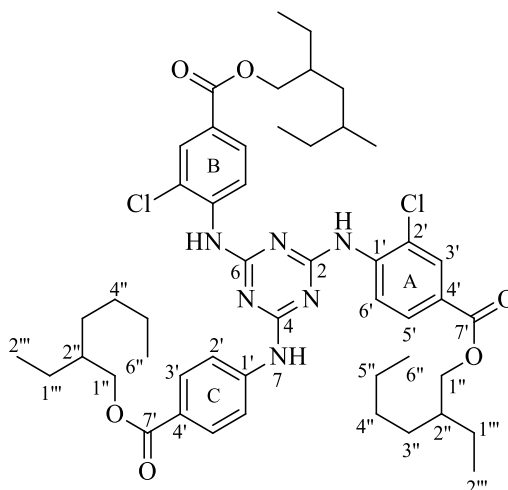


Table SMb4. Data obtained from the mono- and two-dimensional NMR analysis of DBP3, in deuterated chloroform (CDCl₃).

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
2	C	166.51	-		
4/6	C	167.48	-		
1'	C	139.58	-		
2'	C	128.12	-		
3'	CH	132.13	8.14	8.01	166.51, 139.58, 130.20
4'	C	124.89	-		
5'	CH	130.20	8.01	8.54, 8.14	166.51, 139.58, 132.13
6'	CH	120.40	8.54	8.01	130.20, 128.12, 124.89
7'	C	166.51	-		
1''	CH ₂	69.19	4.31, m	1.78	166.51, 31.98, 25.42
2''	CH	40.34	1.78, m	4.31, 1.51, 1.38	30.42, 12.47
3''	CH ₂	31.98	1.51, m	1.78, 1.45	69.19, 25.42, 24.38
4''	CH ₂	30.42	1.45, m	1.51, 1.38	40.34, 15.45
5''	CH ₂	24.38	1.38, m	1.45, 0.96	31.98
6''	CH ₃	15.45	0.96, m	1.38	30.42
1'''	CH ₂	25.42	1.38, m	1.78, 1.01	69.19, 31.98
2'''	CH ₃	12.47	1.01, m	1.38	40.34
1'	C	143.02	-		
2'/6'	CH	121.54	7.73	8.10	143.02, 127.53, 121.54

3'/5'	CH	132.29	8.10	7.73	167.48, 143.02, 132.29
4'	C	127.53	-		
7'	C	167.48	-		
1''	CH ₂	68.81	4.31, m	1.78	167.48, 32.03, 25.45
2''	CH	40.40	1.78, m	4.31, 1.51, 1.38	30.42, 12.47
3''	CH ₂	32.03	1.51, m	1.78, 1.45	68.81, 25.45, 24.38
4''	CH ₂	30.42	1.45, m	1.51, 1.38	40.40, 15.45
5''	CH ₂	24.38	1.38, m	1.45, 0.96	32.03
6''	CH ₃	15.45	0.96, m	1.38	30.42
1'''	CH ₂	25.45	1.38, m	1.78, 1.01	68.81, 32.03
2'''	CH ₃	12.47	1.01, m	1.38	40.40

^aChemical shifts in ppm.

DBP4: 2-ethylhexyl 4-((4-amino-6-(((2-ethylhexyl)oxy)carbonyl)-2-hydroxyphenyl)amino)-1,3,5-triazin-2-yl)amino)-2-chlorobenzoate. White powder. ¹H and ¹³C NMR: see Table S5; MS-TOF (positive ions): *m/z* calculated for C₃₃H₄₅ClN₆O₅ 640.31 [M]⁺; found 641.35 [M + H]⁺ (62%), 643.35 [M + 2 + H]⁺ (18%), 642.35 [M + 1 + H]⁺ (2017%).

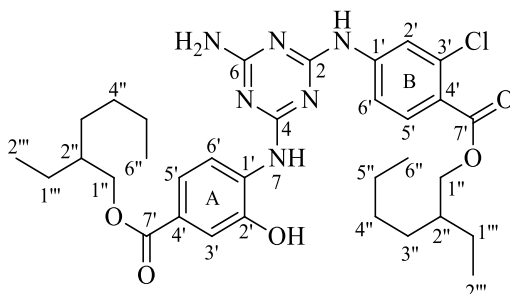


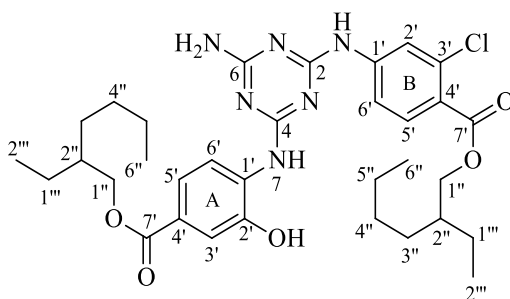
Table SMb5. Data obtained from the mono- and two-dimensional NMR analysis of DBP4, in deuterated chloroform (CDCl₃).

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
2	C	170.50	-		
4	C	165.19	-		
6	C	170.74	-		
1'	C	123.21	-		
2'	C	147.11	-		
3'	CH	130.80	8.19	8.02	165.28, 128.88, 123.21
4'	C	138.53	-		
5'	CH	128.88	8.02	8.19, 7.58	165.28, 130.80, 123.21
6'	CH	119.15	7.58	8.02	147.11, 138.53
7'	C	165.28	-		
1''	CH ₂	67.79	4.35, m	1.80	165.28, 31.80, 23.97
2''	CH	38.85	1.80, m	4.35, 1.50, 1.41	67.79, 30.55, 14.09
3''	CH ₂	31.80	1.50, m	1.80, 1.40	67.79, 23.97, 23.90
4''	CH ₂	30.55	1.40, m	1.50, 1.38	38.85, 14.09

5''	CH ₂	23.90	1.38, m	1.40, 0.96	31.80, 14.09
6''	CH ₃	14.09	0.96, m	1.38	30.55
1'''	CH ₂	23.97	1.41, m	1.80, 1.05	67.79, 31.80, 14.09
2'''	CH ₃	14.09	1.05, m	1.41	38.85, 23.97
1'	C	123.31	-		
2'	CH	124.37	7.40	7.16	147.68, 123.96, 126.37
3'	C	126.37	-		
4'	C	147.68	-		
5'	CH	121.72	8.52	7.16	167.70, 126.37, 123.31
6'	CH	123.96	7.16	8.52, 7.40	147.68, 124.37
7'	C	167.70	-		
1''	CH ₂	65.60	4.39, m	1.80	167.70, 31.79, 23.10
2''	CH	39.25	1.80, m	4.39, 1.51, 1.38	29.21, 11.10
3''	CH ₂	31.79	1.51, m	1.80, 1.40	65.60, 23.10
4''	CH ₂	29.21	1.40, m	1.51, 1.38	39.25, 11.10
5''	CH ₂	23.10	1.38, m	1.40, 0.99	31.79
6''	CH ₃	11.10	0.99, m	1.38	29.21
1'''	CH ₂	23.10	1.38, m	1.80, 1.51, 1.06	65.60, 31.79, 11.10
2'''	CH ₃	11.10	1.06, m	1.38	39.25, 23.10

^aChemical shifts in ppm.

DBP4: 2-ethylhexyl 4-((4-amino-6-(((2-ethylhexyl)oxy)carbonyl)-2-hydroxyphenyl)amino)-1,3,5-triazin-2-yl)amino)-2-chlorobenzoate. White powder. ¹H and ¹³C NMR: see Table S5; MS-TOF (positive ions): *m/z* calculated for C₃₃H₄₅ClN₆O₅ 640.31 [M]⁺; found 641.35 [M + H]⁺ (62%), 643.35 [M + 2 + H]⁺ (18%), 642.35 [M + 1 + H]⁺ (2017%).



SMC-Supporting Materials C- Nimesulide

Table SM1c. Sewage Treatment Plant Water (STPW) physico-chemical characteristics.

Parameters	STPW
pH	7.0
UV ₂₅₄	0.062
IC (mgC /L)	73
TOC (mgC /L)	2.3
Cl ⁻ (mg /L)	100
SO ₄ ²⁻ (mg /L)	53
NO ₃ ⁻ (mg /L)	59

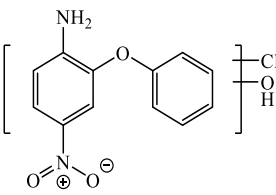
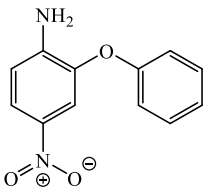
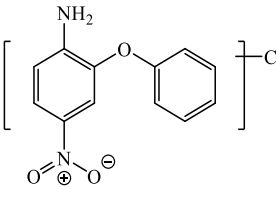
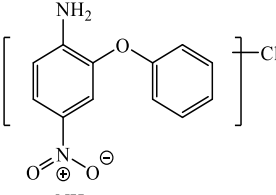
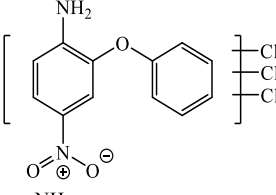
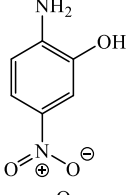
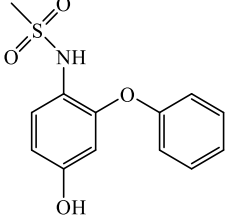
Table SM2c. Reactivity of HO[•] and Cl[•] with HOCl/ClO⁻

$HO^{\bullet} + HOCl \rightarrow ClO^{\bullet} + H_2O$	$k_{HO^{\bullet},HOCl} = 8.46 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$	Buxton et al., 1988
$HO^{\bullet} + ClO^- \rightarrow ClO^{\bullet} + HO^-$	$k_{HO^{\bullet},ClO^-} = 8.8 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$	Watts et al., 2007
$Cl^{\bullet} + HOCl \rightarrow ClO^{\bullet} + Cl^- + H^+$	$k_{Cl^{\bullet},HOCl} = 3.0 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$	Kläning et al., 1985
$Cl^{\bullet} + ClO^- \rightarrow ClO^{\bullet} + Cl^-$	$k_{Cl^{\bullet},ClO^-} = 8.2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$	Kläning et al., 1985

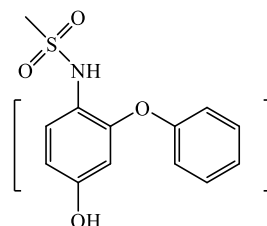
Table SM3c. LC-MS data of NIM disinfection by-products under SSR and Dark in the presenc of FAC

NIM SSR + FAC

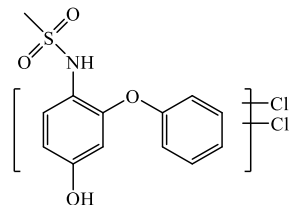
Compound	Rt (min)	Exp [M-H] ⁻	Theo [M-H] ⁻	Δmmu	Molecular formula	Proposed Structure
NIM-Cl	4.81	341.0001	340.9999	-0.23	C ₁₃ H ₁₁ ClN ₂ O ₅ S	
NIM-2Cl	4.98	374.9614	374.9615	0.28	C ₁₃ H ₁₀ Cl ₂ N ₂ O ₅ S	
NIM-3Cl	5.2	408.9224	408.9219	0.44	C ₁₃ H ₉ Cl ₃ N ₂ O ₅ S	
P1	4.14	323.0339	323.0338	0.15	C ₁₃ H ₁₁ N ₂ O ₆ S	
P2	4.42	356.9955	356.9948	0.59	C ₁₃ H ₁₁ ClN ₂ O ₆ S	

P3	4.39	279.0177	279.0173	0.41	$C_{12}H_9ClN_2O_4$	
P4	4.79	229.0615	229.0613	0.18	$C_{12}H_{10}N_2O_3$	
P5	5.12	263.0225	263.0223	0.16	$C_{12}H_9ClN_2O_3$	
P6	5.53	296.9838	296.9834	0.45	$C_{12}H_8Cl_2N_2O_3$	
P7	5.86	330.9447	330.9444	0.39	$C_{12}H_7Cl_3N_2O_3$	
P8	2.81	153.0293	153.0300	0.74	$C_6H_5N_2O_3$	
P9	2.58	278.0493	278.0487	0.67	$C_{13}H_{13}NO_4S$	

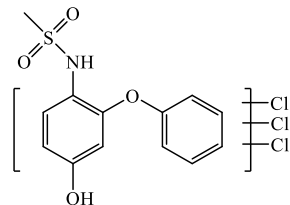
P10 3.83 312.0103 312.0097 0.55 C₁₃H₁₁ClNO₄S



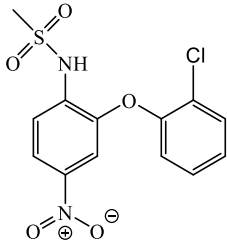
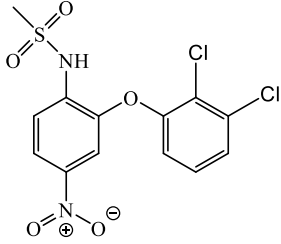
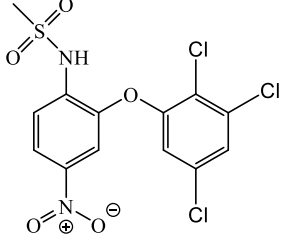
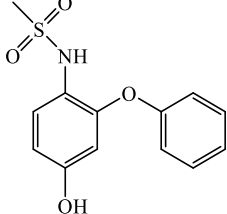
P11 3.57 345.9713 345.9708 0.52 C₁₃H₁₁Cl₂NO₄S



P12 4.70 379.9324 379.9318 0.56 C₁₃H₉Cl₃NO₄S



NIM DARK + FAC

Compound	Rt (min)	Exp [M-H] ⁻	Theor [M-H] ⁻	Δmmu	Molecular formula	Proposed Structure
NIM-Cl	4.81	340.9999	340.9999	0.03	C ₁₃ H ₁₁ ClN ₂ O ₅ S	
NIM-2Cl	5.15	374.9613	374.9615	0.38	C ₁₃ H ₁₀ Cl ₂ N ₂ O ₅ S	
NIM-3Cl	5.57	408.9225	408.9219	0.60	C ₁₃ H ₉ Cl ₃ N ₂ O ₅ S	
P9	4.79	229.0611	229.0613	-0.90	C ₁₂ H ₁₀ N ₂ O ₃	

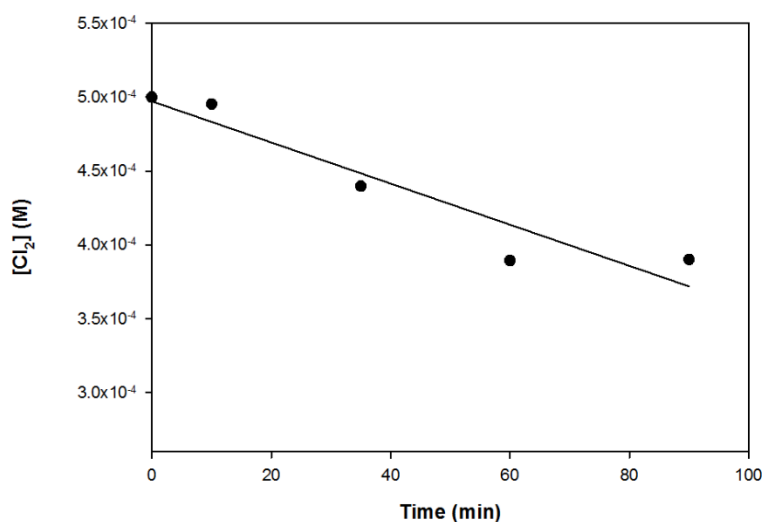


Figure SM1c: Cl_2 degradation under sun-simulated irradiation at pH 7.5

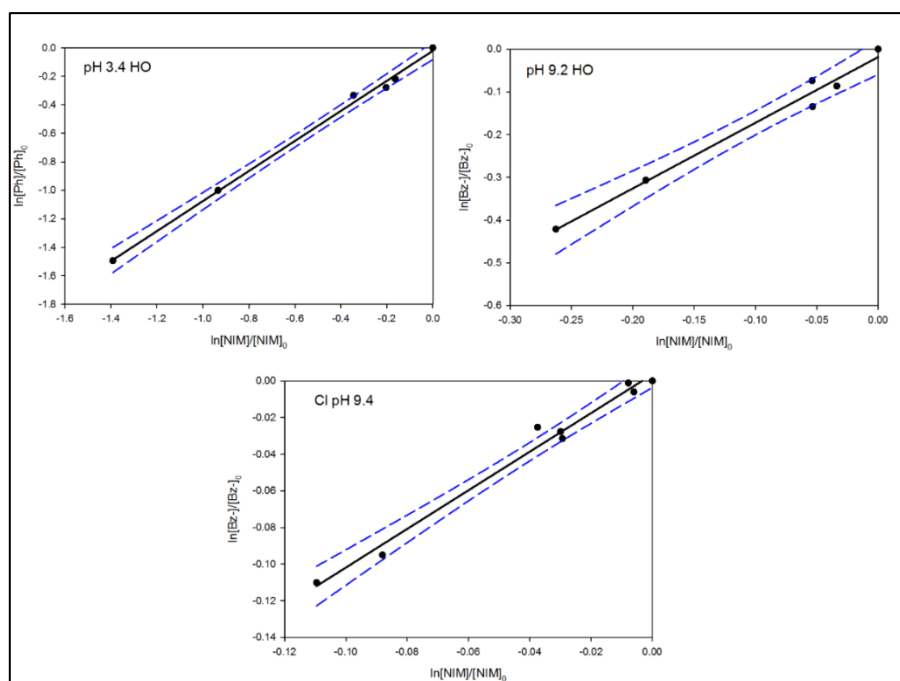


Figure SM2c: Plot of $\ln[\text{NIM}]/[\text{NIM}]_0$ vs. $\ln[\text{Ph}]/[\text{Ph}]_0$ and $\ln[\text{Bz}^-]/[\text{Bz}^-]_0$ for the reactivity constant determination with $\text{HO}^\bullet$ and $\text{Cl}^\bullet$ under acidic and alkaline pH values. Dashed lines denote the 95% confidence of the linear fit.

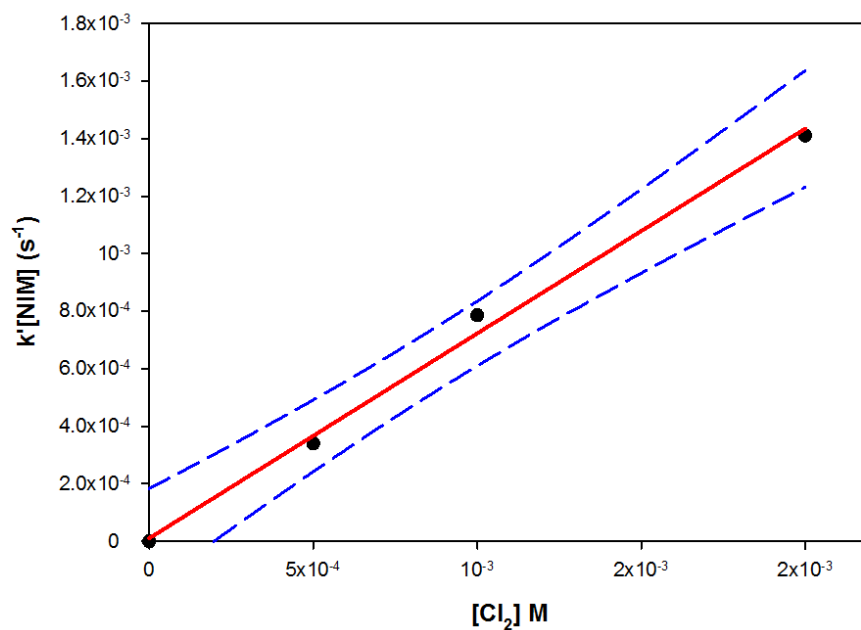


Figure SM3c: k'_{NIM} vs different HOCl concentration for the reactivity constant between NIM and HOCl determination, at pH 3.0.

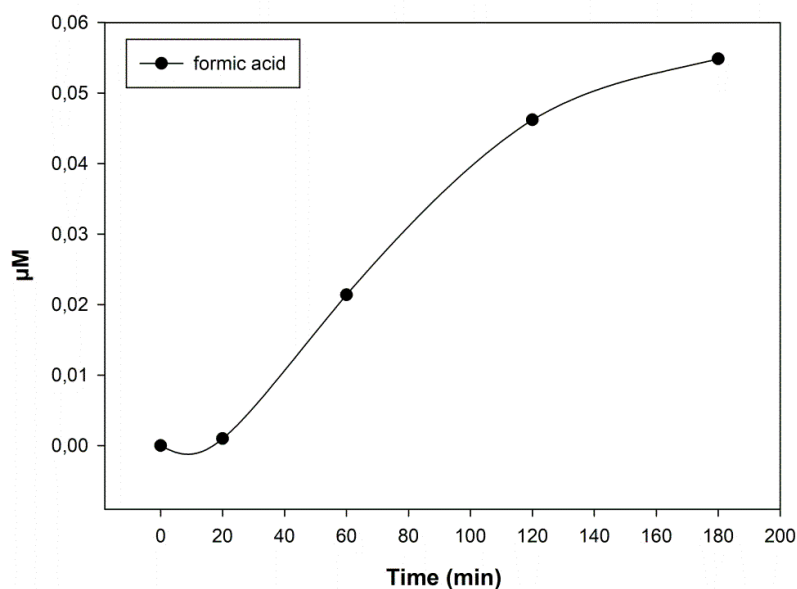


Figure SM4c: Formic acid formation during reaction time under irradiation. Conditions are: $[NIM]$ 25 μM , $[Cl_2] = 0.5$ mM and SSR at pH 7.5

References

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- Klänning, U. K., Wolff, T., 1985. Laser flash photolysis of $HClO$, ClO^- , $HBrO$, and BrO^- in aqueous solution. Reactions of $Cl\cdot$ and $Br\cdot$ atoms. *Berichte der Bunsengesellschaft für physikalische Chemie*, 89(3), 243–245. <https://doi.org/10.1002/bbpc.19850890309>
- Watts, M. J., Linden, K. G., 2007. Chlorine photolysis and subsequent OH radical production during UV treatment of chlorinated water. *Water Research Journal* 41(13), 2871–2878. <https://doi.org/10.1016/j.watres.2007.03.032>

SMD-Supporting Materials D- Irbesartan

IDBP7: Tetrazolo[1,5-*f*]phenanthridine-6-carbaldehyde; white powder; ^1H and ^{13}C NMR in CDCl_3 ; MS-TOF (positive ions): m/z calculated for $\text{C}_{14}\text{H}_8\text{N}_4\text{O}$ 248.07 $[\text{M}]^+$; found 249.11 $[\text{M} + \text{H}]^+$ (72%); 220.23 (15%).

IDBP8: 1-Amino-*N*-(2-hydroxypentanoyl)-*N*-(tetrazolo[1,5-*f*]phenanthridin-6-ylmethyl)cyclopentane carboxamide; white powder; ^1H and ^{13}C NMR in $\text{CDCl}_3/\text{CD}_3\text{OD}$ 9:1; MS-TOF (positive ions): m/z calculated for $\text{C}_{25}\text{H}_{28}\text{N}_6\text{O}_3$ 460.22 $[\text{M}]^+$; found 461.23 $[\text{M} + \text{H}]^+$ (58%); 443.26 (18%).

IDBP9: 9-((2'-(2H-tetrazol-5-yl)-[1,1'-biphenyl]-4-yl)methyl)-7-hydroxy-7-propyl-6,9-diazaspiro[4.5]decane-8,10-dione; white powder; ^1H and ^{13}C NMR in CD_3OD ; MS-TOF (positive ions): m/z calculated for $\text{C}_{25}\text{H}_{28}\text{N}_6\text{O}_3$ 460.22 $[\text{M}]^+$; found 461.24 $[\text{M} + \text{H}]^+$ (78%).

IDBP10: *N*-(2'-(2H-Tetrazol-5-yl)-[1,1'-biphenyl]-4-yl)methyl)-1-amino-*N*-(2-hydroxypentanoyl)cyclopentanecarboxamide; white powder; ^1H and ^{13}C NMR in CDCl_3 ; MS-TOF (positive ions): m/z calculated for $\text{C}_{25}\text{H}_{30}\text{N}_6\text{O}_3$ 462.24 $[\text{M}]^+$; found 463.26 $[\text{M} + \text{H}]^+$ (61%); 645.27 (33%).

IDBP11: 1-Amino-*N*-pentanoyl-*N*-((2'-(trichloromethyl)-[1,1'-biphenyl]-4-yl)methyl)-cyclopentane carboxamide; white powder; ^1H and ^{13}C NMR in $\text{CDCl}_3/\text{CD}_3\text{OD}$ 9:1; MS-TOF (positive ions): m/z calculated for $\text{C}_{25}\text{H}_{29}\text{Cl}_3\text{N}_2\text{O}_2$ 494.13 $[\text{M}]^+$; found 500.17 $[\text{M} + 6]^+$ (2%); 499.13 $[\text{M} + 5]^+$ (7%); 498.13 $[\text{M} + 4]^+$ (27%); 497.14 $[\text{M} + 3]^+$ (28%); 496.15 $[\text{M} + 2]^+$ (92%); 495.88 $[\text{M} + \text{H}]^+$ (27%); 494.13 $[\text{M}]^+$ (87%).

IDBP12: *N*-((2'-(2H-Tetrazol-5-yl)-[1,1'-biphenyl]-4-yl)methyl)-1-amino-*N*-pentanoylcyclopentane carboxamide; white powder; ^1H and ^{13}C NMR in $\text{CDCl}_3/\text{CD}_3\text{OD}$ 9:1; MS-TOF (positive ions): m/z calculated for $\text{C}_{25}\text{H}_{30}\text{N}_6\text{O}_2$ 446.24 $[\text{M}]^+$; found 447.25 $[\text{M} + \text{H}]^+$ (57%); 361.26 (12%).

IDBP13: *N*-(1-Aminocyclopentanecarbonyl)-2'-(2H-tetrazol-5-yl)-[1,1'-biphenyl]-4-carboxamide; white powder; ^1H and ^{13}C NMR in CD_3OD ; MS-TOF (positive ions): m/z calculated for $\text{C}_{20}\text{H}_{20}\text{N}_6\text{O}_2$ 376.16 $[\text{M}]^+$; found 377.18 $[\text{M} + \text{H}]^+$ (38%).

IDBP14: 2'-(2H-Tetrazol-5-yl)-[1,1'-biphenyl]-4-carbaldehyde; White powder; ^1H and ^{13}C NMR in CDCl_3 ; MS-TOF (positive ions): m/z calculated for $\text{C}_{14}\text{H}_{10}\text{N}_4\text{O}$ 250.09 $[\text{M}]^+$; found 251.11 $[\text{M} + \text{H}]^+$ (68%); 222.12 (25%).

IDBP15: *N*-((2'-(2H-Tetrazol-5-yl)-[1,1'-biphenyl]-4-yl)(hydroxy)methyl)-1-pentanamidocyclopentane carboxamide; white powder; ^1H and ^{13}C NMR in CDCl_3 ; MS-TOF (positive ions): m/z calculated for $\text{C}_{25}\text{H}_{30}\text{N}_6\text{O}_3$ 462.24 $[\text{M}]^+$; found 463.26 $[\text{M} + \text{H}]^+$ (37%).

IDBP16: *N*-(1-Pentanamidocyclopentanecarbonyl)-2'-(2H-tetrazol-5-yl)-[1,1'-biphenyl]-4-carboxamide; white powder; ^1H and ^{13}C NMR in CDCl_3 ; MS-TOF (positive ions): m/z calculated for $\text{C}_{25}\text{H}_{28}\text{N}_6\text{O}_3$ 460.22 $[\text{M}]^+$; found 461.23 $[\text{M} + \text{H}]^+$ (31%).

IDBP17: *N*-((2'-(2H-Tetrazol-5-yl)-[1,1'-biphenyl]-4-yl)(hydroxy)methyl)pentanamide; white powder; NMR spectra conform to those recorded for the available standard (Luongo et al., 2020).

IDBP18: 4'-((2-Butyl-4-oxo-1,3-diazaspiro[4.4]non-1-en-3-yl)methyl)-[1,1'-biphenyl]-2-carboxamide; white powder; ^1H and ^{13}C NMR in CDCl_3 ; MS-TOF (positive ions): m/z calculated for $\text{C}_{25}\text{H}_{29}\text{N}_3\text{O}_2$ 403.52 $[\text{M}]^+$; found 404.54 $[\text{M} + \text{H}]^+$ (42%).

IDBP19: 3-((2'-(2H-Tetrazol-5-yl)-[1,1'-biphenyl]-4-yl)methyl)-2-(4-hydroxybutyl)-1,3-diazaspiro[4.4]non-1-en-4-one.; MS-TOF (positive ions): m/z calculated for $\text{C}_{25}\text{H}_{28}\text{N}_6\text{O}_2$ 444.23 $[\text{M}]^+$; found 445.23 $[\text{M} + \text{H}]^+$ (63%).

IDBP19: 3-((2'-(2H-Tetrazol-5-yl)-[1,1'-biphenyl]-4-yl)methyl)-2-(4-hydroxybutyl)-1,3-diazaspiro[4.4]non-1-en-4-one.; MS-TOF (positive ions): m/z calculated for $\text{C}_{25}\text{H}_{28}\text{N}_6\text{O}_2$ 444.23 $[\text{M}]^+$; found 445.23 $[\text{M} + \text{H}]^+$ (63%).

IDBP20: 3-((2'-(2H-Tetrazol-5-yl)-[1,1'-biphenyl]-4-yl)methyl)-2-(3-hydroxybutyl)-1,3-diazaspiro[4.4]non-1-en-4-one; MS-TOF (positive ions): m/z calculated for $\text{C}_{25}\text{H}_{28}\text{N}_6\text{O}_2$ 444.23 $[\text{M}]^+$; found 445.24 $[\text{M} + \text{H}]^+$ (59%).

IDBP21: 3-((2'-(2H-Tetrazol-5-yl)-[1,1'-biphenyl]-4-yl)methyl)-2-butyl-6-hydroxy-1,3-diazaspiro[4.4]non-1-en-4-one; MS-TOF (positive ions): m/z calculated for $\text{C}_{25}\text{H}_{28}\text{N}_6\text{O}_2$ 444.23 $[\text{M}]^+$; found 445.24 $[\text{M} + \text{H}]^+$ (66%).

IDBP22: 3-((2'-(2H-Tetrazol-5-yl)-[1,1'-biphenyl]-4-yl)methyl)-2-butyl-6-hydroxy-1,3-diazaspiro[4.4]non-1-en-4-one; MS-TOF (positive ions): m/z calculated for $\text{C}_{25}\text{H}_{28}\text{N}_6\text{O}_2$ 444.23 $[\text{M}]^+$; found 445.25 $[\text{M} + \text{H}]^+$ (48%).

IDBP23: 2-Butyl-3-((2-hydroxy-2'-(2H-Tetrazol-5-yl)-[1,1'-biphenyl]-4-yl)methyl)-1,3-diazaspiro[4.4]non-1-en-4-one; MS-TOF (positive ions): m/z calculated for $\text{C}_{25}\text{H}_{28}\text{N}_6\text{O}_2$ 444.23 $[\text{M}]^+$; found 445.27 $[\text{M} + \text{H}]^+$ (71%).

IDBP24: 2-Butyl-3-((5'-hydroxy-2'-(2H-Tetrazol-5-yl)-[1,1'-biphenyl]-4-yl)methyl)-1,3-diazaspiro[4.4]non-1-en-4-one; MS-TOF (positive ions): m/z calculated for $\text{C}_{25}\text{H}_{28}\text{N}_6\text{O}_2$ 444.23 $[\text{M}]^+$; found 445.24 $[\text{M} + \text{H}]^+$ (57%).

IDBP25: 2-Butyl-3-((4'-hydroxy-2'-(2H-Tetrazol-5-yl)-[1,1'-biphenyl]-4-yl)methyl)-1,3-diazaspiro[4.4]non-1-en-4-one; MS-TOF (positive ions): m/z calculated for $C_{25}H_{28}N_6O_2$ 444.23 $[M]^+$; found 445.24 $[M + H]^+$ (71%).

IDBP26: *Valsartanic acid*. Identified by comparison with an authentic sample.

DBP27: 2-Butyl-3-(Tetrazolo[1,5-*f*]phenanthridin-6-ylmethyl)-1,3-diazaspiro[4.4]non-1-en-4-one. White powder. ^1H and ^{13}C NMR in CD_3OD ; MS-TOF (positive ions): m/z calculated for $C_{25}H_{26}N_6O$ 426.22 $[M]^+$; found 427.23 $[M + H]^+$ (63%).

IDBP28: 2-(2-Butyl-4-oxo-1,3-diazaspiro[4.4]non-1-en-3-yl)acetic acid. White powder. ^1H and ^{13}C NMR in CD_3OD ; MS-TOF (positive ions): m/z calculated for $C_{13}H_{20}N_2O_3$ 252.15 $[M]^+$; found 253.16 $[M + H]^+$ (35%).

IDBP29: *Pentanoic acid*. Identified by comparison with an authentic sample.

Table SMd1. ^1H , ^{13}C and 2D NMR data of IDBP7

Position	Residue	$^{13}\text{C}^a$	$^1\text{H}^a$, multiplicity	^1H - ^1H COSY	^1H - ^{13}C HMBC
11	CH	190.01	10.30, s	8.28	129.68, 127.19, 125.41
12	C	129.68			
13	CH	127.19			
14	C	148.81			
15	C	127.19	8.73, d	8.28	125.41
16	CH	126.57			
17	CH	125.41			
18	C	136.92			
19	CH	120.18	8.61, d	7.98, 7.92	127.19, 136.92, 130.95, 119.70
20	CH	125.09			
21	CH	130.95			
22	CH	124.03			
23	C	119.70	8.86, dd	7.98, 7.92	136.92, 125.09, 119.70, 150.62
24	C	150.62			

^aChemical shifts in ppm.**Table SMd2.** ^1H , ^{13}C and 2D NMR data of IDBP8

Position	Residue	$^{13}\text{C}^a$	$^1\text{H}^a$, multiplicity	^1H - ^1H COSY	^1H - ^{13}C HMBC
1,2	CH ₂	25.82	1.89 m, 1.91 m	1.91	25.82, 37.00, 77.20
3,5	CH ₂	37.00			
4	C	77.20			
7	C	159.43			
9	C	186.43	4.94, d, 5.16, d	5.16, 4.94	159.43, 186.43, 114.89, 125.77
11	CH ₂	43.30			
12	C	138.78			
13	CH	114.89			
14	C	129.20	8.36, d	8.45	43.30, 125.77
15	C	121.86			
16	CH	124.93			
17	CH	125.77			
18	C	129.40	8.49, d	8.45	138.78, 129.20, 121.86, 129.40
19	CH	123.06			
20	CH	132.23			
21	CH	129.58			
22	CH	126.38	8.45, dd	8.36, 8.49	43.30, 114.89, 121.86
23	C	118.05			
24	C	147.16			
29	CH	52.50			
30	CH ₂	35.52	7.50, d	7.74	121.86, 129.58, 118.05
31	CH ₂	19.37			
32	CH ₃	12.71			
			7.74, t	7.50, 7.85	129.40, 126.38
			7.85, t	7.74, 8.64	123.06, 118.05
			8.6, d	7.85	129.40, 132.23, 147.16
			4.34, t	1.27	159.43, 35.52, 19.37
			1.27, m	4.34, 1.15	159.43, 52.50, 19.37, 12.71
			1.15, m	1.27, 0.69	52.50, 35.52, 12.71
			0.69, t	1.15	19.37, 12.71

^aChemical shifts in ppm.

Table SMd3. ^1H , ^{13}C and 2D NMR data of IDBP9

Position	Residue	$^{13}\text{C}^a$	$^1\text{H}^a$, multiplicity	^1H - ^1H COSY	^1H - ^{13}C HMBC
1,2	CH_2	26.11	2.02, m	1.89	26.11, 37.72, 77.0
3,5	CH_2	37.72	1.89, m, 2.02, m	2.02 1.89	26.11, 37.72, 187.48
4	C	77.0			
7	C	158.02			
9	C	187.48			
11	CH_2	46.84	5.25, s		158.02, 187.48, 127.48, 127.48
12	C	137.21			
13,17	CH	127.48	7.08, d	7.07	46.84, 138.26, 127.48
14,16	CH	129.07	7.07, d	7.08	137.21, 129.07, 140.47
15	C	138.26			
18	C	140.47			
19	CH	130.80	7.46, d	7.62	138.26, 128.53, 122.03
20	CH	131.49	7.62, t	7.46, 7.57	140.47, 130.62
21	CH	128.53	7.57, t	7.62, 8.23	130.80, 122.03
22	CH	130.62	8.23, d	7.57	140.47, 131.49, 154.38
23	C	122.03			
24	C	154.38			
29	C	83.09			
30	CH_2	46.84	2.67, m	1.88	158.02, 18.23, 13.54
31	CH_2	18.23	1.88, m	2.67, 1.09	83.09, 46.84, 13.54
32	CH_3	13.54	1.09, t	1.88	46.84, 18.23

^aChemical shifts in ppm.**Table SMd4.** ^1H , ^{13}C and 2D NMR data of IDBP10

Position	Residue	$^{13}\text{C}^a$	$^1\text{H}^a$, multiplicity	^1H - ^1H COSY	^1H - ^{13}C HMBC
1,2	CH_2	26.14	1.81, m, 1.93, m	1.93 1.81	26.14, 37.46, 77.0
3,5	CH_2	37.46	1.93, m	1.81	26.14, 37.46, 186.67
4	C	77.0			
7	C	159.67			
9	C	186.67			
11	CH_2	43.48	4.66, d 4.98, d	4.98, 4.66	159.67, 186.67, 126.74
12	C	135.90			
13,17	CH	126.74	7.06, d	7.09	43.48, 126.74, 139.12
14,16	CH	129.76	7.09, d	7.06	135.90, 129.76, 140.90
15	C	139.12			
18	C	140.90			
19	CH	131.19	7.41, dd	7.57, 7.48	139.12, 128.13, 123.04
20	CH	131.70	7.57, dt	7.41, 7.48	140.90, 131.19
21	CH	128.13	7.48, dt	7.57, 7.79	131.19, 123.04
22	CH	131.19	7.79, d	7.48	140.90, 131.70, 155.60
23	C	123.04			
24	C	155.60			

29	CH	52.81	4.31, dd	2.03, 1.39	159.67, 19.54
30	CH ₂	35.71	2.03, m	4.31, 1.28, 1.39	159.67, 13.10
31	CH ₂	19.54	1.28, sest, 1.39, sest	2.03, 0.78	52.81, 13.10
32	CH ₃	13.10	0.78 t	1.28, 1.39	19.54, 35.71

^aChemical shifts in ppm.

Table SMd5. ¹H, ¹³C and 2D NMR data of IDBP11

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
1,2	CH ₂	26.93	1.99, m	1.96, 2.12	38.41, 73.05
3,5	CH ₂	38.41	1.96, m, 2.12, m	1.99, 2.12, 1.96, 1.99	26.93, 73.05, 180.05
4	C	73.05			
7	C	176.01			
9	C	180.05			
11	CH ₂	44.40	4.93, s		126.98, 134.75, 176.01, 180.05
12	C	134.75			
13,17	CH	126.98	7.23, d	7.41	44.40, 126.98, 141.54
14,16	CH	132.14	7.41, d	7.23	132.14, 134.75, 141.89
15	C	141.51			
18	C	141.89			
19	CH	131.47	7.50, m	7.19, 7.51	127.05, 128.82, 141.51
20	CH	134.75	7.51, m	7.19, 7.50	127.72, 141.89
21	CH	128.82	7.19, m	7.51, 8.24	127.05, 131.47
22	CH	127.72	8.24, d	7.19	97.40, 134.75, 141.89
23	C	127.05			
24	C	97.40			
29	CH ₂	37.85	2.49, t	1.35, 1.54	23.19, 28.32, 176.01
30	CH ₂	28.32	1.54, t	1.35, 2.49	13.93, 23.19, 176.01
31	CH ₂	23.19	1.35, sest	0.89, 1.54	13.93, 28.32, 37.85
32	CH ₃	13.93	0.81, t	1.35, 1.54	23.19, 28.32

^aChemical shifts in ppm.

Table SMd6. ¹H, ¹³C and 2D NMR data of IDBP12

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
1,2	CH ₂	28.34	1.65, m	2.05, 2.20	28.34, 39.32, 79.3
3,5	CH ₂	39.32	2.05, m, 2.20, m	1.65, 2.20 1.65, 2.05	28.34, 39.32, 178.59
4	C	79.3			
7	C	180.55			
9	C	178.59			
11	CH ₂	47.06	4.31, s		180.55, 178.59, 131.42
12	C	142.16			
13,17	CH	131.42	6.96, d	7.15	47.06, 145.44, 131.42
14,16	CH	132.99	7.15, d	6.96	142.16, 132.99, 141.88
15	C	145.44			

18	C	141.88			
19	CH	131.76	7.43, d	7.58	145.44, 134.62, 126.65
20	CH	135.19	7.58, t	7.43, 7.50	141.88, 134.52
21	CH	134.62	7.50, t	7.58, 7.85	131.76, 126.65
22	CH	134.52	7.85, d	7.50	141.88, 135.19, 159.06
23	C	126.65			
24	C	159.06			
29	CH ₂	37.38	2.41, t	1.50	180.55, 30.40, 26.31
30	CH ₂	30.40	1.50, quint	2.41, 1.29	37.38, 30.40, 17.56
31	CH ₂	26.31	1.29, sest	1.50, 0.84	37.38, 30.40, 17.56
32	CH ₃	17.56	0.84, t	1.29	30.40, 26.31

^aChemical shifts in ppm.

Table SMd7. ¹H, ¹³C and 2D NMR data of IDBP13

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
1,2	CH ₂	27.06	1.94, m	1.92, 1.99	27.06, 38.67, 77.27
3,5	CH ₂	38.67	1.92, m, 1.99, m	1.94, 1.99 1.92, 1.94	27.06, 38.67, 192.25
4	C	77.27			
9	C	192.25			
11	C	166.70			
12	C	127.40			
13,17	CH	128.61	7.84, d	7.28	166.70, 146.21, 128.61
14,16	CH	130.87	7.28, d	7.84	127.40, 130.87, 142.10
15	C	146.21			
18	C	142.10			
19	CH	131.49	7.56, d	7.63	146.21, 129.37, 128.05
20	CH	131.58	7.63, d	7.56, 7.55	142.10, 131.84
21	CH	129.37	7.55, t	7.63, 7.65	131.49, 128.05
22	CH	131.84	7.65, d	7.55	142.10, 131.58, 159.35
23	C	128.05			
24	C	159.35			

^aChemical shifts in ppm.

Table SMd8. ¹H, ¹³C and 2D NMR data of IDBP14

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
11	CH	191.54	10.06, s		135.88, 130.67
12	C	135.88			
13,17	CH	130.67	7.94, d	7.44	191.54, 145.57, 130.67
14,16	CH	129.83	7.44, d	7.94	135.88, 129.83, 139.93
15	C	145.57			
18	C	139.93			
19	CH	130.67	7.51, d	7.69	145.57, 129.14, 122.37
20	CH	131.61	7.69, t	7.51, 7.64	139.93, 130.88
21	CH	129.14	7.64, t	7.69, 8.13	130.67, 122.37

22	CH	130.88	8.13, d	7.64	139.93, 131.61, 155.09
23	C	122.37			
24	C	155.09			

^aChemical shifts in ppm.

Table SMd9. ¹H, ¹³C and 2D NMR data of IDBP15

Position	residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
1,2	CH ₂	27.52	1.62, m	1.94	27.52, 31.88, 77.0
3,5	CH ₂	31.88	1.94, m	1.62	27.52, 31.88, 169.00
4	C	77.0			
7	C	172.37			
9	C	169.00			
11	CH	83.04	6.66, s		172.37, 169.00, 135.15, 127.53
12	C	135.15			
13,17	CH	127.53	7.26, d	7.28	83.04, 133.02, 127.53
14,16	CH	129.63	7.28, d	7.26	135.15, 129.63, 140.15
15	C	133.02			
18	C	140.15			
19	CH	130.83	7.43, dd	7.61, 7.56	133.02, 128.73, 122.01
20	CH	131.54	7.61, dd	7.43, 7.56	140.15, 130.83
21	CH	128.73	7.56, dd	7.61, 8.19	130.83, 122.01
22	CH	130.83	8.19, dd	7.56, 7.61	140.15, 131.54, 153.90
23	C	122.01			
24	C	153.90			
29	CH ₂	36.13	2.95, t	1.62	172.37, 25.80, 22.16
30	CH ₂	25.80	1.62, m	2.95, 1.36	172.37, 36.13, 22.16, 13.74
31	CH ₂	22.16	1.36, quint	1.62, 0.92	36.13, 25.80, 13.74
32	CH ₃	13.74	0.92, t	1.36	25.80, 22.16

^aChemical shifts in ppm.

Table SMd10. ¹H, ¹³C and 2D NMR data of IDBP16

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
1,2	CH ₂	24.20	1.72, m	1.98, 2.33	24.20, 36.16, 68.29
3,5	CH ₂	36.16	1.98, m, 2.33, m	1.72, 2.33 1.72, 1.98	24.20, 36.16, 168.48
4	C	68.29			
7	C	176.73			
9	C	168.48			
11	C	168.42			
12	C	132.36			
13,17	CH	127.53	7.60, d	7.11	168.42, 143.15, 127.53
14,16	CH	129.20	7.11, d	7.60	132.36, 129.20, 140.52
15	C	143.15			
18	C	140.52			
19	CH	130.53	7.35, d	7.58	143.15, 127.62, 123.32

20	CH	130.77	7.58, dd	7.35, 7.45	140.52, 131.15
21	CH	127.62	7.45, dd	7.58, 7.77	130.53, 123.32
22	CH	131.15	7.77, dd	7.45, 7.58	140.52, 130.77, 154.13
23	C	123.32			
24	C	154.13			
29	CH ₂	37.13	2.71, t	1.54	176.73, 26.21, 22.17
30	CH ₂	26.21	1.54, quint	2.71, 1.30	176.73, 37.13, 22.17, 13.75
31	CH ₂	22.17	1.30, sest	1.54, 0.86	37.13, 26.21, 13.75
32	CH ₃	13.75	0.86, t	1.30	26.21, 22.17

^aChemical shifts in ppm.

Table SMd11. ¹H, ¹³C and 2D NMR data of IDBP17

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
1,2	CH ₂	27.04	1.98, m	1.81, 2.02	77.55
3,5	CH ₂	38.41	1.81, m, 2.02, m	1.98, 2.02, 1.81, 1.98	77.55, 187.92
4	C	77.55			
7	C	165.32			
9	C	187.92			
11	C	44.22			
12	C	137.01			
13,17	CH	127.62	7.23, d	7.29	44.22, 127.62, 142.36
14,16	CH	130.08	7.29, d	7.23	130.08, 137.01, 143.02
15	C	142.36			
18	C	143.02			
19	CH	131.74	7.38, d	7.45, 7.58	128.52, 137.01, 142.36
20	CH	132.56	7.58, d	7.38, 7.45	130.73, 143.02
21	CH	128.52	7.45, d	7.58, 7.78	131.74, 137.01
22	CH	130.73	7.78, d	7.45, 7.58	132.56, 143.02, 170.51
23	C	137.01			
24	C	170.51			
29	CH ₂	29.50	2.50, t	1.31, 1.52	23.73, 29.45, 165.32
30	CH ₂	29.45	1.52, quint	1.31, 2.50	13.97, 23.73, 29.50
31	CH ₂	23.73	1.31, sest	0.89, 1.52	13.97, 29.45
32	CH ₃	13.97	0.89, t	1.31, 1.52	23.73, 29.45

^aChemical shifts in ppm.

Table SMd12. ¹H, ¹³C and 2D NMR data of DBP18

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
1,2	CH ₂	27.04	1.98, m	1.81, 2.02	77.55
3,5	CH ₂	38.41	1.81, m, 2.02, m	1.98, 2.02 1.81, 1.98	77.55, 187.92
4	C	77.55			
7	C	165.32			
9	C	187.92			
11	C	44.22			

12	C	137.01			
13,17	CH	127.62	7.23, d	7.29	44.22, 127.62, 142.36
14,16	CH	130.08	7.29, d	7.23	130.08, 137.01, 143.02
15	C	142.36			
18	C	143.02			
19	CH	131.74	7.38, d	7.45, 7.58	128.52, 137.01, 142.36
20	CH	132.56	7.58, t	7.38, 7.45	130.73, 143.02
21	CH	128.52	7.45, t	7.58, 7.78	131.74, 137.01
22	CH	130.73	7.78, d	7.45, 7.58	132.56, 143.02, 170.51
23	C	137.01			
24	C	170.51			
29	CH ₂	29.50	2.50, t	1.31, 1.52	23.73, 29.45, 165.32
30	CH ₂	29.45	1.52, quint	1.31, 2.50	13.97, 23.73, 29.50
31	CH ₂	23.73	1.31, sest	0.89, 1.52	13.97, 29.45
32	CH ₃	13.97	0.89, t	1.31, 1.52	23.73, 29.45

Table SMd13. ¹H, ¹³C and 2D NMR data of IDBP19

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
1,2	CH ₂	25.62	1.96, m	1.80	76.20, 25.62
3,5	CH ₂	36.96	1.80, m, 1.96, m	1.96 1.80	186.39, 36.96 186.39, 36.96
4	C	76.20	-		
7	C	163.90	-		
9	C	186.39	-		
11	CH ₂	42.95	4.75, s		186.39, 163.90, 135.81, 126.45
12	C		-		
13,17	CH	126.45	7.12, s		141.37, 139.62, 135.81,
14,16	CH	129.72	7.12, s		129.72, 126.45, 42.95
15	C	139.62	-		
18	C	141.37	-		
19	CH	129.75	7.53, m	7.62	139.62, 127.45, 125.17
20	CH	130.30	7.62, m	7.53, 7.52	141.37, 130.27
21	CH	127.45	7.52, m	7.62	129.75, 125.17
22	CH	130.27	7.62, m	7.52	157.21, 141.37, 130.30
23	C	125.17	-		
24	C	157.21	-		
29	CH ₂	29.52	1.34, m	1.59	31.20
30	CH ₂	21.40	1.59, m	1.52, 1.34	163.90, 60.87
31	CH ₂	31.20	1.52, m	3.51, 1.59	29.52, 21.40
32	CH ₂	60.87	3.51, t	1.52	31.20, 21.40

^aChemical shifts in ppm.

Table SMd14. ¹H, ¹³C and 2D NMR data of IDBP20.

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
1,2	CH ₂	25.30	1.96, m	1.79	76.17, 25.30
3,5	CH ₂	37.50	1.79, m, 1.96, m	1.96, 1.79	186.44, 37.50

						186.44, 37.50
4	C	76.17	-			
7	C	164.03	-			
9	C	186.44	-			
11	CH ₂	42.60	4.75, s			186.44, 164.03, 135.62, 126.41
12	C	135.62	-			
13,17	CH	126.41	7.13, s			141.36, 139.68, 135.62, 129.43, 126.41, 42.60
14,16	CH	129.43	7.13, s			
15	C	139.68	-			
18	C	141.36	-			
19	CH	130.14	7.53, m	7.64		139.68, 127.40, 125.33
20	CH	130.30	7.64, m	7.53		141.36, 130.20
21	CH	127.40	7.53, m	7.64		130.14, 125.33
22	CH	130.20	7.64, m	7.53		157.63, 141.36, 130.30
23	C	125.33	-			
24	C	157.63	-			
29	CH ₂	22.18	2.45, m, 2.54, m	2.54, 1.71, 1.65 2.45, 1.71, 1.65 3.74, 2.54, 2.45, 1.71		66.41, 33.80 66.41, 33.80
30	CH ₂	33.80	1.65, m, 1.71, m	3.74, 2.54, 2.45, 1.65		164.03, 22.18 164.03, 22.18
31	CH	66.41	3.74, m	1.71, 1.65, 1.14		22.18
32	CH ₃	22.18	1.14, d	3.74		66.41, 33.80

^aChemical shifts in ppm.

Table SMd15. ¹H, ¹³C and 2D NMR data of DBP21.

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
1	CH ₂	34.60	2.22, m	2.09, 1.98, 1.89, 1.71	74.90, 72.75
2	CH ₂	34.60	1.71, m, 1.89, m	4.54, 2.22, 1.89 4.54, 2.22, 1.71	74.90, 44.60 74.90, 44.60
3	CH	72.75	4.54, t	1.89, 1.71	185.70, 44.60, 34.60
4	C	74.90	-		
5	CH ₂	44.60	1.98, m, 2.09, m	2.22, 2.09 2.22, 1.98	185.70, 72.75, 34.60 185.70, 72.75, 34.60
7	C	164.10	-		
9	C	185.70	-		
11	CH ₂	42.90	4.77, s		185.70, 164.10, 135.70, 126.35
12	C	135.70	-		
13,17	CH	126.35	7.11, s	7.12	139.58, 126.35, 42.90
14,16	CH	129.50	7.12, s	7.11	141.17, 135.70, 129.50
15	C	139.58	-		
18	C	141.17	-		

19	CH	130.06	7.53, m	7.62	139.58, 127.55, 124.64
20	CH	130.70	7.62, m	7.54, 7.53	141.17, 130.20
21	CH	127.55	7.54, m	7.62	130.06, 124.64
22	CH	130.20	7.62, m	7.54	157.09, 141.17, 130.70
23	C	124.64	-		
24	C	157.09	-		
29	CH ₂	25.60	1.96, m	1.50	26.55, 21.56,
30	CH ₂	26.55	1.50, quint	1.96, 1.32	164.10, 13.05
31	CH ₂	21.56	1.32, sest	1.50, 0.86	25.60
32	CH ₃	13.05	0.86, t	1.32	26.55, 21.56

^aChemical shifts in ppm.

Table SMd16. ¹H, ¹³C and 2D NMR data of DBP22.

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
1	CH ₂	34.31	2.23, m	2.07, 1.98, 1.89, 1.75	74.80, 72.70
2	CH ₂	34.44	1.75, m, 1.89, m	4.53, 2.23, 1.89	74.80, 44.10
3	CH	72.70	4.53, t	4.53, 2.23, 1.75	74.80, 44.10
4	C	74.80		1.89, 1.75	185.75, 34.31
5	CH ₂	44.60	1.98, m, 2.07, m	2.23, 2.07 2.23, 1.98	185.75, 72.70, 34.44 185.75, 72.70, 34.44
7	C	164.30	-		
9	C	185.75	-		
11	CH ₂	42.95	4.70, s		185.75, 164.30, 134.40, 125.70
12	C	134.40	-		
13,17	CH	127.70	7.03, d	7.11	140.75, 125.70, 42.95
14,16	CH	129.65	7.11, d	7.03	141.35, 134.40, 129.65
15	C	140.75	-		
18	C	141.35	-		
19	CH	130.98	7.42, dd	7.48	140.75, 129.50, 126.30
20	CH	128.60	7.48, dt	7.42, 7.41	141.35, 130.40
21	CH	129.50	7.41, dt	7.53, 7.48	130.98, 126.30
22	CH	130.40	7.53, dd	7.41	161.10, 141.35, 128.60
23	C	126.30	-		
24	C	161.10	-		
29	CH ₂	27.50	2.37, t	1.51	27.25, 21.83
30	CH ₂	27.25	1.51, quint	2.37, 1.33	164.30, 12.45
31	CH ₂	21.83	1.33, m	1.51, 0.86	27.50
32	CH ₃	12.45	0.86, t	1.33	27.25, 21.83

^aChemical shifts in ppm.

Table SMd17. ^1H , ^{13}C and 2D NMR data of DBP23.

Position	Residue	$^{13}\text{C}^a$	$^1\text{H}^a$, multiplicity	^1H - ^1H COSY	^1H - ^{13}C HMBC
1,2	CH_2	25.60	1.99, m	1.83	76.10, 37.10, 25.60
3,5	CH_2	37.10	1.99, m, 1.83, m	1.83, 1.99	186.40, 37.10, 25.60 186.40, 37.10, 25.60
4	C	76.10	-		
7	C	164.05	-		
9	C	186.40	-		
11	CH_2	42.80	4.68, s		186.40, 164.05, 137.95, 117.70, 113.05
12	C	137.95	-		
13	CH	113.05	6.51, d	6.73	126.60, 117.70, 42.80
14	C	154.40	-		
15	C	126.60	-		
16	CH	131.50	7.17, d	6.73	154.40, 138.45, 137.95
17	CH	117.70	6.73, dd	7.17, 6.51	126.60, 113.05, 42.80
18	C	138.45	-		
19	CH	131.10	7.48, d	7.63	127.20, 126.60, 125.03
20	CH	130.20	7.63 dt	7.67, 7.48	138.45, 129.25
21	CH	127.20	7.48, t	7.67, 7.63	131.10, 125.03
22	CH	129.25	7.67, d	7.48	138.45, 130.20
23	C	125.03	-		
24	C	156.60	-		
29	CH_2	27.33	2.41, t	1.55	27.05, 21.80
30	CH_2	27.05	1.55, quint	2.41, 1.35	164.05, 12.50
31	CH_2	21.80	1.35, sest	1.55, 0.91	27.33, 12.50
32	CH_3	12.50	0.91, t	1.35	27.05, 21.80

^aChemical shifts in ppm.**Table SMd18.** ^1H , ^{13}C and 2D NMR data of DBP24.

Position	Residue	$^{13}\text{C}^a$	$^1\text{H}^a$, multiplicity	^1H - ^1H COSY	^1H - ^{13}C HMBC
1,2	CH_2	25.65	1.94, m	1.80	76.10, 25.65
3,5	CH_2	37.03	1.80, m, 1.94, m	1.94, 1.80	186.48, 37.03, 25.65 186.48, 37.03, 25.65
4	C	76.10	-		
7	C	163.90	-		
9	C	186.48	-		
11	CH_2	42.70	4.72, s		186.48, 163.90, 134.71, 125.73
12	C	134.71	-		
13,17	CH	125.73	7.01, d	7.10	140.90, 125.73, 42.70
14,16	CH	129.25	7.10, d	7.01	142.80, 134.71, 129.25
15	C	140.90	-		
18	C	142.80	-		
19	CH	116.39	6.85, d	6.83	140.90, 120.42, 113.68
20	C	157.90	-		
21	CH	113.68	6.83, dd	7.35, 6.85	120.42, 116.39
22	CH	131.55	7.35, d	6.83	161.27, 157.90, 142.80
23	C	120.42	-		
24	C	161.27	-		

29	CH ₂	27.64	2.39, t	1.51	27.40, 21.90
30	CH ₂	27.40	1.51, quint	2.39, 1.33	163.90, 12.70
31	CH ₂	21.90	1.33, sest	1.51, 0.89	27.64, 12.70
32	CH ₃	12.70	0.89, t	1.33	27.40, 21.90

^aChemical shifts in ppm.

Table SMd19. ¹H, ¹³C and 2D NMR data of DBP25.

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
1,2	CH ₂	26.93	1.96, m	1.97, 1.78	77.33, 38.29, 26.93
3,5	CH ₂	38.29	1.78, m, 1.97, m	1.97, 1.96 1.96, 1.78	187.64, 38.29, 26.93 187.64, 38.29, 26.93
4	C	77.33	-		
7	C	165.41	-		
9	C	187.64	-		
11	CH ₂	44.06	4.73, s		187.64, 165.41, 136.54, 127.69
12	C	136.54	-		
13,17	CH	127.69	7.09, d	7.06	140.60, 127.69, 44.06
14,16	CH	130.68	7.06, d	7.09	136.54, 133.82, 130.68
15	C	140.60	-		
18	C	133.82	-		
19	CH	132.97	7.34, d	7.05	157.43, 140.60, 125.96
20	CH	117.99	7.05, d	7.34	133.82, 119.07
21	C	157.43	-		
22	CH	119.07	7.04, s		157.41, 133.82, 117.99
23	C	125.96	-		
24	C	157.41	-		
29	CH ₂	29.45	1.31, m	1.50, 1.32	28.44, 23.06
30	CH ₂	28.44	1.50, quint	1.32, 1.31	165.41, 13.91
31	CH ₂	23.06	1.32, sest	1.50, 0.87	29.45, 13.91
32	CH ₃	13.91	0.87, t	1.32	28.44, 23.06

^aChemical shifts in ppm.

Table SMd20 ¹H, ¹³C and 2D NMR data of DBP27.

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
1,2	CH ₂	25.26	2.06, m	2.08, 1.91	77.12, 36.64, 25.26
3,5	CH ₂	36.64	1.91, m 2.08, m	2.08, 2.06 2.06, 1.91	186.21, 36.64, 25.26 186.21, 36.64, 25.26
4	C	77.12	-		
7	C	163.35	-		
9	C	186.21	-		
11	CH ₂	42.70	5.10, s		186.21, 163.35, 139.80, 126.30, 114.80
12	C	139.80	-		

13	CH	114.80	8.47, s	7.66	126.30, 121.78, 42.70
14	C	129.62	-		
15	C	1217.78	-		
16	CH	124.48	8.76, d	7.66	139.80, 129.80, 129.62
17	CH	126.30	7.66, dd	8.76, 8.47	121.78, 114.80, 42.70
18	C	129.80	-		
19	CH	123.24	8.72, d	7.98	129.67, 121.78, 118.05
20	CH	134.43	7.98, d	8.72, 7.88	129.80, 124.71
21	CH	129.67	7.88, d	8.68, 7.98	123.24, 118.05
22	CH	124.71	8.68, d	7.88	147.51, 134.43, 129.80
23	C	118.05	-		
24	C	147.51	-		
29	CH ₂	27.80	1.31, t	1.58	26.81, 21.60
30	CH ₂	26.81	1.58, quint	1.35, 1.31	163.35, 11.98
31	CH ₂	21.60	1.35, sest	1.58, 0.82	27.80
32	CH ₃	11.98	0.82, t	1.35	26.81, 21.60

^aChemical shifts in ppm.

Table SMd21. ¹H, ¹³C and 2D NMR data of DBP28.

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
1,2	CH ₂	25.30	1.92, m	1.80	75.60, 25.30
3,5	CH ₂	36.75	1.80, m 1.92, m	1.92, 1.80	185.47, 36.75 185.47, 36.75
4	C	75.60	-		
7	C	164.76	-		
9	C	185.47	-		
11	CH ₂	43.05	4.21, s		185.47, 170.71, 164.76
12	C	170.71	-		
13	CH ₂	27.25	1.42, t	1.66	27.10, 21.90
14	CH ₂	27.10	1.66, quint	1.45, 1.42	164.76, 12.50
15	CH ₂	21.90	1.45, sest	1.66, 0.96	27.25
16	CH ₃	12.50	0.96, t	1.45	27.10, 21.90

^aChemical shifts in ppm.

Table SMd22 ¹H, ¹³C and 2D NMR data of Irbesartan.

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
1,2	CH ₂	26.99	1.96, m	1.80	77.24, 38.36, 26.99
3,5	CH ₂	38.36	1.96, m 1.80, m	1.80 1.96	187.42, 77.24, 38.36, 26.99 187.42, 77.24, 38.36, 26.99
4	C	77.24	-		
7	C	165.76	-		
9	C	187.42	-		
11	CH ₂	44.13	4.76, s		187.42, 165.76, 137.37, 127.94
12	C	137.37	-		
13,17	CH	127.94	7.15, m	7.13	140.49, 127.94, 44.13
14,16	CH	130.74	7.13, m	7.15	142.88, 137.37, 130.74
15	C	140.49	-		
18	C	142.88	-		

19	CH	131.64	7.54, dt	7.66, 7.56	140.49, 129.08, 124.76
20	CH	132.39	7.66, dt	7.56, 7.54	142.88, 131.76
21	CH	129.08	7.56, dt	7.66, 7.65	131.64, 124.76
22	CH	131.76	7.65, t	7.66, 7.56	157.03, 142.88, 132.39
23	C	124.76	-		
24	C	157.03	-		
29	CH ₂	28.53	2.39, m	1.50, 1.33	165.76, 28.46, 23.13
30	CH ₂	28.46	1.50, q	2.39, 1.33	165.76, 28.53, 13.98
31	CH ₂	23.13	1.33, sest	1.50, 0.87	28.53, 13.98
32	CH ₃	13.98	0.87, t	1.33	28.46, 23.13

SME-Supporting Materials E- Valsartan

Valsartan: 2-(N-((2'-(2H-tetrazol-5-yl)-[1,1'-biphenyl]-4-yl)methyl)pentanamido)-3-methyl butanoic acid. White powder. ¹H- and ¹³C-NMR, see Table SMe1. MS-TOF (positive ions): *m/z* calculated for C₂₄H₂₉N₅O₃ *m/z* 435.23 [M]⁺; found 436.53 [M + H]⁺ (60%). UV (CH₃CN) λ_{max}/nm 254 (ε/dm³ mol⁻¹ cm⁻¹, 6,600).

VDBP1: Valsartan N-oxide. White powder. ¹H- and ¹³C-NMR, see Table SMe2. MS-TOF (positive ions): *m/z* calculated for C₂₄H₂₉N₅O₄ *m/z* 451.22 [M]⁺; found 452.53 [M + H]⁺ (45%). UV (CH₃CN) λ_{max}/nm 259 (ε/dm³ mol⁻¹ cm⁻¹, 11,000).

VDBP2: 2-(N-((2'-(2H-tetrazol-5-yl)-[1,1'-biphenyl]-4-yl)-(hydroxy)methyl)pentanamido)-3-methylbutanoic acid. White powder. ¹H- and ¹³C-NMR, see Table SMe3. MS-TOF (positive ions): *m/z* calculated for C₂₄H₂₉N₅O₄ *m/z* 451.22 [M]⁺; found 452.54 [M + H]⁺ (54%). UV (CH₃CN) λ_{max}/nm 248 (ε/dm³ mol⁻¹ cm⁻¹, 13,600).

VDBP3: 2'-(2H-tetrazol-5-yl)-[1,1'-biphenyl]-4-carboxylic acid. Identified by comparison with an authentic sample.

VDBP4: N-((2'-(2H-tetrazol-5-yl)-[1,1'-biphenyl]-4-yl)methyl)pentanamide. White powder. ¹H- and ¹³C-NMR, see Table SMe4. MS-TOF (positive ions): *m/z* calculated for C₁₉H₂₁N₅O *m/z* 335.17 [M]⁺; found 336.41 [M + H]⁺. UV (CH₃CN) λ_{max}/nm 248 (ε/dm³ mol⁻¹ cm⁻¹, 20,000), 241 (15,100).

VDBP5: N-(tetrazolo[1,5-f]phenanthridin-6-ylmethyl)pentanamide. White powder. ¹H- and ¹³C-NMR, see Table SMe5. MS-TOF (positive ions): *m/z* calculated for C₁₉H₁₉N₅O *m/z* 333.16 [M]⁺; found 334.38 [M + H]⁺. UV (CH₃CN) λ_{max}/nm 248 (ε/dm³ mol⁻¹ cm⁻¹, 21,000), 241 (16,000).

VDBP6: 3-methyl-2-(N-(tetrazolo[1,5-f]phenanthridin-6-ylmethyl)pentanamido)butanoic acid. White powder. ^1H - and ^{13}C -NMR, see Table SMe6. MS-TOF (positive ions): m/z calculated for $\text{C}_{24}\text{H}_{27}\text{N}_5\text{O}_3$ m/z 433.21 $[\text{M}]^+$; found 434.50 $[\text{M} + \text{H}]^+$. UV (CH_3CN) $\lambda_{\text{max}}/\text{nm}$ 249 ($\epsilon/\text{dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$, 22,000), 241 (18,000).

VDBP7: 3-methyl-2-(N-(tetrazolo[1,5-f]phenanthridin-6-ylmethyl)pentanamido)butanoic acid N-oxide. White powder. ^1H - and ^{13}C -NMR, see Table SMe7. MS-TOF (positive ions): m/z calculated for $\text{C}_{24}\text{H}_{27}\text{N}_5\text{O}_4$ m/z 449.21 $[\text{M}]^+$; found 450.52 $[\text{M} + \text{H}]^+$. UV (CH_3CN) $\lambda_{\text{max}}/\text{nm}$ 249 ($\epsilon/\text{dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$, 21,000), 241 (17,000).

VDBP8: N-isobutyryl-N-(tetrazolo[1,5-f]phenanthridin-6-ylmethyl)pentanamide. White powder. ^1H - and ^{13}C -NMR, see Table SMe8. MS-TOF (positive ions): m/z calculated for $\text{C}_{23}\text{H}_{25}\text{N}_5\text{O}_2$ m/z 403.20 $[\text{M}]^+$; found 404.49 $[\text{M} + \text{H}]^+$. UV (CH_3CN) $\lambda_{\text{max}}/\text{nm}$ 248 ($\epsilon/\text{dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$, 26,600), 241 (22,000).

VDBP9: N-formyl-N-(tetrazolo[1,5-f]phenanthridin-6-ylmethyl)pentanamide. White powder. ^1H - and ^{13}C -NMR, see Table SMe9. MS-TOF (positive ions): m/z calculated for $\text{C}_{20}\text{H}_{19}\text{N}_5\text{O}_2$ m/z 361.15 $[\text{M}]^+$; found 362.40 $[\text{M} + \text{H}]^+$. UV (CH_3CN) $\lambda_{\text{max}}/\text{nm}$ 248 ($\epsilon/\text{dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$, 38,000), 241 (31,000).

VDBP10: 2-(((2'-(2H-tetrazol-5-yl)-[1,1'-biphenyl]-4-yl)methyl)amino)-3-methylbutanoic acid. White powder. ^1H - and ^{13}C -NMR, see Table SMe10. MS-TOF (positive ions): m/z calculated for $\text{C}_{19}\text{H}_{21}\text{N}_5\text{O}_2$ m/z 351.17 $[\text{M}]^+$; found 352.29 $[\text{M} + \text{H}]^+$.

VDBP11: 2-(((2'-(2H-tetrazol-5-yl)-[1,1'-biphenyl]-4-yl)methyl)(hydroxyamino)-3-methyl butanoic acid. White powder. ^1H - and ^{13}C -NMR, see Table S11. MS-TOF (positive ions): m/z calculated for $\text{C}_{19}\text{H}_{21}\text{N}_5\text{O}_3$ m/z 367.16 $[\text{M}]^+$; found 368.31 $[\text{M} + \text{H}]^+$.

VDBP12: Benzoic acid. Identified by comparison with an authentic sample.

VDBP13: Phenol. Identified by comparison with an authentic sample.

VDBP14: 4-Hydroxybenzoic acid. Identified by comparison with an authentic sample.

Table SMe1. ^1H , ^{13}C and 2D NMR data of Valsartan in CD_3OD .

Position	Residue	$^{13}\text{C}^a$	$^1\text{H}^a$, multiplicity	^1H - ^1H COSY	^1H - ^{13}C HMBC
1	CH_3	14.23	0.94, t	1.37	23.45, 28.60
2	CH_2	23.45	1.37, sest	1.65, 0.94	34.42, 28.60, 14.23
3	CH_2	28.60	1.65, m	2.62, 2.50, 1.37	176.99, 34.42, 23.45, 14.23
4	CH_2	34.42	2.62, m 2.50, m	2.50, 1.65 2.62, 1.65	28.60, 23.45
5	C	176.99	-		
6	CH	67.96	4.13, d	2.28	176.99, 172.99, 47.37, 29.26, 20.02, 19.29
7	CH	29.26	2.28, m	4.13, 1.02, 0.83	172.99, 67.96, 20.02, 19.29
8	CH_3	19.29	0.83, d	2.28	67.96, 29.26, 20.02
9	CH_3	20.02	1.02, d	2.28	67.96, 29.26, 19.29
10	C	172.99	-		
11	CH_2	47.37	4.61, s		176.99, 67.96, 138.96, 128.68
12	C	138.96	-		
13/17	CH	128.68	7.18, d	7.18	139.52, 128.68, 47.37
14/16	CH	129.00	7.02, d	7.02	143.28, 138.96, 129.00
15	C	139.52	-		
18	C	143.28	-		
19	C	124.22	-		
20	CH	132.52	7.68, dd	7.64, 7.52	156.65, 143.28, 131.89
21	CH	128.87	7.52, dt	7.68, 7.64, 7.56	131.68, 124.22
22	CH	131.89	7.64, dt	7.68, 7.56, 7.52	143.28, 132.52
23	CH	131.68	7.56, dd	7.64, 7.52	139.52, 128.87, 124.22
24	C	156.65	-		

^aChemical shifts in ppm.**Table SMe2.** ^1H , ^{13}C and 2D NMR data of VDBP1 in CD_3OD .

Position	Residue	$^{13}\text{C}^a$	$^1\text{H}^a$, multiplicity	^1H - ^1H COSY	^1H - ^{13}C HMBC
1	CH_3	14.00	0.82, t	1.23	23.38, 28.49
2	CH_2	23.38	1.23, sest	1.51, 0.82	34.52, 28.49, 14.00
3	CH_2	28.49	1.51, m	2.32, 2.18, 1.23	177.20, 34.52, 23.38, 14.00
4	CH_2	34.52	2.18, m 2.32, m	2.32, 1.51 2.18, 1.51	28.49, 23.38
5	C	177.20	-		
6	CH	64.97	4.57, d	2.29	177.20, 173.62, 50.61, 29.21, 20.61, 19.28
7	CH	29.21	2.29, m	4.57, 1.00, 0.84	173.62, 64.97, 20.61, 19.28
8	CH_3	19.28	0.84, d	2.29	64.97, 29.21, 20.61
9	CH_3	20.61	1.00, d	2.29	64.97, 29.21, 19.28
10	C	173.62	-		
11	CH_2	50.60	4.71, s		177.20, 64.97, 138.79, 127.81
12	C	138.79	-		
13/17	CH	127.81	7.24, d	7.10	139.72, 127.81, 50.60

14/16	CH	130.37	7.10, d	7.24	143.15, 138.79, 130.37
15	C	139.72	-		
18	C	143.15	-		
19	C	124.40	-		
20	CH	132.52	7.68, dd	7.52	156.77, 143.15, 131.82
21	CH	129.85	7.52, dt	7.68, 7.64	131.64, 124.40
22	CH	131.82	7.64, dt	7.56, 7.52	143.15, 132.52
23	CH	131.64	7.56, dd	7.64	139.72, 129.85, 124.40
24	C	156.77	-		

^aChemical shifts in ppm.

Table SMe3. ¹H, ¹³C and 2D NMR data of VDBP2 in CDCl₃.

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
1	CH ₃	13.37	0.92, t	1.32	26.53, 21.91
2	CH ₂	21.91	1.32, sest	1.57, 0.92	34.54, 26.53, 13.37
3	CH ₂	26.53	1.57, quint	2.34, 1.32	177.05, 34.54, 21.91, 13.37
4	CH ₂	34.54	2.34, m	1.57	26.53, 21.91
5	C	177.05	-		
6	CH	61.05	4.51, d	2.43	177.05, 168.85, 90.45, 33.06, 18.15, 18.12
7	CH	33.06	2.43, m	4.51, 1.32, 1.31	168.85, 61.05, 18.15, 18.12
8	CH ₃	18.12	1.31, d	2.43	61.05, 33.06, 18.15
9	CH ₃	18.15	1.32, d	2.43	61.05, 33.06, 18.12
10	C	168.85	-		
11	CH ₂	90.45	6.53, s		177.05, 61.05, 136.57, 127.10
12	C	136.57	-		
13/17	CH	127.10	7.36, d	7.25	140.79, 127.10, 90.45
14/16	CH	129.61	7.25, d	7.36	140.02, 136.57, 129.61
15	C	140.79	-		
18	C	140.02	-		
19	C	122.51	-		
20	CH	131.20	8.10, d	7.58	154.10, 140.02, 131.11
21	CH	128.25	7.58, t	8.10, 7.63	130.91, 122.51
22	CH	131.11	7.63, dt	7.58, 7.47	140.02, 131.20
23	CH	130.91	7.47, d	7.63, 7.58	140.79, 128.25, 122.51
24	C	154.10	-		

^aChemical shifts in ppm.

Table SMe4. ¹H, ¹³C and 2D NMR data of VDBP4 in DMSO-*d*₆.

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity (J in Hz)	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
1	CH ₃	15.60	0.92, t	1.35	28.35, 23.70
2	CH ₂	23.70	1.35, sest	1.59, 0.92	34.88, 28.35, 15.60
3	CH ₂	28.35	1.59, quint	2.25, 1.35	176.60, 34.88, 23.70, 15.60

4	CH ₂	34.88	2.25, t	1.59	28.35, 23.70
5	C	176.60	-		
6	CH ₂	43.45	4.78, s		176.60, 138.60, 128.90
7	C	138.60	-		
8/12	CH	128.90	7.15, d	7.04	140.00, 128.90, 43.45
9/11	CH	130.50	7.04, d	7.15	142.30, 138.60, 130.50
10	C	140.00	-		
13	C	142.30	-		
14	CH	132.75	7.53, d	7.67, 7.57	140.00, 129.90, 125.44
15	CH	133.00	7.67, dt	8.64, 7.57, 7.53	142.30, 133.02
16	CH	129.90	7.57, dt	8.64, 7.67, 7.53	132.75, 125.44
17	CH	133.02	8.64, d	7.67, 7.57	157.60, 142.30, 133.00
18	C	125.44	-		
19	C	157.60	-		

^aChemical shifts in ppm.

Table SMe5. ¹H, ¹³C and 2D NMR data of VDBP5 in DMSO-*d*6.

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
1	CH ₃	15.62	0.92, t	1.35	29.37, 23.72
2	CH ₂	23.72	1.35, sest	1.59, 0.92	36.98, 29.37, 15.62
3	CH ₂	29.37	1.59, quint	2.25, 1.35	174.44, 36.98, 23.72, 15.62
4	CH ₂	36.98	2.25, t	1.59	29.37, 23.72
5	C	174.44	-		
6	CH ₂	43.53	4.58, s		174.44, 144.98, 116.68
7	C	144.98	-		
8	CH	116.68	8.47, d	7.72	129.28, 122.57, 43.53
9	C	130.74	-		
10	C	122.57	-		
11	CH	126.81	8.81, d	7.72	144.98, 131.56, 130.74
12	CH	129.28	7.72, dd	8.81, 8.47	122.57, 116.68, 43.53
13	C	131.56	-		
14	CH	125.74	8.81, d	8.01, 7.90	131.30, 122.57, 119.71
15	CH	134.12	8.01, dt	8.81, 8.64, 7.90	131.56, 127.10
16	CH	131.30	7.90, dt	8.81, 8.64, 8.01	125.74, 119.71
17	CH	127.10	8.64, dd	8.01, 7.90	148.85, 134.12, 131.56
18	C	119.71	-		

19	C	148.85	-
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^aChemical shifts in ppm.

Table SMe6. ¹H, ¹³C and 2D NMR data of VDBP6 in DMSO-*d*₆.

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
1	CH ₃	15.69	0.88, t	1.36	28.95, 23.72
2	CH ₂	23.72	1.36, sest	1.44, 0.88	34.37, 28.95, 15.69
3	CH ₂	28.95	1.44, quint	2.64, 2.26, 1.36	173.43, 34.37, 23.72, 15.69
4	CH ₂	34.37	2.64, m 2.26, m	2.26, 1.44 2.64, 1.44	28.95, 23.72
5	C	173.43	-		
6	CH	64.71	4.60, d	2.39	173.43, 175.40, 29.44, 21.25, 20.64
7	CH	29.44	2.39, m	4.60, 0.94, 0.82	175.40, 64.71, 21.25, 20.64
8	CH ₃	20.64	0.82, d	4.60, 0.94	64.71, 29.44, 21.25
9	CH ₃	21.25	0.94, d	4.60, 0.82	64.71, 29.44, 20.64
10	C	175.40	-		
11	CH ₂	50.48	4.95, s		173.43, 64.71, 143.66, 128.43, 116.29
12	C	143.66	-		
13	CH	116.29	8.51, s	7.71	128.43, 122.68, 50.48
14	C	130.78	-		
15	C	122.68	-		
16	CH	127.04	8.77, d	7.71	143.66, 131.37, 130.78
17	CH	128.43	7.71, d	8.77, 8.51	122.68, 116.29, 50.48
18	C	131.37	-		
19	C	119.67	-		
20	CH	130.78	8.57, d	8.72, 7.96, 7.84	148.82, 134.15, 131.37
21	CH	131.45	7.84, t	8.72, 8.57, 7.96	125.72, 119.67
22	CH	134.15	7.96, t	8.72, 8.57, 7.84	131.37, 130.78
23	CH	125.72	8.72, d	7.96, 7.84	131.45, 131.37, 122.68, 119.67
24	C	148.82	-		

^aChemical shifts in ppm.

Table SMe7. ¹H, ¹³C and 2D NMR data of VDBP7 in DMSO-*d*₆.

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
1	CH ₃	15.54	0.68, t	1.36	28.76, 23.55
2	CH ₂	23.55	1.36, sest	1.35, 0.68	34.31, 28.76, 15.54

3	CH ₂	28.76	1.35, quint	2.74, 2.48, 1.36	175.63, 34.31, 23.55, 15.54
4	CH ₂	34.31	2.74, m 2.48, m	2.48, 1.35 2.74, 1.35	28.95, 23.55
5	C	175.63	-		
6	CH	67.60	4.16, d	2.60	175.63, 175.40, 29.45, 21.25, 20.64
7	CH	29.45	2.60, m	4.16, 0.95, 0.80	175.40, 64.71, 21.96, 20.35
8	CH ₃	20.35	0.80, d	4.60, 0.94	64.71, 29.45, 21.96
9	CH ₃	21.96	0.95, d	4.60, 0.82	64.71, 29.45, 20.35
10	C	173.63	-		
11	CH ₂	47.44	4.88, d 4.63, d	4.63 4.88	173.63, 64.71, 143.66, 128.43, 116.29
12	C	144.02	-		
13	CH	116.83	8.35, s	7.61	128.43, 122.68, 47.44
14	C	130.46	-		
15	C	122.19	-		
16	CH	127.08	8.67, d	7.61	143.66, 131.37, 130.78
17	CH	129.30	7.61, d	8.67, 8.35	122.68, 116.83, 47.44
18	C	131.18	-		
19	C	119.53	-		
20	CH	130.46	8.57, d	8.72, 8.57, 7.96	148.82, 134.15, 131.37
21	CH	131.52	7.82, t	8.72, 8.57, 7.96	125.72, 119.67
22	CH	134.67	7.96, t	8.72, 8.57, 7.82	131.37, 130.78
23	CH	125.61	8.72, d	7.96, 7.82	131.45, 131.37, 122.68, 119.67
24	C	148.72	-		

^aChemical shifts in ppm.

Table SMe8. ¹H, ¹³C and 2D NMR data of VDBP8 in CDCl₃.

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
1	CH ₃	13.45	0.90, t	1.36	26.59, 21.74
2	CH ₂	21.74	1.36, sest	1.68, 0.90	37.80, 26.59, 13.45
3	CH ₂	26.59	1.68, quint	2.76, 1.68	176.80, 37.84, 21.74, 13.45
4	CH ₂	37.80	2.76, t	1.68	26.59, 21.74
5	C	176.80	-		
6	C	181.40	-		
7	CH	35.27	3.35, sept	1.20	181.40, 19.94
8/9	CH ₃	19.94	1.20, d	3.35	181.40, 35.27
10	CH ₂	46.90	5.22, s		181.40, 176.80, 140.31, 126.10, 114.87
11	C	140.31	-		
12	CH	114.87	8.50, s	7.53	126.10, 121.70, 46.90
13	C	129.60	-		

14	C	121.70	-		
15	CH	123.04	8.52, d	7.53	140.31, 130.05, 129.60
16	CH	126.10	7.53, dd		
17	C	130.05	-	8.51, 8.48	121.70, 114.87, 46.90
18	CH	124.68	8.48, d	7.90, 7.81	129.80, 121.70, 118.40
19	C	147.41	-		
20	C	118.40	-		
21	CH	126.13	8.78, dd	7.90, 7.81	147.41, 132.03, 130.05
22	CH	129.80	7.81, dt	8.78, 8.48, 7.90	124.68, 118.40
23	CH	132.03	7.90, dt	8.78, 8.48, 7.81	130.05, 126.13

^aChemical shifts in ppm.

Table SMe9. ¹H, ¹³C and 2D NMR data of VDBP9 in CDCl₃.

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
1	CH ₃	13.70	0.94, t	1.41	26.64, 22.13
2	CH ₂	22.13	1.41, sest	1.75, 0.94	34.75, 26.64, 13.70
3	CH ₂	26.64	1.75, quint	2.75, 1.41	173.82, 34.75, 22.13, 13.70
4	CH ₂	34.75	2.75, t	1.75	26.64, 22.13
5	C	173.82	-		
6	CH	162.20	9.41, s		173.82, 42.50
7	CH ₂	42.50	5.13, s		173.82, 162.20, 139.51, 128.05, 115.50
8	C	139.51	-		
9	CH	115.50	8.51, d	7.62	128.05, 121.45, 42.50
10	C	129.54	-		
11	C	121.45	-		
12	CH	123.10	8.33, d	7.62	129.57, 129.54, 139.51
13	CH	128.05	7.62, dd		
14	C	129.57	-	8.51, 8.33	121.45, 115.50, 42.50
15	CH	122.90	8.35, d	7.82, 7.74	129.54, 121.45, 118.45
16	CH	131.76	7.82, dt	8.69, 8.35, 7.74	129.57, 126.15
17	CH	129.54	7.74, dt	8.69, 8.35, 7.82	122.90, 118.45
18	CH	126.15	8.69, dd	7.82, 7.74	147.27, 131.76, 129.57
19	C	118.45	-		
20	C	147.27	-		

^aChemical shifts in ppm.

Table SMe10. ¹H, ¹³C and 2D NMR data of VDBP10 in DMSO-*d*₆.

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
1	CH ₃	18.96	0.72, d	2.17	63.68, 29.87, 20.09
2	CH	29.87	2.17, m	4.40, 0.88, 0.72	172.64, 63.68, 20.09, 18.96
3	CH ₃	20.09	0.88, d	2.17	63.68, 29.87, 18.96
4	CH	63.68	4.40, d	2.17	172.64, 29.87, 20.09, 18.96
5	C	172.64	-		
6	CH ₂	49.01	4.64, s		137.82, 126.57, 63.68

7	C	137.82	-		
8/12	CH	126.57	7.19, d	7.05	138.73, 126.57, 49.01
9/11	CH	129.35	7.05, d	7.19	141.31, 137.82, 129.35
10	C	138.73	-		
13	C	141.31	-		
14	CH	128.04	7.49, d	7.57	138.73, 130.94, 125.79
15	CH	131.28	7.57, m	7.49-7.46	141.31, 130.41
16	CH	130.94	7.46, t	7.59-7.57	128.04, 125.79
17	CH	130.41	7.59, m	7.46	156.78, 141.31, 131.28
18	C	125.79	-		
19	C	156.78	-		

^aChemical shifts in ppm.

Table SMe11. ¹H, ¹³C and 2D NMR data of VDBP11 in DMSO-*d*₆.

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
1	CH ₃	19.41	0.72, d	2.17	65.63, 29.90, 20.31
2	CH	29.90	2.17, m	4.08, 0.91, 0.72	172.56, 65.63, 20.31, 19.41
3	CH ₃	20.31	0.91, d	2.17	65.63, 29.90, 19.41
4	CH	65.63	4.08, d	2.17	172.56, 29.90, 20.31, 19.41
5	C	172.56	-		
6	CH ₂	45.91	4.43, s		138.09, 126.77, 65.63
7	C	138.09	-		
8/12	CH	126.77	7.03, d	6.94	139.11, 126.57, 45.91
9/11	CH	128.96	6.94, d	7.03	141.57, 138.09, 128.96
10	C	139.11	-		
13	C	141.57	-		
14	CH	128.86	7.50, d	7.57	139.11, 130.86, 125.75
15	CH	131.28	7.57, m	7.50, 7.46	141.57, 130.44
16	CH	130.86	7.46, t	7.59, 7.57	128.86, 125.75
17	CH	130.44	7.59, m	7.46	156.77, 141.57, 131.28
18	C	125.75	-		
19	C	156.77	-		

^aChemical shifts in ppm.

SMF-Supporting Materials F- Losartan

Losartan: 1-((2'-(2H-Tetrazol-5-yl)-[1,1'-biphenyl]-4-yl)methyl)-2-butyl-4-chloro-1H-imidazol-5-yl) methanol. White powder. MS-TOF (positive ions): m/z calculated for $C_{22}H_{23}ClN_6O$ 422.16 $[M]^+$; found 423.17 $[M + H]^+$ (84%), 425.17 (26%), 424.18 (21%), 426.17 (7%).

LDBP1: 1-((2'-(2H-Tetrazol-5-yl)-[1,1'-biphenyl]-4-yl)methyl)-2-butyl-4-chloro-1H-imidazole-5-carbaldehyde. Gray powder. MS-TOF (negative ions): m/z calculated for $C_{22}H_{21}ClN_6O$ 420.15 $[M]^+$; found 419.15 $[M - H]^-$ (90%), 420.16 (20%), 421.15 (30%), 422.15 (7%).

LDBP2: 2-butyl-4-chloro-1-((2'-(2-hydroxy-2H-tetrazol-5-yl)-[1,1'-biphenyl]-4-yl)methyl)-1H-imidazole-5-carbaldehyde. Gray powder. MS-TOF (positive ions): m/z calculated for $C_{22}H_{21}ClN_6O_2$ 436.14 $[M]^+$; found 437.15 $[M + H]^+$ (88%), 439.14 (28%), 438.15 (24%), 440.17 (6%).

LDBP3: ((2'-(2H-Tetrazol-5-yl)-[1,1'-biphenyl]-4-yl)methyl)(1-acetamido-1-hydroxypentyl) carbamic acid. Gray powder. MS-TOF (positive ions): m/z calculated for $C_{22}H_{26}N_6O_4$ 438.20 $[M]^+$; found 461.21 $[M + Na]^+$ (48%), 462.21 (10%).

LDBP4: *N*-((2'-(2H-Tetrazol-5-yl)-[1,1'-biphenyl]-4-yl)methyl)pentanimidamide. White powder. MS-TOF (positive ions): m/z calculated for $C_{19}H_{22}N_6$ 334.19 $[M]^+$; found 335.20 $[M + H]^+$ (90%), 336.20 (22%).

LDBP5: (2-Butyl-4-chloro-1-(tetrazolo [1,5-*f*]phenanthridin-6-ylmethyl)-1H-imidazol-5-yl) methanol. Gray powder. MS-TOF (positive ions): m/z calculated for $C_{22}H_{21}ClN_6O$ 420.15 $[M]^+$; found 421.16 $[M + H]^+$ (85%), 423.16 (24.0%), 422.16 (18%).

LDBP6: 2-Butyl-4-chloro-1-(tetrazolo [1,5-*f*]phenanthridin-6-ylmethyl)-1H-imidazole-5-carbaldehyde. Gray powder. MS-TOF (positive ions): m/z calculated for $C_{22}H_{19}ClN_6O$ 418.13 $[M]^+$; found 419.13 $[M + H]^+$ (100%), 421.14 (30%), 420.14 (24%).

LDBP7: 2-Butyl-4-chloro-1-((2-hydroxy-2,3-dihydro-1H-tetrazolo[1,5-*f*]phenanthridin-6-yl)methyl)-1H-imidazole-5-carbaldehyde. Gray powder. MS-TOF (positive ions): m/z calculated for $C_{22}H_{21}ClN_6O_2$ 436.14 $[M]^+$; found 437.15 $[M + H]^+$ (95%), 439.16 (31%), 438.17 (22%), 440.15 (5%).

LDBP8: Methyl 2-butyl-4-chloro-1-(tetrazolo [1,5-*f*]phenanthridin-6-ylmethyl)-1H-imidazole-5-carboxylate. Gray powder. MS-TOF (positive ions): m/z calculated for $C_{23}H_{21}ClN_6O_2$ 448.14 $[M]^+$; found 449.15 $[M + H]^+$ (100%), 451.15 (32%), 450.16 (25%), 452.16 (7%).

LDBP9: *N*-(Tetrazolo [1,5-*f*]phenanthridin-6-ylmethyl)pentanamide. Gray powder. MS-TOF (positive ions): m/z calculated for $C_{19}H_{19}N_5O$ 333.16 $[M]^+$; found 334.16 $[M + H]^+$ (87%), 335.17 (21%).

Table S1. Data obtained from the mono- and two-dimensional NMR analysis of Losartan, in deuterated methanol (CD₃OH).

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
2	C	150.01	-		
4	C	127.39	-		
5	C	126.85	-		
6	CH ₂	53.01	4.43, s		127.39, 126.85
7	CH ₂	27.40	2.57, t	1.53	150.01, 30.95, 23.24
8	CH ₂	30.95	1.53, quint	2.57, 1.31	150.01, 23.24, 14.03
9	CH ₂	23.24	1.31, sest	1.53, 0.86	30.95, 27.40, 14.03
10	CH ₃	14.03	0.86, t	1.31	30.95, 23.24
11	CH ₂	48.29	5.25, s		150.01, 136.08, 126.85, 126.70
12	C	136.08	-		
13/17	CH	126.70	6.91, d	7.11	142.24, 126.70, 48.29
14/18	CH	130.84	7.11, d	6.91	142.62, 136.08, 130.84
15	C	142.24	-		
18	C	142.62	-		
19	CH	131.17	7.45, dd	7.49, 7.43	142.24, 131.11, 128.19
20	CH	130.02	7.49, t	7.45, 7.43	142.62, 131.82
21	CH	128.19	7.43, t	7.53, 7.49	131.17, 131.11
22	CH	131.82	7.53, dd	7.49, 7.43	162.71, 142.62, 130.02
23	C	131.11	-		
24	C	162.71	-		

^aChemical shifts in ppm.

Table S2. Data obtained from the mono- and two-dimensional NMR analysis of DP1, in deuterated chloroform (CDCl₃).

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
2	C	154.80	-		
4	C	143.23	-		
5	C	124.19	-		
6	CH	177.99	9.55, s		143.23, 124.19
7	CH ₂	26.39	2.59, t	1.62	154.80, 29.22, 22.30
8	CH ₂	29.22	1.62, quint	2.59, 1.30	154.80, 29.22, 22.30, 13.61
9	CH ₂	22.30	1.30, sest	1.62, 0.85	29.22, 26.39, 13.61
10	CH ₃	13.61	0.85, t	1.30	29.22, 22.30
11	CH ₂	47.94	5.49, s		154.80, 126.73, 124.19
12	C	135.42	-		
13/17	CH	126.73	6.97, d	7.09	139.17, 126.73, 47.94
14/18	CH	129.57	7.09, d	6.97	140.58, 139.17, 135.42, 129.57
15	C	139.17	-		
18	C	140.58	-		
19	CH	130.74	7.40, dd	7.58, 7.50	139.17, 128.30, 122.65
20	CH	131.32	7.58, dt	7.50, 7.40	140.58, 130.74

21	CH	128.30	7.50, dt	7.89, 7.58	130.74, 122.65
22	CH	130.74	7.89, dd	7.58, 7.50	154.85, 140.58, 131.32
23	C	122.65	-		
24	C	154.85	-		

^aChemical shifts in ppm.

Table S3. Data obtained from the mono- and two-dimensional NMR analysis of DP2, in deuterated chloroform (CDCl₃).

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
2	C	154.64	-		
4	C	124.21	-		
5	C	124.42	-		
6	CH	177.77	9.72, s		124.42, 124.21
7	CH ₂	25.69	2.66, t	1.57	154.64, 28.82, 21.82
8	CH ₂	28.82	1.57, quint	2.66, 1.31	154.64, 12.56
9	CH ₂	21.82	1.31, sest	1.57, 0.87	25.69, 12.56
10	CH ₃	12.56	0.87, t	1.31	28.82, 21.82
11	CH ₂	47.41	5.61, s		154.64, 124.42, 126.16
12	C	135.35	-		
13/17	CH	126.16	7.03, d	7.08	139.37, 126.16, 4741
14/18	CH	129.24	7.08, d	7.03	141.77, 135.35, 129.24
15	C	139.37	-		
18	C	141.77	-		
19	CH	130.52	7.49, d	7.60	139.37, 127.48, 124.42
20	CH	130.26	7.60, t	7.52, 7.49	141.77, 130.26
21	CH	127.48	7.52, t	7.62, 7.60	130.52, 124.42
22	CH	130.26	7.62, d	7.52	156.80, 141.77, 130.26
23	C	124.42	-		
24	C	156.80	-		

^aChemical shifts in ppm.

Table S4. Data obtained from the mono- and two-dimensional NMR analysis of DP3, in deuterated methanol (CD₃OD).

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
1	CH ₃	19.97	2.01, s		175.40
2	C	175.40	-		
4	C	101.22	-		
5	CH ₂	31.27	1.75, m 1.66, m	1.09, 0.75	101.22, 24.27, 21.54
6	CH ₂	24.27	1.09, m 0.75, m	1.75, 1.66, 1.07, 0.87	101.22, 31.27, 21.54, 12.64
7	CH ₂	21.54	1.07, m 0.87, m	1.09, 0.75, 0.65	31.27, 24.27, 12.64

8	CH ₃	12.64	0.65, t	1.07, 0.87	24.27, 21.54
10	C	160.55	-		
11	CH ₂	42.41	4.96, overlapped 4.17, d	4.17 4.96	160.55, 101.22, 128.13
12	C	135.30	-		
13/17	CH	128.13	7.32, d	7.14	139.95, 128.13, 42.41
14/16	CH	129.15	7.14, d	7.32	141.35, 135.30, 129.15
15	C	139.95	-		
18	C	141.35	-		
19	CH	130.45	7.48, d	7.61	139.95, 127.18, 126.19
20	CH	129.92	7.61, m	7.52, 7.48	141.35, 131.1
21	CH	127.18	7.52, t	7.62, 7.61	130.45, 126.19
22	CH	131.01	7.62, m	7.61, 7.52	158.35, 141.35, 129.92
23	C	126.19	-		
24	C	158.35	-		

^aChemical shifts in ppm.

Table SMf5. Data obtained from the mono- and two-dimensional NMR analysis of DP4, in deuterated methanol (CD₃OD).

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
1	C	168.35	-		
2	CH ₂	32.39	2.48, t	1.68	168.35, 29.19, 21.67
3	CH ₂	29.19	1.68, quint	2.48, 1.41	168.35, 32.39, 21.67, 12.42
4	CH ₂	21.67	1.41, sest	1.68, 0.97	32.39, 29.19, 12.42
5	CH ₃	12.42	0.97, t	1.41	29.19, 21.67
7	CH ₂	45.48	4.41, s		168.35, 132.56, 127.16
8	C	132.56	-		
9/13	CH	127.16	7.19, d	7.15	141.13, 127.16, 45.48
10/12	CH	129.53	7.15, d	7.19	141.34, 132.56, 129.53
11	C	141.13	-		
14	C	141.34	-		
15	CH	129.92	7.44, d	7.53	141.13, 128.33, 127.48
16	CH	128.91	7.53, t	7.46, 7.44	141.34, 130.55
17	CH	128.33	7.46, dt	7.57, 7.52	129.92, 127.48
18	CH	130.55	7.57, d	7.52, 7.45	160.41, 141.34, 128.91
19	C	127.48	-		
20	C	160.41	-		

^aChemical shifts in ppm.

Table SMf6. Data obtained from the mono- and two-dimensional NMR analysis of DP5, in deuterated dimethyl sulfoxide (CD₃SOCD₃).

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
2	C	148.88	-		
4	C	127.16	-		
5	C	123.16	-		
6	CH ₂	53.18	4.41, s		127.16, 123.16
7	CH ₂	27.66	2.54, t	1.48	148.88, 23.45
8	CH ₂	30.88	1.48, quint	2.54, 1.23	148.88, 15.41

9	CH ₂	23.45	1.23, sest	1.48, 0.72	27.66, 15.41
10	CH ₃	15.41	0.72, t	1.23	30.88, 23.45
11	CH ₂	48.28	5.58, s		148.88, 127.75, 123.16, 116.56
12	C	141.86	-		
13	CH	116.56	8.30, s		127.75, 123.16, 48.28
14	C	131.42	-		
15	C	123.16	-		
16	CH	125.79	8.78, d	7.48	141.86, 131.63, 131.42
17	CH	127.75	7.48, d	8.78	123.16, 116.56, 48.28
18	C	131.63	-		
19	CH	125.84	8.73, d	7.98	131.35, 123.16, 119.83
20	CH	134.24	7.98, t	8.73, 7.85	131.63, 127.21
21	CH	131.35	7.85, t	8.57, 7.98	125.84, 119.83
22	CH	127.21	8.57, d	7.85	149.43, 134.24, 131.63
23	C	119.83	-		
24	C	149.43	-		

^aChemical shifts in ppm.

Table SMf7. Data obtained from the mono- and two-dimensional NMR analysis of DP6, in deuterated chloroform (CDCl₃).

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
2	C	154.67	-		
4	C	143.50	-		
5	C	124.39	-		
6	CH	178.09	9.81, s		143.50, 124.39
7	CH ₂	29.31	2.76, t	1.74	22.41
8	CH ₂	26.59	1.74, quint	2.76, 1.40	154.67, 13.67
9	CH ₂	22.41	1.40, sest	1.74, 0.90	29.31
10	CH ₃	13.67	0.90, t	1.40	26.59, 22.41
11	CH ₂	47.94	5.83, s		154.67, 126.21, 124.39, 115.38
12	C	138.35	-		
13	CH	115.38	8.42, s		126.21, 122.08, 47.94
14	C	129.38	-		
15	C	122.08	-		
16	CH	124.96	8.49, d	7.40	138.35, 129.58, 129.38
17	CH	126.21	7.40, d	8.49	122.08, 115.38, 47.94
18	C	129.58	-		
19	CH	123.06	8.45, dd	7.91, 7.83	129.73, 122.08, 118.69
20	CH	132.16	7.91, t	8.45, 7.83	129.58, 126.08
21	CH	129.73	7.83, t	8.77, 7.91	123.06, 118.69
22	CH	126.08	8.77, dd	7.91, 7.83	147.38, 132.16, 129.58
23	C	118.69	-		
24	C	147.38	-		

^aChemical shifts in ppm.

Table SMf8. Data obtained from the mono- and two-dimensional NMR analysis of DP7, in deuterated dimethyl sulfoxide (CD₃SOCD₃).

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
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2	C	155.07	-		
4	C	141.71	-		
5	C	125.86	-		
6	CH	178.50	9.71, s		141.71, 125.86
7	CH ₂	29.09	2.75, t	1.57, 1.27	155.07, 25.87, 22.02
8	CH ₂	25.87	1.57, quint	2.75, 1.27	155.07, 29.09, 22.02, 13.94
9	CH ₂	22.02	1.27, sest	1.57, 0.77	29.09, 25.87, 13.94
10	CH ₃	13.94	0.77, t	1.57, 1.27	25.87, 22.02
11	CH ₂	47.40	5.89, s		155.07, 126.46, 125.86, 114.84
12	C	139.60	-		
13	CH	114.84	8.31, d	8.82	126.46, 121.91, 47.40
14	C	129.45	-		
15	C	121.91	-		
16	CH	126.34	8.82, d	8.31, 7.50	129.51, 129.45, 139.60
17	CH	126.46	7.50, dd	8.82	121.91, 114.84, 47.40
18	C	129.51	-		
19	CH	124.64	8.78, d	7.99, 7.88	129.81, 121.91, 118.49
20	CH	132.85	7.99, t	8.78, 7.88	129.51, 126.22
21	CH	129.81	7.88, t	8.63, 7.99	124.64, 118.49
22	CH	126.22	8.63, d	7.99, 7.88	147.49, 132.85, 129.51
23	C	118.49	-		
24	C	147.49	-		

^aChemical shifts in ppm.

Table SM9. Data obtained from the mono- and two-dimensional NMR analysis of DP8, in deuterated chloroform (CDCl₃).

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
2	C	156.41	-		
4	C	127.50	-		
5	C	124.44	-		
6	C	161.25	-		
7	CH ₂	25.50	2.33, t	1.63	22.05
8	CH ₂	29.67	1.63, quint	2.33, 1.35	156.41, 14.09
9	CH ₂	22.05	1.35, sest	1.63, 0.87	25.50
10	CH ₃	14.09	0.87, t	1.35	29.67, 22.05
11	CH ₂	43.35	4.83, s		156.41, 124.44, 127.73, 116.79
12	C	139.60	-		
13	CH	116.79	8.62, s		127.73, 121.89, 43.35
14	C	129.50	-		
15	C	121.89	-		
16	CH	125.00	8.52, d	7.72	139.60, 129.50, 129.47
17	CH	127.73	7.72, d	8.52	121.89, 116.79, 43.35
18	C	129.47	-		
19	CH	123.12	8.50, dd	7.92, 7.83	129.50, 121.89, 118.57
20	CH	132.31	7.92, t	8.50, 7.83	129.47, 126.25
21	CH	129.50	7.83, t	8.79, 7.92	123.12, 118.57
22	CH	126.25	8.79, dd	7.92, 7.83	147.46, 132.31, 129.47
23	C	118.57	-		
24	C	147.46	-		
OCH ₃	CH ₃	53.88	3.96, s		161.25

^aChemical shifts in ppm.

Table SMf10. Data obtained from the mono- and two-dimensional NMR analysis of LDP9, in deuterated methanol (CD₃OD).

Position	Residue	¹³ C ^a	¹ H ^a , multiplicity	¹ H- ¹ H COSY	¹ H- ¹³ C HMBC
1	C	170.20	-		
2	CH ₂	34.92	2.61, t	1.78	170.20, 30.51, 22.50
3	CH ₂	30.51	1.78, quint	2.61, 1.49	170.20, 34.92, 22.50, 13.10
4	CH ₂	22.50	1.49, sest	1.78, 1.02	34.92, 30.51, 13.10
5	CH ₃	13.10	1.02, t	1.49	30.51, 22.50,
7	CH ₂	46.28	4.60, s		170.20, 128.74, 115.85
8	C	138.87	-		
9	CH	115.85	8.68, d	8.84	128.74, 123.59, 130.80, 46.28
10	C	130.80			
11	C	123.59			
12	CH	126.55	8.84, d	8.68, 7.82	138.87, 130.80, 123.59
13	CH	128.74	7.82, dd	8.84	123.59, 115.85, 46.28
14	C	130.80	-		
15	CH	124.80	8.78, dd	8.02	130.86, 123.59, 119.54
16	CH	133.56	8.02, dt	8.78, 7.91	130.80, 126.45
17	CH	130.86	7.91, t	8.73, 8.02	124.80, 119.54
18	CH	126.45	8.73, d	7.91	148.47, 133.56, 130.80
19	C	119.54	-		
20	C	148.47	-		

^aChemical shifts in ppm.



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