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Ecotoxicological Study of the Pharmaceutical Active Substance Isotretinoin

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1. Introduction and Objectives

Pharmaceuticals are essential in modern healthcare, but their release into the environment has become an increasing concern in recent decades. After administration, many active substances are excreted in either unmetabolised or partially metabolised forms, and traces of these substances may enter aquatic systems through various pathways such as wastewater and improper disposal (Wilkinson et al., 2022; Ortúzar et al., 2022).

One pharmaceutical of interest is isotretinoin, a synthetic derivative of vitamin A (13-cis-retinoic acid). It is primarily prescribed in dermatology for the treatment of severe acne vulgaris, especially when patients do not respond adequately to conventional therapies. Acne can result in substantial psychosocial disturbance, driving demand for effective treatment options. Isotretinoin is widely considered the most effective therapy for severe or treatment-resistant acne and has been associated with long-lasting remission and improved patient quality of life (Cyrulnik et al., 2012). Public attention toward isotretinoin has also increased, with social media platforms shaping awareness and perceptions surrounding acne treatment (Asare et al., 2025).

Like most pharmaceuticals, isotretinoin can enter natural waters mainly through patient excretion. A portion of the compound and its metabolites may pass through wastewater systems and reach surface waters (Yeung et al., 2020; Pello Alfonso-Muniozguren et al., 2021). Once released, its environmental fate depends on physical and chemical conditions that influence transformation processes. Although isotretinoin can undergo light-driven degradation and isomerisation, these reactions are often incomplete, allowing residues to persist long enough to exert biological effects (Ioele et al., 2005; Hejna et al., 2022).

The ecological relevance of isotretinoin lies in its biological activity. As a retinoid, it regulates processes such as cell differentiation and development in humans, and similar mechanisms may occur in aquatic species (Yeung et al., 2020).

Pharmaceutical residues have been shown to affect aquatic organisms at concentrations far below therapeutic levels. Primary producers such as algae are particularly sensitive to contaminants that interfere with photosynthesis or growth. Because of their high sensitivity and ecological importance, algae are often used as test organisms in ecotoxicological research (Ortúzar et al., 2022).

Compared with widely studied pharmaceutical groups such as antibiotics and hormones, data on isotretinoin's environmental occurrence and effects are still scarce, particularly concerning

primary producers (Yeung et al., 2020). This knowledge gap limits current understanding of its environmental behaviour and potential ecological risks.

The present thesis addresses this gap by investigating the effects of isotretinoin on freshwater microalgae. The study follows the OECD 201 algal growth inhibition test guideline to assess its impact on the growth of *Desmodesmus subspicatus*. In addition to growth rate, chlorophyll-a content and chlorophyll-fluorescence parameters are evaluated to detect potential sub-lethal effects on photosynthetic performance, thereby contributing new data on the environmental relevance of isotretinoin.

The objective of this thesis is to investigate the potential effects of isotretinoin on freshwater primary producers, focusing on the green microalga *Desmodesmus subspicatus*. This study aims to:

- Assess whether isotretinoin exposure alters algal growth under controlled laboratory conditions following the OECD 201 guideline.
- Evaluate photosynthetic responses through chlorophyll-a content and chlorophyll-fluorescence parameters to identify potential early sub-lethal physiological effects.
- Generate ecotoxicological data contributing to a more informed understanding of isotretinoin's environmental relevance.

2. Literature review

2.1. Introduction to Pharmaceutical Pollution

Active pharmaceutical ingredients (APIs), also known as pharmaceutical micropollutants, are a distinctive class of environmental contaminants due to their continuous discharge into the environment and their inherent biological activity (Daughton, 2016; Gupta et al., 2024). Unlike conventional pollutants such as potentially toxic elements (PTEs) and pesticides, pharmaceuticals are regularly introduced into aquatic systems through multiple anthropogenic pathways. Most of this release originates from human and livestock excreta as well as pharmaceutical manufacturing effluents, which are ultimately directed to wastewater treatment plants (WWTPs) (Daughton, 2016; Samal et al., 2022). Since these substances are specifically formulated to act at low concentrations, they are capable of disrupting normal physiological and hormonal functions in non-target aquatic organisms, such as fish and amphibians, even at trace levels in the parts-per-trillion range (Daughton, 2016; Gupta et al., 2024). Conventional WWTPs are not primarily designed to remove these bioactive compounds, leading to their persistence or accumulation in treated effluent and sludge. As a result, APIs can enter receiving water bodies where they may undergo limited degradation or partition into sediments and biota, contributing to long-term ecological exposure (Ranjan et al., 2022; Samal et al., 2022). These substances may cause a range of adverse effects in aquatic organisms, including endocrine disruption, reproductive impairment, behavioral changes, and increased toxicity due to the combined presence of multiple compounds (Gupta et al., 2024; Ranjan et al., 2022; Rzymski et al., 2017).

The occurrence of pharmaceutical micropollutants in aquatic ecosystems has become a growing global concern. Their presence has been widely documented in surface waters, groundwater, sewage treatment influent and effluent, sludge, and even drinking water in some regions (Gupta et al., 2024; Ranjan et al., 2022). This widespread detection reflects both continuous emissions and the environmental persistence of certain APIs, particularly those resistant to conventional treatment or prone to bioaccumulation (Geissen et al., 2015; Samal et al., 2022).

The increasing consumption of pharmaceuticals has intensified the global scale of pharmaceutical pollution. The expansion of the pharmaceutical industry and rising demand for medications have resulted in greater quantities entering aquatic environments (Rzymski et al., 2017; Samal et al., 2022). Although only a fraction of all pharmaceuticals currently in use are

detected in the environment, the number of compounds identified continues to increase as analytical technologies advance (Gupta et al., 2024; Rzymski et al., 2017). The presence of multiple pharmaceuticals in mixtures makes it challenging to evaluate their ecological impacts, as interactions between compounds—including additive, synergistic, or antagonistic effects—can significantly alter overall toxicity (Ranjan et al., 2022).

Pharmaceutical micropollutants are often classified as "emerging pollutants" or "contaminants of emerging concern" due to their bioactivity, persistence, and limited regulatory oversight (Geissen et al., 2015; Samal et al., 2022; Gupta et al., 2024). These substances are not yet comprehensively regulated or routinely monitored, yet evidence demonstrates their potential to cause adverse ecological effects (see Section 2.3.3. for further discussion of current pharmaceutical regulations.) Geissen et al. (2015) point out that emerging pollutants create management challenges due to gaps in our knowledge about their fate and toxicity. This highlights the need to include them in future regulations. The absence of many pharmaceuticals from monitoring lists under policies like the European Union Water Framework Directive (WFD; 2000/60/EC) shows a regulatory delay. This results in minimal requirements for wastewater treatment plants and manufacturers to manage pharmaceutical discharge (Geissen et al., 2015).

One of the major challenges in addressing pharmaceutical pollution is understanding the fate, transport, and ecological effects of these contaminants to accurately assess environmental risk. Once a pharmaceutical enters an aquatic system, it may undergo transformation processes such as photolysis, hydrolysis, or microbial degradation; it may also adsorb onto sediments or partition into biological tissues (Geissen et al., 2015; Rzymski et al., 2017). Some APIs can persist under environmentally relevant conditions, while others form metabolites that retain pharmacological activity and may exhibit distinct ecotoxicological properties (Gupta et al., 2024; Ranjan et al., 2022). Chronic exposure to low concentrations of APIs can lead to reproductive disruption, altered hormone signaling, behavioral changes, and broader ecological impacts at the community level (Ranjan et al., 2022; Rzymski et al., 2017).

Pharmaceuticals reach aquatic systems through several well-established pathways, including patient excretion, improper disposal of unused or expired medication, and incomplete removal during wastewater treatment (Daughton, 2016; Rzymski et al., 2017). After administration, a portion of the active substance is metabolized, while another fraction is excreted unchanged or as conjugated metabolites in urine and feces. These waste streams enter municipal sewage systems and are conveyed to WWTPs (Gupta et al., 2024). WWTPs were originally designed

to remove conventional pollutants such as suspended solids, biodegradable organic matter, and nutrients, rather than trace concentrations of biologically active micropollutants; consequently, many pharmaceuticals are only partially removed and are discharged in treated effluent to surface waters (Samal et al., 2022; Ranjan et al., 2022). Samal et al. (2022) report that conventional WWTPs can convert some pharmaceuticals into transformation products rather than fully mineralizing them, adding complexity to environmental fate.

Improper disposal of unused medicines also contributes to pharmaceutical pollution, although this typically occurs as episodic rather than continuous input. Surveys consistently show that households often dispose of expired or unused medication through sinks, toilets, or general waste, leading to temporary spikes of pharmaceutical contamination in wastewater or landfill leachate (Rzymski et al., 2017).

2.2. Retinoids and Isotretinoin as Active Pharmaceutical Ingredients

2.2.1. Classification and Function of Retinoids

Retinoids are a group of compounds derived from vitamin A that are essential in regulating cell growth and development. They include both naturally occurring molecules, such as retinol and retinoic acid, and synthetic derivatives, such as isotretinoin. These compounds exert their biological effects mainly by binding to specific nuclear receptors, which act as transcription factors that influence gene expression and cellular function (Layton, 2009; Zouboulis, 2001).

Retinoids are traditionally classified into generations based on their chemical structure. First-generation retinoids include naturally occurring forms (retinol, retinal, and tretinoin) as well as the synthetic retinoid isotretinoin, which is commonly used in acne therapy. Second-generation retinoids, such as etretinate and acitretin, are modified to improve stability and reduce side effects. Third-generation retinoids, including adapalene and bexarotene, were developed with more complex structures to increase receptor selectivity and enhance therapeutic outcomes (Zouboulis, 2001).

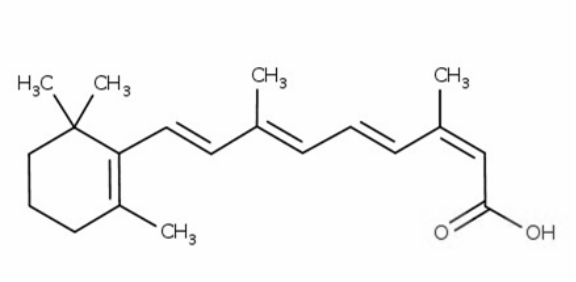
Biologically, retinoids play a central role in regulating the life cycle of epithelial cells. They promote proper cell differentiation while preventing abnormal proliferation, a mechanism that is crucial in conditions involving hyperkeratinization such as acne. Retinoids also reduce sebum production by shrinking sebaceous glands and exhibit anti-inflammatory effects by modulating immune pathways (Layton, 2009). These combined actions explain their wide therapeutic use

in dermatology, particularly for acne, psoriasis, and certain skin cancers, as well as their continued evaluation for future clinical applications (Zouboulis, 2001).

2.2.2. Chemical Structure, Physicochemical Properties, and Behaviour of Isotretinoin

Isotretinoin (13-cis-retinoic acid) is a first-generation synthetic retinoid structurally related to vitamin A derivatives. It is an isomer of all-trans-retinoic acid, differing only in the configuration of the double bond at the 13th carbon, a variation that significantly influences its biological activity and pharmacokinetic profile (Bremner, 2021). Isotretinoin has the molecular formula $C_{20}H_{28}O_2$ and a molecular weight of 300.44 g/mol. Its chemical structure consists of a conjugated polyene chain and a terminal carboxyl group as it can be seen on Figure 1, which confer both lipophilicity and sensitivity to light (Bremner, 2021; ECHA, 2024; Layton, 2009).

Figure 1. Chemical structure of Isotretinoin (Source: ECHA, 2024)



According to the European Chemicals Agency (ECHA, Substance ID 100.022.996), isotretinoin is registered under CAS number 4759-48-2 and EC number 225-296-0. Although it does not possess harmonized classification under the CLP Regulation, several manufacturers have notified isotretinoin as hazardous to the aquatic environment (Aquatic Chronic 2). It exhibits low water solubility (<0.1 mg/L at 25 °C), a high log Pow of approximately 6.3 indicative of strong lipophilicity, and is reported as not readily biodegradable, suggesting potential persistence and bioaccumulation (ECHA, 2024). These physicochemical properties explain both its pharmacological distribution in lipid-rich tissues and its tendency to partition into organic matter in environmental compartments (Layton, 2009).

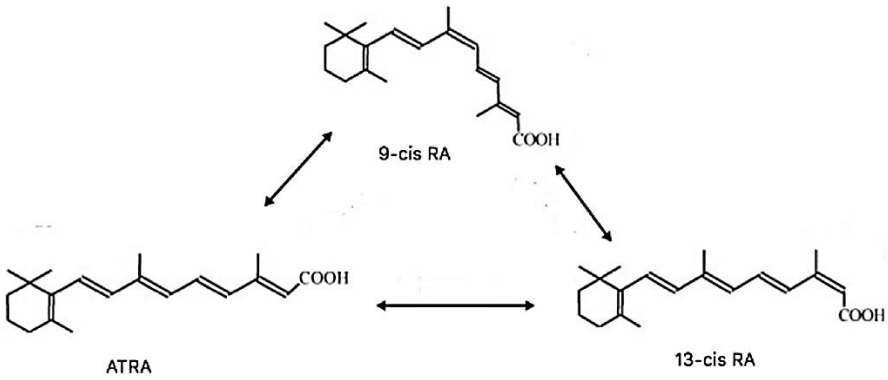
In pharmacological use, isotretinoin acts primarily through modulation of retinoid receptors, influencing epithelial differentiation, sebum production, and inflammation. It reduces sebaceous gland activity, normalizes desquamation, and downregulates *Cutibacterium acnes* proliferation (Layton, 2009; Zouboulis, 2001). These effects are mediated through interactions with retinoic acid receptors and retinoid X receptors, which regulate gene expression and cellular differentiation (Layton, 2009; Zouboulis, 2001).

2.2.3. Known Transformation and Degradation Products

In both clinical and environmental contexts, isotretinoin undergoes transformation processes that influence its persistence and biological activity. *In vivo*, isotretinoin is primarily metabolized in the liver via oxidation and isomerization, generating active metabolites including 4-oxo-isotretinoin, all-trans-retinoic acid, and 9-cis-retinoic acid (Layton, 2009; Bremner, 2021). These metabolites contribute to therapeutic efficacy but may also be excreted in active or conjugated forms, entering municipal wastewater systems (F. Hoffmann-La Roche Ltd, 2024).

In the environment, isotretinoin is chemically unstable under light exposure due to its conjugated double-bond system. Photodegradation leads to isomerization, producing all-trans-retinoic acid (ATRA) and 9-cis-retinoic acid (9-cis RA), both of which retain affinity for retinoid receptors and are therefore environmentally relevant (Layton, 2009; Zouboulis, 2001).

Figure 2. Metabolites of isotretinoin: all-trans-retinoic acid (ATRA), 9-cis-retinoic acid (9-cis RA) and 13-cis retinoic acid (13-cis RA) (Source: Bouriez, Giraud, Gronnier, & Varon, 2018)



These metabolites are known regulators of gene expression and can potentially induce differentiation or apoptosis in non-target organisms. Their receptor-binding capabilities raise concerns regarding endocrine disruption in aquatic biota (Bremner, 2021; Zouboulis, 2001). In addition, isotretinoin's high lipophilicity contributes to adsorption onto particulate matter and sediments, leading to potential accumulation in benthic organisms (ECHA, 2024; Layton, 2009). Environmental assessments indicate strong sorption to sludge during wastewater treatment, suggesting sediments may act as reservoirs from which isotretinoin or its metabolites may be released under changing environmental conditions (F. Hoffmann-La Roche Ltd, 2024).

Despite this, data on the long-term environmental persistence of isotretinoin and its derivatives remain limited, indicating a need for further investigation to understand their ultimate fate in aquatic ecosystems (F. Hoffmann-La Roche Ltd, 2024).

2.2.4 Therapeutic Use, Dosage, and Consumption Trends of Isotretinoin

Isotretinoin is marketed in Hungary under several brand names such as Roaccutan (internationally known as Accutane), Inerta, Medinac, Sotret Neo (or Sotret), Aknenormin, and Isotretinoin-Teva. It is a systemic retinoid prescribed for the treatment of severe, recalcitrant acne that does not respond to conventional therapies such as oral antibiotics or topical agents (Bagatin et al., 2020; Layton, 2009). It is typically administered orally at a dosage of 0.5–1 mg/kg/day, with treatment protocols designed to reach a cumulative dose of 120–150 mg/kg to minimize the risk of relapse (Layton, 2009). Owing to its high lipophilicity and potent biological activity, isotretinoin reduces sebaceous gland size, decreases sebum production, normalizes follicular keratinization, and exerts anti-inflammatory effects, which together account for its exceptional therapeutic efficacy in acne management (Bagatin et al., 2020; Layton, 2009). However, this clinical effectiveness is accompanied by significant safety concerns, most notably its teratogenicity, necessitating strict regulatory controls and mandatory pregnancy prevention programs in all prescribing countries (Duboust, 2024; Reinold et al., 2024).

Recent pharmacovigilance data indicate a continued increase in isotretinoin prescribing globally, not only in dermatological practice but also in primary care settings, reflecting expanding access and rising demand for acne treatment across broader patient populations (Bagatin et al., 2020; Reinold et al., 2024). A 2024 population-based study reported a substantial rise in isotretinoin use among adolescents and women of reproductive age, raising ongoing concerns regarding fetal exposure despite existing prevention programs (Reinold et al., 2024).

In Hungary, the national pharmaceutical sales data published by the National Health Insurance Fund Administration (NEAK) indicate a steady increase in dispensed isotretinoin units over recent years, which is demonstrated both on Figure 3 and Figure 4. While Figure 3 presents the quantity of isotretinoin sold in kilograms, it only accounts for the capsule formulations. Figure 4, on the other hand, also includes isotretinoin sold in gel form, which explains the slightly higher values observed in this figure. However, from 2017 onward, the NEAK database contains records exclusively for the capsule formulation, as sales data for the gel form are no longer available.

Figure 3. Trends in the Sales of Isotretinoin Capsules of Various Brands in Hungary (Source: Graph created based on NEAK Data from 2014–2024)

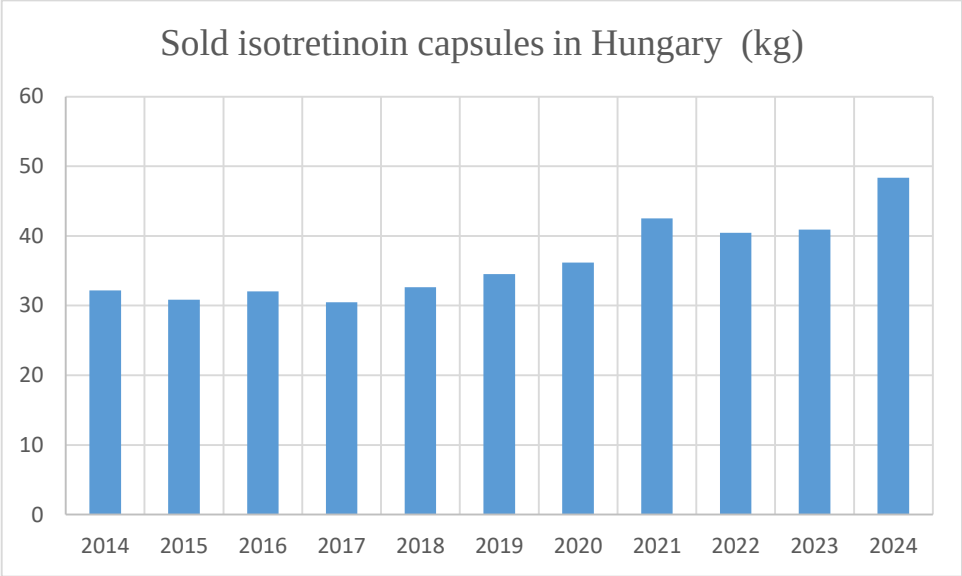
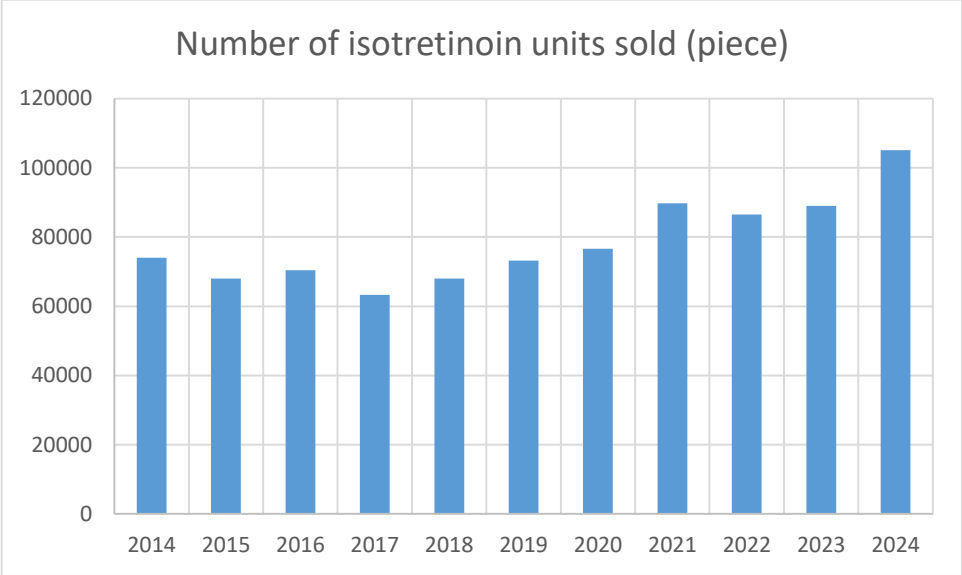


Figure 4. Number of isotretinoin units sold (boxes of medicine and tubes) (Source: Graph created based on NEAK Data from 2014–2024)



Pharmaceutical consumption trends are a useful predictor of such environmental inputs. Welch et al. (2022) demonstrated that national pharmaceutical wholesales data can effectively predict concentrations of active pharmaceutical ingredients in surface waters. Their model, based on Norwegian sales statistics, showed that predicted environmental concentrations closely aligned with monitoring data, confirming that medicine sales correlate strongly with freshwater pharmaceutical loads (Welch et al., 2022).

2.3. Isotretinoin from an Ecotoxicological Perspective

2.3.1. Environmental Behaviour of Isotretinoin

As previously mentioned, isotretinoin is a lipophilic retinoid with very low water solubility. For retinoic acid (a close chemical relative of isotretinoin) Szuts and Harosi (1991) reported measured solubilities on the order of 0.1–0.2 μM under neutral conditions; because isotretinoin has similar structural features (a conjugated polyene chain and a terminal carboxyl group) it behaves as a poorly water-soluble compound in natural waters. Roche's environmental risk assessment summary reports calculated log D values that are pH-dependent (for example log D ≈ 2.8 at pH 7, higher at lower pH), consistent with the molecule's weakly acidic character and propensity to be largely non-ionic under environmentally relevant pH ranges (F. Hoffmann-La Roche Ltd, 2024). The practical consequence is that isotretinoin will preferentially sorb to suspended particulate matter, organic detritus and sediments, and may be taken up and retained by lipid-rich tissues in aquatic organisms.

Photodegradation and isomerization are important environmental transformation pathways for retinoids. Retinoids are well known to be photolabile and chemically unstable in the presence of light, oxygen, and heat (Barua & Furr, 1998). Isotretinoin readily undergoes isomerization (for example to all-trans-retinoic acid and 9-cis isomers) and other photoproducts when exposed to UV/visible light. These phototransformations can occur rapidly in surface waters exposed to sunlight, producing isomers and breakdown products that often retain biological activity (Barua & Furr, 1998; F. Hoffmann-La Roche Ltd, 2024). Photodegradation therefore does not necessarily result in detoxification; instead, it may shift the chemical mixture toward different retinoid isomers and oxidation products that still interact with retinoid receptors or other biological targets. The presence of dissolved organic matter, turbidity, and sorption to particles will modify the photolysis rate by attenuating light exposure and by partitioning the compound into microenvironments where photoreactions are slower.

Sorption to organic matter and partitioning into sediments are predicted consequences of isotretinoin's lipophilicity. Because the compound prefers organic phases, sediments and particulate organic carbon become important sinks (Barua & Furr, 1998; F. Hoffmann-La Roche Ltd, 2024). Sorbed fractions are less available for direct aqueous exposure but may act as long-term reservoirs that slowly release compound back to overlying water or become bioavailable to benthic organisms. Sorption also affects the efficiency of removal in wastewater

treatment: compounds that partition strongly to sludge can be partially removed via primary settling and sludge handling, while dissolved fractions may pass through biological treatment units if they are not degraded.

Biodegradability and persistence of isotretinoin have been investigated using standard laboratory protocols and are summarized in the manufacturer's environmental risk assessment. In an inherent biodegradability test (OECD 302C, modified MITI or similar), isotretinoin showed 59% biodegradation (measured as biological oxygen demand (BOD)/theoretical oxyen demand (ThOD)) after 28 days (F. Hoffmann-La Roche Ltd, 2024). This result indicates that isotretinoin is partly biodegradable under adapted laboratory conditions, with an initially higher degradation rate that declined over time; the test report noted an adaptation phase and a maximum degradation rate during the early period. However, inherent biodegradability tests reflect potential under favourable microbial adaptation and nutrient conditions and do not always translate to rapid removal in full-scale wastewater treatment plants or in the environment, where microbial communities, contact times, and environmental conditions vary (F. Hoffmann-La Roche Ltd, 2024).

The balance of photochemical, chemical and biological transformation pathways determines isotretinoin's environmental persistence. Photolysis at the surface can be relatively fast under direct sunlight, producing isomers and oxidation products; microbial transformation may proceed in the water column and in sediments but can be slow for lipophilic molecules that are sequestered in particulate or solid phases. Laboratory evidence and modeling (F. Hoffmann-La Roche Ltd, 2024) suggest that isotretinoin is not extremely persistent in ideal degradation tests but that environmental persistence is plausible in sediments and zones with low light and limited microbial activity. The ratio of predictive environmental concentration (PEC) and predicted non-effective concentration (PNEC) calculated by F. Hoffmann-La Roche Ltd (2024) for a conservative European scenario (PEC ≈ 0.016 µg/L; PNEC derived from aquatic toxicity data) yielded a ratio below 1 (≈0.55), indicating limited overall risk at the scenario scale.

Measured concentrations of 13-cis-retinoic acid in surface waters and sewage treatment systems have been reported in several Asian countries. According to Yeung et al. (2020), 13c-RA concentrations in rivers adjacent to Liaodong Bay, China, ranged from below 0.03 to 0.41 ng/L (Wu et al., 2010). In Japan, concentrations in sewage treatment plant effluents in Osaka reached up to 11.5 ng/L, while influent levels ranged from 2.3 to 104.9 ng/L (Inoue et al., 2013; Sawada et al., 2012). In Hong Kong, effluent concentrations were around 1.0 ng/L, while influent levels

ranged from 2.00 to 4.11 ng/L (Zhou et al., 2019). In Beijing, influent concentrations ranged from 2.3 to 7.1 ng/L, and effluent concentrations were below 1.1 ng/L (Zhen et al., 2009). These values are several orders of magnitude lower than laboratory-based acute-effect thresholds (mg/L range) but show continuous environmental input from pharmaceutical sources. Yeung et al. (2020) noted that retinoic acids and their metabolites can act additively or synergistically with other endocrine-active compounds, as all-trans-, 9-cis-, and 13-cis-retinoic acids share nuclear receptor targets involved in thyroid and steroid hormone regulation.

2.3.2. Known and Suspected Ecotoxicological Effects

Isotretinoin poses an environmental concern because of its strong biological activity and discharge into aquatic systems through wastewater effluents. Retinoid signaling pathways are highly conserved among vertebrates and regulate embryogenesis, cell differentiation, and morphogenesis (Zouboulis, 2001). As a known human teratogen, isotretinoin can cause comparable developmental and reproductive effects in aquatic organisms.

According to F. Hoffmann-La Roche Ltd (2024), isotretinoin shows measurable toxicity in organisms representing different trophic levels. In a 72-hour algal growth inhibition test with *Raphidocelis subcapitata* (OECD 201), the 72 h EC_{50} values were 14.4 mg/L for growth rate and 1.39 mg/L for biomass, with a NOEC of 0.8 mg/L. The 48 h EC_{50} in *Daphnia magna* (OECD 202) was 5.4 mg/L, while an FDA No. 4.08 assay reported an EC_{50} of 2.06 mg/L. For fish (*Oncorhynchus mykiss*, OECD 203), the 96 h LC_{50} was 0.52 mg/L and the NOEC was 0.05 mg/L. The frog embryo teratogenesis assay with *Xenopus laevis* (FETAX) reported a 96 h LC_{50} (mortality) of 0.030 mg/L and a 96 h EC_{50} (developmental effects) of 0.003 mg/L (DeYoung, Bantle, & Fort, 2008). DeYoung and colleagues tested a pharmaceutical formulation containing isotretinoin rather than the pure active ingredient, which may influence the results.

Fish and amphibians are the most sensitive organisms tested. Isotretinoin and its active isomer, all-trans-retinoic acid, interfere with gene networks that control tissue differentiation and organ development (Zouboulis, 2001). In zebra fish, embryonic exposure can lead to spinal curvature, craniofacial deformities, pericardial oedema, and neural tube defects (DeYoung et al., 2008). Retinoids can also affect endocrine systems by interacting with nuclear receptors that regulate thyroid and steroid hormone signaling (Yeung et al., 2020).

While wastewater discharge and human pharmaceutical use represent major pathways for isotretinoin and related retinoids to enter aquatic systems, natural sources can also contribute to environmental exposure. Cyanobacteria are capable of producing retinoid compounds, and field

cyanobacterial blooms have been shown to induce developmental toxicity in aquatic vertebrates. For example, Pípal et al. (2020) demonstrated that zebrafish (*Danio rerio*) embryos exposed to bloom-derived retinoids developed characteristic malformations, including spinal curvature, craniofacial deformities, pericardial edema, and impaired hatching.

In addition to cyanobacteria, algae of the genus *Desmodesmus* have also been identified as natural producers of retinoid compounds. Laboratory analyses have confirmed the presence of retinoic acids, including all-trans-retinoic acid (ATRA) and 9-cis-retinoic acid, in cultures of *Desmodesmus quadricauda*, a closely related species to *D. subspicatus* (Bittner et al., 2021).

Despite the above mentioned toxicological effects, the ecotoxicological studies focusing on 13-cis-retinoic acid remain limited. A search in the U.S. EPA ECOTOX Knowledgebase (http1) returned only 10 entries for 13-cis-retinoic acid (CAS No. 4759-48-2), derived from just two studies—one on a pharmaceutical formulation (DeYoung et al., 1991) and one on the active compound (Alzualde et al., 2018). Complementary searches in Scopus (http2), using combinations of "isotretinoin," "13-cis-retinoic acid," "aquatic," "environment," and "ecotoxicology," yielded between none and 26 search results, the majority concerning clinical pharmacology or human toxicology rather than environmental effects. Searches in PubMed (http3) produced over a hundred results for similar keyword combinations, yet most references focused on medical applications or therapeutic mechanisms, and the environmental context was largely absent. Across these three databases, approximately four articles had actually any ecotoxicological relevance.

2.3.3. Regulatory Context and Knowledge Gaps

As mentioned in Section 2.3.2, research on the environmental effects of isotretinoin is still limited, and the regulations addressing its presence in water environments are also sparse. Right now, isotretinoin is not included in the major regulated pollutants list of either the U.S. Environmental Protection Agency (EPA) or the European Union's Watch List as defined by the Water Framework Directive (WFD; 2000/60/EC), which is a gap in the regulation regarding its potential environmental impact (European Comission, 2023; U.S. EPA, 2015; 2023).

The EPA Priority Pollutant List, developed under the Clean Water Act, includes 126 substances considered toxic or harmful to aquatic life. This list features heavy metals, certain pesticides, chlorinated hydrocarbons, and other organic pollutants. It serves as a basis for U.S. water quality standards and industrial discharge limits. However, it does not cover new contaminants,

such as pharmaceuticals or personal care products, unless clear evidence of environmental harm is provided (EPA, 2015).

In the European Union, pharmaceuticals are seen as emerging contaminants. This means they are recognized as pollutants of concern, but they are not yet subject to strict legal environmental standards. The main piece of EU water legislation, the Water Framework Directive (2000/60/EC), sets a broad goal of achieving "good chemical and ecological status" for all surface and groundwater in the EU. It outlines a framework for pollution control, which is supported by later directives that establish Environmental Quality Standards (EQS) for specific substances (European Parliament and Council, 2000).

Directive 2013/39/EU, which updates both the Water Framework Directive and the Environmental Quality Standards Directive (2008/105/EC), sets EQS values for a group of priority and priority hazardous substances based on their toxicity, persistence, and ability to accumulate in organisms. This includes 33 priority substances with defined concentration limits to protect aquatic ecosystems. For instance, EQS values are set at 0.0065 µg/L for benzo[a]pyrene, 0.01 µg/L for nonylphenol, and 0.1 µg/L for individual pesticides (Directive 2013/39/EU, Annex I). However, there are no EQS values for pharmaceuticals like isotretinoin. Only a few drugs, such as diclofenac, 17β-estradiol, and 17α-ethinylestradiol, have been added to the EU Surface Water Watch List for monitoring (European Commission, 2022). This Watch List allows member states to gather data on emerging substances, which may later be considered for priority pollutant status if there is enough evidence of risk.

Similarly, the Drinking Water Directive (EU 2020/2184), which replaced Directive 98/83/EC, takes a risk-based approach to ensure the safety of drinking water. While this Directive recognizes pharmaceuticals as contaminants of concern, it does not set specific limits for them. Instead, Article 13 introduces a watch list system for substances "likely to be present in water intended for human consumption and which pose a potential risk to human health" (Official Journal of the European Union L 435/1, 2020). The current watch list includes β-estradiol, nonylphenol, and perfluorooctane sulfonate (PFOS), which are all known or suspected endocrine disruptors. The European Commission must review and, if needed, update this list at least every six years (European Commission, 2017). Although the Directive imposes strict limits on conventional chemical parameters (e.g., arsenic ≤ 10 µg/L, benzene ≤ 1 µg/L), it has not defined similar thresholds for pharmaceutical residues (Directive 2020/2184, Annex I).

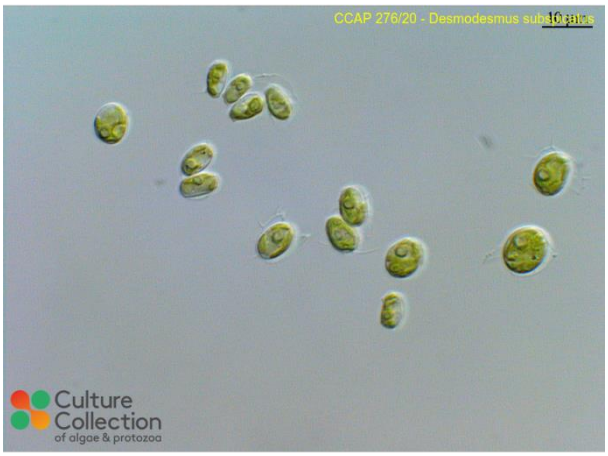
The lack of isotretinoin in these existing regulations may be partly due to how rarely it is detected in the environment, as retinoids are typically found at very low levels in surface waters (a few ng/L or less) (Yeung et al., 2020). Therefore, isotretinoin and similar compounds have not drawn enough regulatory interest to warrant inclusion in pollutant monitoring programs. Additionally, no EU directive currently addresses retinoids as a chemical group, and isotretinoin is missing from both the priority lists of the Water Framework Directive and the Drinking Water Directive.

Kubickova et al. (2021) note that retinoid compounds interact with key nuclear receptor pathways that traditional endocrine-disruptor screening often overlooks. Current regulations mainly focus on classical endocrine disruptors, such as estrogens, bisphenol A, and some pesticides, which act through well-understood hormonal mechanisms. In contrast, the signaling pathways affected by isotretinoin and related compounds represent a different action mode, possibly needing a new regulatory strategy to evaluate their ecological and health effects.

2.4. Characteristics of the Aquatic Test Organism *Desmodesmus subspicatus*

Desmodesmus subspicatus is a unicellular freshwater green alga belonging to the division Chlorophyta and class Chlorophyceae. It is recognized in international ecotoxicological standards as a recommended test species for evaluating the effects of chemicals on primary producers in aquatic ecosystems. The OECD Guideline 201 (2011) and ISO 8692 (2012) both list *Desmodesmus subspicatus* as a valid test organism for freshwater algal growth inhibition studies. According to the SAG database, *D. subspicatus* forms non-motile coenobia typically consisting of four cells, though two- and eight-celled forms may also occur. The cells possess a rigid cell wall and a parietal chloroplast containing a single pyrenoid, which facilitates carbon fixation during photosynthesis. In this study, the strain CCAP 276/20 (Culture Collection of Algae and Protozoa) is used (see Figure 5), which is maintained as an axenic freshwater culture and recommended for ecotoxicity testing in standard assays.

Figure 5. Desmodesmus subspicatus cells (strain CCAP 276/20) under light microscopy (Source: Culture Collection of Algae and Protozoa, CCAP 276/20)



Taxonomically, *Desmodesmus subspicatus* is classified as follows (SAG 86.81):

- **Kingdom:** Plantae
- **Division:** Chlorophyta
- **Class:** Chlorophyceae
- **Order:** Sphaeropleales
- **Family:** Scenedesmaceae
- **Genus:** *Desmodesmus*
- **Species:** *subspicatus*

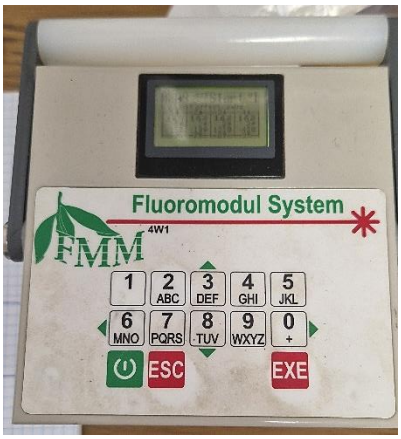
As a photosynthetic primary producer, *D. subspicatus* is ecologically important in freshwater systems. They contribute to oxygen production and nutrient cycling, while supporting the base of the aquatic food web. Their rapid growth rate and sensitivity to environmental changes make them ideal indicators of water quality and suitable organisms for toxicity testing (ISO 8692, 2012). The OECD 201 guideline recommends *Desmodesmus subspicatus* due to its stable growth characteristics, sensitivity to pollutants, and ability to provide quantifiable endpoints within a standard 72-hour exposure period (OECD, 2011).

Species within the genus *Desmodesmus* have also been shown to naturally produce biologically active compounds. Laboratory studies have identified retinoids, including all-trans-retinoic acid and 9-cis-retinoic acid, in cultures of *Desmodesmus quadricauda*, a closely related species (Bittner et al., 2021).

2.5. The FluoroMeter Module

Chlorophyll-a molecules are present in all algal species; therefore, the quantity of algal cells can also be estimated using techniques based on chlorophyll-a fluorescence. One such instrument suitable for determining induced chlorophyll-a fluorescence is the FluoroMeter Module (FMM), a portable, modular fluorometer adapted from a plant-leaf fluorescence system for use with microalgal samples. A photograph of the FluoroMeter Module instrument is shown in Figure 6.

Figure 6. FluoroMeter Module (FMM) instrument used for chlorophyll-a fluorescence measurements in the algal growth inhibition test (Source: own picture)



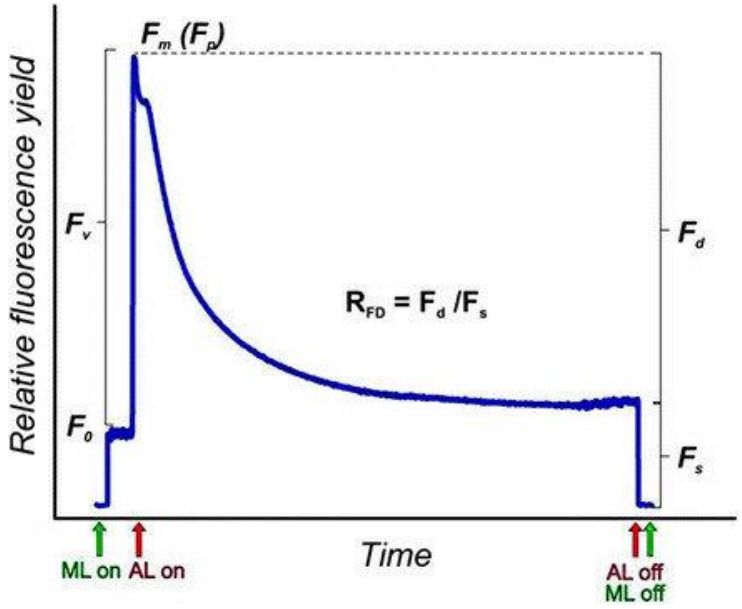
The FMM employs a 635 nm laser diode as the actinic light source, with a 256-step adjustable power control (0–100%) and a maximum output of 5.6 mW. Excitation and detection are handled by a fiber-optic system, in which a central laser-guiding fiber directs the beam to the microplate wells, while surrounding fibers collect emitted fluorescence from approximately 1 mm above the sample surface. The emission is filtered at 690 nm and 730 nm to remove scattered light, and fluorescence is detected by low-noise PIN photodiodes. The signal is digitized with a 12-bit analog-to-digital converter (4095 relative fluorescence units or RFUs), and the instrument is operated and controlled via a microprocessor system with USB or serial data transfer (Lázár et al., 2023). This setup has been used in various algal ecotoxicity studies. It ensures that the results are comparable and methodologically consistent with those reported earlier (Lázár et al., 2023; Klátyik et al., 2024; Takács et al., 2024).

Before the measurement, algal suspensions were placed in a 96-well microplate and dark-adapted for approximately 10 minutes in a specialized sample holder to stabilize photosynthetic activity and ensure uniform baseline fluorescence before excitation (Klátyik et al., 2024; Takács et al., 2024). During measurement, fluorescence emission was recorded simultaneously at 690

nm and 735 nm, corresponding to the two main chlorophyll-a fluorescence maxima (Lázár et al., 2023; Klátyik et al., 2024; Takács et al., 2024). Carotenoids themselves do not fluoresce but transfer absorbed energy to chlorophyll-a molecules, thereby contributing to the fluorescence signal (Barócsi et al., 2009).

The principle of the measurement is based on the Kautsky effect, according to which the photochemical processes of photosynthesis are temporarily inhibited in dark-adapted plant samples. When the samples are suddenly exposed to high-intensity laser excitation, the excess light energy is re-emitted by the chlorophyll molecules in the form of fluorescence, as the conditions required for photosynthesis take longer to re-establish. After a few minutes, the fluorescence intensity gradually decreases and stabilizes at a lower level (Kautsky et al., 1931; Lenk et al., 2016).

Figure 7. Schematic representation of the Kautsky effect in a dark-adapted sample upon illumination. The parameters shown on the curve are explained in Table 1. ML refers to modulated measuring light, while AL refers to actinic light. (Source: Brestic & Zivcak, 2013)



The photochemical parameter provides a more sensitive endpoint compared to conventional biomass-based indicators such as optical density, chlorophyll-a content, or cell number determined using a Bürker chamber (Jakab et al., 2023). After each measurement, the FMM immediately transmits the recorded data to a computer, where it is displayed graphically using the FluorMeas software. In the graphs, the x-axis represents time (seconds), while the y-axis

shows the intensity of the fluorescence signal. In the kinetic curves, data measured at 690 nm are indicated in red, while those measured at 735 nm are shown in purple.

The software also displays values directly readable from the kinetic curve, as well as derived parameters that characterize photosynthetic activity (see Table 1).

Table 1. Summary of main fluorescence parameters and their meaning (Source: Barócsi et al., 2009; Klátyik et al., 2024)

Symbol	Name	Meaning
F_0	Minimal fluorescence yield	Non-variable, baseline level fluorescence, when all PSII centres are open.
F_p	Peak fluorescence	Peak fluorescence when all PSII centres are closed.
F_s	Steady-state fluorescence	Fluorescence during continuous illumination.
$T_{1/2}$	Half-time of fluorescence rise	Rise time to $(F\text{-}F_0)=0.5(F_p\text{-}F_0)$
F_v/F_0	Variable to minimal fluorescence ratio	$(F_p-F_o)/F_o$; indicates potential PSII activity.
F_v/F_p	Maximum quantum yield of PSII photochemistry	$(F_p-F_o)/F_p$; efficiency of PSII photochemistry.
Rfd	Vitality index	$(F_p-F_s)/F_s$; indicator of photosynthetic vitality and stress.
A_p	Performance index	Area between fluorescence curve and F_p; reflects overall photosynthetic performance.

3. Materials and methods

The algal growth inhibition tests were conducted in accordance with OECD Guideline 201 (Freshwater Alga and Cyanobacteria, Growth Inhibition Test). The objective of this test is to assess the inhibitory effects of isotretinoin on the growth of freshwater microalgae under controlled laboratory conditions. Each experiment was performed three times independently to ensure reproducibility and reliability of the results.

In this method, exponentially growing algal cultures are exposed to a range of test-substance concentrations for 72 hours. The test evaluates the substance's ability to inhibit algal growth relative to untreated controls. Algal growth inhibition was assessed based on optical density measurements at 750 nm, and chlorophyll-a content was additionally determined following ethanol extraction. Additionally, the photosynthetic activity was assessed using the FluoroMeter Module, which was applied as a complementary analysis outside the standard OECD 201 protocol to evaluate photosynthetic performance and stress responses.

3.1. Origin of test compound and algal species

The test compound, isotretinoin, and the materials used to make the Zehnder-8 liquid medium were obtained from Sigma Aldrich Kft. The additional *Desmodesmus subspicatus*, Hegewald & Schmidt (CCAP 276/20), was derived from the Scottish Culture Collection of Algae and Protozoa.

3.2. The test organism and the culture medium

Desmodesmus subspicatus cultures were maintained under sterile conditions in 500 mL Erlenmeyer flasks at room temperature, under continuous cool-white illumination (7760±1100 lux). Subculturing was carried out weekly to prevent contamination by bacteria or other algal species. The inoculum was diluted to approximately 1:100, and the freshly prepared culture reached the exponential growth phase within approximately 11 days, a prerequisite for initiating the test.

Synchronous cultures were maintained in Zehnder-8 (Z8) liquid medium (Zehnder & Gorham, 1960). The medium was prepared from four stock solutions as follows:

Stock solution I (in 300 mL distilled water):

- 46.7 g $NaNO_3$

- 5.9 g $Ca(NO_3)_2 \cdot 4H_2O$
- 2.5 g $MgSO_4 \cdot 7H_2O$

Stock solution II (in 300 mL distilled water):

- 39.3 g K_2HPO_4
- 6.3 g Na_2CO_3

Stock solution III (prepared separately):

- Fe solution: 1.4 g $FeCl_3 \cdot 6H_2O$ dissolved in 150 mL 0.1 N HCl
- EDTA solution: 2.2 g EDTA-Na_2 dissolved in 150 mL 0.1 N NaOH
- 50 mL of each solution was combined and made up to 490 mL with distilled water

Stock solution IV (in 500 mL distilled water):

- 25 mg $Na_2SiO_4 \cdot 2H_2O$
- 44 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$
- 59.6 mg KBr
- 143.5 mg $ZnSO_4 \cdot 7H_2O$
- 73 mg $Co(NO_3)_2 \cdot 6H_2O$
- 62.5 mg $CuSO_4 \cdot 5H_2O$
- 237 mg $Al_2(SO_4)_3$
- 1550 mg H_3BO_3
- 25 mg $LiCl \cdot H_2O$
- 1115 mg $MnCl_2 \cdot 4H_2O$

For the final medium, 3 mL of stock solution I, 1 mL of stock solution II, 10 mL of stock solution III, and 0.08 mL of stock solution IV were combined and diluted to 1 L with distilled water, followed by autoclave sterilization.

3.3. Test Substance and Preparation of Solutions

The test substance used in this study was isotretinoin, a retinoid pharmaceutical compound widely used in dermatological applications. Due to its low water solubility, ethanol was used as a solvent (<0.1 mg/L at 25 °C). Five mg of isotretinoin was measured and mixed with 500 µL of ethanol. Complete dissolution of isotretinoin required mechanical mixing and sonication. The mixture was vortexed with a Phoenix Instrument RS-VA10 vortex mixer (RK Tech) and then ultrasonicated in a TESLA UC 005 AJ1 ultrasonic bath to ensure complete solubilization

of the compound. The resulting solution was transferred into a 500 mL volumetric flask containing 50 mL of tenfold diluted Z8 medium (prepared at a ratio of 100 mL Z8 stock to 1000 mL distilled water), and the flask was then filled to the calibration mark with distilled water. This solution represented the highest test concentration (T5), corresponding to 10 mg/L isotretinoin.

A geometric (1:2) dilution series was prepared from the stock solution to obtain five nominal test concentrations:

- T1 = 0.625 mg/L
- T2 = 1.25 mg/L
- T3 = 2.5 mg/L
- T4 = 5 mg/L
- T5 = 10 mg/L

Each test concentration was prepared at a total volume of 200 mL using a tenfold-diluted Z8 medium.

For each concentration level, the prepared 200 mL solution was divided into four identical 300 mL Erlenmeyer flasks, with 50 mL added to each. Three flasks were assigned as replicates, with algae added later. One flask served as a concentration-specific blank containing no algae to account for background absorbance and potential solvent interference.

The solvent control was prepared by adding 50 mL of tenfold diluted Z8 medium and 500 µL of ethanol to a 500 mL volumetric flask, which was then filled to the calibration mark with distilled water. The solution was sealed with parafilm and gently mixed to ensure homogeneity. The resulting 500 mL solution was then divided into four 300 mL Erlenmeyer flasks (S0–S3), each containing 50 mL: one blank without algae (S0) and three biological replicates (S1–S3).

The negative control consisted solely of tenfold diluted Z8 medium. Similarly to the treatment groups and solvent control, a total volume of 200 mL was prepared and distributed into four 300 mL Erlenmeyer flasks, each containing 50 mL. Three flasks (C1–C3) were inoculated with algae, while one flask (C0) served as the blank.

3.4. Test Design and Experimental Procedure

3.4.1. Inoculation process

The test was initiated by inoculating each flask with an exponentially growing *Desmodesmus subspicatus* culture to achieve a target optical density (OD) of 0.030 at 750 nm in 50 mL of test solution. OD was measured with a Camspec M330 UV–Visible Spectrophotometer at 750 nm, blanked with a tenfold dilution of Z8 (set as aborbance value of 0.000).

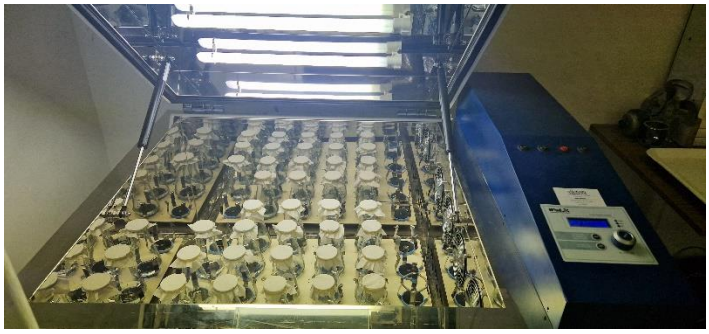
To determine the precise inoculum volume required to reach the target OD, *Desmodesmus subspicatus* culture was added gradually in small amounts to 50 mL of Z8 medium. The OD was measured after each addition until a value of approximately 0.030 was achieved. This calibration process was conducted separately for each experimental run. The final volumes required to reach the target OD were as follows:

- 1st measurement: 270 µL to reach OD = 0.030
- 2nd measurement: 210 µL to reach OD = 0.029
- 3rd measurement: 200 µL to reach OD = 0.030

For each run, the exact volume determined (270, 210, or 200 µL) was added to each Erlenmeyer flask in that run, excluding the blanks.

After inoculation, flasks were covered with paper towels secured with rubber bands and placed in the incubator (Witeg WIS-10RL, Wertheim, Germany) under continuous operation (23 °C, 100 rpm) and and uniform cool-white illumination (approximately 7760±1100 lux) as seen on Figure 8. The exposure period was 72 h. Flasks were gently mixed once during incubation and returned to the incubator in randomized order to minimize positional effects.

Figure 8. Algal cultures in the Witeg WIS-10RL incubator (Source: own picture)



3.4.2. Growth Measurement (Optical Density)

After 72 hours, the optical density (OD) was measured at 750 nm using a Camspec M330 UV–Visible Spectrophotometer. For each concentration, the instrument was blanked with the corresponding concentration-specific blank (test solution without algae) to correct for background absorbance and any solvent effects.

Because the final OD values were high, the replicates were diluted prior to measurement to improve precision. Specifically, samples were diluted at a 1:4 ratio (1 part sample : 4 parts blank). OD was measured on the diluted samples, and values were then back-calculated to undiluted equivalents by multiplying by four. The values were recorded for all treatments and controls and used for subsequent growth inhibition calculations.

Following OD determination, the percent inhibition (or stimulation) of algal growth was calculated for each treatment relative to the control. Since specific growth rates were not calculated, optical density values were used as a proxy for biomass according to a modified form of the OECD 201 equation:

$$\%\mathbf{I} = \frac{\boldsymbol{OD_C - OD_T}}{\boldsymbol{OD_C}} \times \mathbf{100}$$

where:

%I = percent inhibition of algal growth (negative values indicate stimulation)

OD_C = mean optical density of the control group

OD_T = optical density measured for the treatment replicate

For each concentration, the back-calculated OD values (corrected for dilution) were averaged across replicates, and %I was computed by comparing the mean treatment value to the mean control value. Negative %I values were interpreted as growth stimulation, while positive values indicated growth inhibition.

3.4.3. Chlorophyll measurement

The procedure for chlorophyll-a determination was based on the ISO 10260:1992 standard (*Water Quality – Measurement of biochemical parameters – Spectrometric determination of the chlorophyll-a concentration*; ISO, 1992). At the end of the 72-hour exposure period, chlorophyll-a content was measured to evaluate algal biomass and photosynthetic activity. The

three replicate samples from each treatment group (T1–T5), the solvent control (S), and the negative control (C) were poured together to create pooled samples (Cx, Sx, T1x, T2x, T3x, T4x, and T5x). After mixing, 50 mL of each pooled sample was transferred into centrifuge tubes. One tube filled with distilled water was used for balance in the centrifuge.

The samples were centrifuged at 3700 rpm for 30 minutes at 4°C using a Hettich Zentrifugen Rotina 46R centrifuge. After centrifugation, the liquid phase (supernatant) was carefully poured off, leaving only the algal pellet. To extract chlorophyll-a, 20 mL of 96% ethanol was added to each tube, and the samples were gently shaken to resuspend the algal cells. The solvent was chosen due to its efficiency in lysing algal cells and releasing chlorophyll-a. The tubes were then placed in a Phoenix Instrument WB-12 water bath at 75°C for 15 minutes to facilitate cellular disruption and pigment extraction.

Once heating was complete, the samples were cooled to room temperature under running tap water and centrifuged again at 4700 rpm for 20 minutes at 4 °C. After centrifugation, the ethanol solution containing the extracted chlorophyll-a was poured into clean beakers for spectrophotometric measurement.

Absorbance was measured at three wavelengths: 750 nm, 666 nm, and 653 nm, using ethanol as the blank. The value at 750 nm (E750) was used to correct for turbidity, while 666 nm (E666) and 653 nm (E653) correspond to chlorophyll-a absorption peaks. For accurate measurement at 666 nm, samples were diluted in a 1:4 ratio (1 mL sample + 3 mL ethanol), and the final value was multiplied by four to obtain the undiluted result.

Chlorophyll-a concentration (Ca) was calculated using the Felföldy (1987) equation:

$$Ca = (17{,}12 * x1 - 8{,}68 * x2) * m * 1000 / M \ [\mu g/l]$$

Where:

- Ca = chlorophyll-a concentration in µg/L
- m = volume of ethanol used for extraction (20 mL)
- M = volume of the filtered sample (mL)
- x1=E666 – E750
- x2=E653 – E750

3.5. Photosynthetic Activity Assessment Using the FluoroMeter Module

Following 72 hours of exposure, 250 µL of each algal sample from all test concentrations and controls was transferred into the wells of a 96-well microplate. To ensure complete dark adaptation of the photosystem II (PSII) reaction centers, the samples were kept in darkness for 10 minutes prior to measurement. After dark adaptation, fluorescence induction was initiated using the instrument's laser excitation source, and fluorescence kinetics were recorded over a 5-minute measurement period. Each sample was measured in triplicate.

Photosynthetic performance was evaluated using two chlorophyll fluorescence parameters: the photochemical efficiency of photosystem II (Fv/Fp) and the vitality index (Rfd). The Fv/Fp parameter indicates the efficiency of PSII photochemistry, while Rfd reflects overall photosynthetic vitality and stress response. For comparative analysis, fluorescence values recorded at 690 nm were used and are presented in the Results section.

3.6. Statistical analysis

All statistical analyses were performed in MATLAB R2025b (MathWorks Inc.) using the Statistics and Machine Learning Toolbox. Prior to hypothesis testing, Grubbs' test was applied to determine the significant outliers at $\alpha = 0.05$ significance level. After the examination of outliers, data were assessed for compliance with parametric test assumptions. Normality was evaluated using the Lilliefors test, and homogeneity of variances was assessed with Levene's test. A significance level of $\alpha = 0.05$ was applied.

For parameters that met both assumptions ($p > 0.05$), a one-way analysis of variance (ANOVA) was conducted to evaluate differences between treatment groups, followed by Tukey's HSD post-hoc test for pairwise comparisons (significance level $\alpha = 0.05$). When the assumptions for ANOVA were not fulfilled, the non-parametric Kruskal–Wallis test was applied instead, followed by post-hoc multiple comparison testing ($\alpha = 0.05$).

4. Results and evaluation

4.1. Culture Performance & Test Validity

According to OECD Guideline 201 (OECD, 2011), the algal growth inhibition test is considered valid when the control cultures show at least a 16-fold increase in biomass, a coefficient of variation among control replicates below thirty-five percent, and a coefficient of variation of the mean specific growth rate within the control of seven percent or less. In the present study, the control cultures exhibited consistent exponential growth with low variability between replicates, fulfilling the required validity criteria. Although pH was not recorded, the stable incubation conditions suggest that major pH fluctuations were unlikely.

4.2. Results of the optical density measurement

Across the tested concentration range (0.625–10 mg/L), no growth inhibition was observed in *Desmodesmus subspicatus* based on OD_{750} measurements. After the exclusion of outlier data determined by Grubbs test at 0.05 significance level, average optical density and standard deviation values were determined. Inhibition % values were calculated based on the OD values (Table 2.). Ethanol was applied as solvent in this ecotoxicological assay at 500 µL/L concentration, thus solvent biological control was also investigated at the highest concentration of 500 µL/L. A stimulation of growth (20.8%) was seen at biological solvent control (S), as reported in previous algal toxicology studies using ethanol as a solvent (Miazek et al., 2017). The statistical analysis resulted in significant difference ($p<0.05$) between the biological control (C) and biological solvent control (S), thus inhibition % values of treated groups were compared to S group. Although significant difference ($p<0.05$) was determined in T1, T2, T3 and T4 isotretinoin treated groups compared to the biological solvent control, this difference may have been the results of the decreasing solvent content and not the effect of isotretinoin.

Table 2. Optical density and inhibition % values in the treatment groups determined at 750 nm wavelength. C – biological control, S – biological solvent control, T1-T5 – concentration series of isotretinoin, Negative inhibition % value mean stimulation of growth.

treatment group	average OD	standard deviation (OD)	average inhibition %	standard deviation of inhibition %
C	1.18	0.05		
S	1.42	0.21	-20.8	3.0

T1 (0.625 mg/l)	1.26	0.07	-7.3	0.4
T2 (1.25 mg/L)	1.28	0.08	-8.7	0.5
T3 (2.5 mg/L)	1.36	0.13	-15.1	14
T4 (5 mg/L)	1.33	0.08	-13.0	0.8
T5 (10 mg/L)	1.39	0.15	-18.4	1.9

In this study results align with the reported EC_{50} of 14.4 mg/L for isotretinoin in algal tests on *Raphidocelis subcapitata* green alga species (F. Hoffmann-La Roche Ltd, 2024), indicating that the highest concentration tested (10 mg/L) was still below the expected effective toxicity threshold.

4.3. Interpretation of Chlorophyll-a Response

Chlorophyll-a content was determined by ISO 10260:1992 standard in biological control, biological solvent control and isotretinoin-treated groups. No outlier data was determined by Grubbs test at significance level of 0.05. Chlorophyll-a content was calculated by the Felföldy equation described in 3.4.3 chapter. Inhibition % values were calculated based on the chlorophyll-a content (Table 3.).

A stimulation in chlorophyll-a content (6.8%) was seen at biological solvent control (S). The statistical analysis resulted in significant difference ($p<0.05$) between the biological control (C) and biological solvent control (S), thus inhibition % values of treated groups were compared to S group. Significant difference ($p<0.05$) was determined in all isotretinoin-treated groups ($p<0.05$).

Table 3. Chlorophyll-a content and inhibition % values in the treatment groups. C – biological control, S – biological solvent control, T1-T5 – concentration series of isotretinoin, negative inhibition % value mean stimulation of chlorophyll-a production.

Treatment group	Average chlorophyll – content (µg/L)	Standard deviation of chlorophyll-a content (µg/L)	Average inhibition %	Standard deviation of inhibition %
C	6814.1	932.8		
S	7274.2	529.7	-6.8	0.5
T1	8232.1	1198.0	-20.8	3,0
T2	8056.0	882.1	-18.2	2,0

T3	8380.5	407.6	-23,0	1,1
T4	9449.4	947.9	-38,7	3,9
T5	31192,87	2806,3	-357,8	-32,2

The stimulation in chlorophyll-a production was concentration-dependent and positively correlated with the concentration. These results indicate a hormetic response, where low doses of a biologically active compound cause stimulation rather than inhibition. It is consistent with literature showing biphasic responses for retinoids in aquatic organisms (Young et al., 2020). Moreover, it also suggests that the observed effect was not merely enhanced biomass, but rather a physiological pigment-accumulation response**.**

Microalgae commonly adjust their pigment content as a stress-acclimation strategy. When exposed to chemical stressors or conditions that reduce photosynthetic efficiency, algae may increase chlorophyll levels to enhance light-harvesting capacity and compensate for reduced photosynthetic performance (Bock et al., 2022). Such responses can occur even when cell division slows, resulting in higher chlorophyll per cell rather than a straightforward increase in biomass (Songserm et al., 2024).

In this experiment, the concentration-dependent chlorophyll-a increase at isotretinoin-treated groups likely reflects a stress-induced pigment upregulation. Isotretinoin's hydrophobicity and potential interaction with cellular membranes may have triggered compensatory photophysiological adjustments, producing elevated chlorophyll-a levels despite only moderate increases in OD. Thus, the isotretinoin active compound appears to have induced physiological stress with chlorophyll-accumulation, rather than enhanced algal performance.

4.4. Photosynthetic Efficiency (Fluorescence Parameters)

Effect of isotretinoin active compound on PSII photochemical system was also investigated and measured by the induced fluorescence-based FluoroMeter Modul (FMM). The instrument is developed to determine the excitation kinetics of chlorophyll-a fluorescence induction besides the traditional Kautsky induction kinetics. Fluorescence-based parameters, as Fp, Fs, T1/2, Fv/F0, Fv/Fp, Rfd, Ap and F0 were determined or calculated (see chapter 2.5, Table 1). Among the investigated parameters T1/2 (half-time of fluorescence rise) and Fv/F0 (variable to minimal fluorescence ratio indicating the potential PSII activity) showed a significant increase and Ap (performance index indicating the overall photosynthetic performance) a significant decrease

at the highest isotretinoin concentration (10 mg/L) (Table 4). Other parameters remained stable, did not show any significant concentration-dependence.

Table 4. Fluorescence-based parameters that showed significant differences compared to the control. C – control, T1-5 – isotretinoin-treated groups at concentration range of 0.625 – 10 mg/L. T1/2 – half-time of fluorescence rise, Fv/F0 – variable to minimal fluorescence ratio, Ap – performance index.

Parameter	**C**	**T1**	**T2**	**T3**	**T4**	**T5**
T1/2	0.02±0,02	0.02±0.02	0.00±0,00	0.01±0.00	0.01±0.01	0.75±0.2
Fv/F0	2.70±0.42	2.86±0.20	2.77±0.80	2.75±0.00	2.64±1.29	3.42±1.84
Ap	0.49±0.37	0.37±0.31	0.29±0.30	0.47±0.52	0.46±0.45	0.03±0.01

Significantly increased chlorophyll-a content, T1/2, Fv/F0 and significantly decreased Ap value at the highest concentration (10 mg/L) are a result of physiological stress of isotretinoin as retinoid-type compound. Some comparative study proved, that a low-level stress (e.g. low concentration of pollutants) can resulted in stimulation of chlorophyll contents as a sign of adaptive conditioning (Agathokleous et al., 2020; Jalal et al., 2021; Xu et al., 2023). The increased T1/2 and decreased Ap values indicate the electron transfer is affected and the coupling/energy transfer changed. Inhibition or slowing of the electron transfer by herbicide or heavy metal is published in various studies (Ospina Calvo et al., 2025; Wang et al., 2022; Zimmermann et al., 2006).

5. Conclusion and recommendations

The results of this study demonstrated that isotretinoin did not inhibit the growth of *Desmodesmus subspicatus* over the tested concentration range of 0.625–10 mg/L. Based on optical density (OD_{750}) measurements, no reduction in algal biomass was observed. In fact, several treatment groups showed moderate stimulation. Statistical evaluation confirmed that these differences were not caused by isotretinoin itself but rather by the biological solvent control containing ethanol. The solvent concentration applied (500 µL/L) induced a measurable enhancement in algal growth compared to the untreated control, which is consistent with previous studies reporting that low ethanol concentrations can serve as an alternative carbon source or stimulate algal metabolism (Miazek et al., 2017). When inhibition percentages were calculated relative to the solvent control, no significant decrease in growth was observed in any of the isotretinoin-treated groups. Therefore, it can be concluded that under the applied acute exposure conditions, isotretinoin did not display measurable toxicity to *D. subspicatus*.

In contrast, chlorophyll-a content showed a more pronounced increase, especially at higher isotretinoin concentrations. This disproportionate rise compared with optical density suggests that the effect was not purely due to biomass growth but rather a physiological adjustment involving increased pigment synthesis. The pattern observed in the chlorophyll-a results may indicate a hormetic response, where low or moderate concentrations of a chemical induce stimulation rather than inhibition. Hormesis represents a biphasic dose–response relationship characterized by beneficial or adaptive responses at sublethal exposure levels, followed by adverse effects at higher concentrations. Biologically, this phenomenon reflects an adaptive stress response in which moderate chemical or environmental stimuli activate cellular protection and repair mechanisms (Mattson, 2008). In this case, the enhancement of pigment content may reflect an adaptive metabolic response to mild chemical stress rather than a direct toxic effect.

The findings of this research are consistent with the broader ecotoxicological understanding of retinoids and other pharmaceutical contaminants. As discussed in the literature, isotretinoin and related retinoid compounds primarily act through receptor-mediated pathways, affecting gene expression and cellular signaling rather than directly interfering with photosynthesis or cell division (Kubickova et al., 2021). This explains the absence of classical growth inhibition, as the compound may instead trigger sublethal or metabolic responses. The present findings also correspond with the data reported by F. Hoffmann-La Roche Ltd (2024), who determined an

EC_{50} value of 14.4 mg/L for algal species exposed to isotretinoin, confirming its low acute toxicity to primary producers. The absence of inhibitory effects in this experiment, even at 10 mg/L, supports this classification and indicates that isotretinoin is unlikely to pose an immediate risk to algal populations in aquatic ecosystems.

During the photosynthetic activity assessment several chlorophyll-a fluorescence parameters related to PSII photochemical performance (Fp, Fs, $T_{1/2}$, F_v/F_0, Fv/Fp, Rfd, Ap, and F_0). Among these, a significant increase in $T_{1/2}$ (half-time of fluorescence rise) and F_v/F_0 (the variable to minimal fluorescence ratio indicating potential PSII activity), together with a significant decrease in Ap (overall photosynthetic performance index), were observed at the highest isotretinoin concentration (10 mg/L). These findings indicate that isotretinoin at elevated levels affects the electron transfer processes within PSII, leading to slower excitation kinetics and reduced energy transfer efficiency. Similar alterations in fluorescence parameters have been reported under herbicide or heavy metal exposure (Zimmermann et al., 2006; Wang et al., 2022; Ospina Calvo et al., 2025), suggesting that isotretinoin induces a comparable stress response pattern.

The combined increase in chlorophyll-a content, $T_{1/2}$, and F_v/F_0, alongside the decreased Ap value, supports the interpretation that isotretinoin acts as a mild physiological stressor rather than a growth inhibitor. These fluorescence changes, consistent with the concept of hormesis, likely reflect an adaptive response of *D. subspicatus* under transient oxidative or metabolic stress caused by isotretinoin. Comparable observations of pigment stimulation and altered PSII dynamics under low-level chemical stress have been described by Agathokleous et al. (2020), Jalal et al. (2021), and Xu et al. (2023), further supporting this interpretation.

Although isotretinoin did not display acute toxicity, the observed physiological and fluorescence-based responses highlight the need for further investigation. The pattern of pigment overproduction, altered PSII fluorescence kinetics, and potential hormesis suggests that sublethal stress mechanisms may be at play, which are not fully captured by standard OECD 201 growth-based endpoints. Fluorescence-based parameters may also be more sensitive than the standard growth or biomass endpoints in OECD 201 tests, allowing early signs of stress to be detected before any measurable growth inhibition occurs. For this reason, additional studies should be conducted at higher isotretinoin concentrations to determine the threshold at which growth inhibition begins. Chronic exposure tests could further reveal long-term physiological and reproductive effects that may not be apparent during short-term assays.

Moreover, evaluating isotretinoin's effects on other trophic levels, such as *Daphnia magna* (large water flea) and *Danio rerio* (zebrafish), would provide a broader ecotoxicological perspective. Such studies could follow internationally recognized testing frameworks, including OECD Test Guideline 202 (*Daphnia sp*. Acute Immobilisation Test) and OECD Test Guideline 203 (Fish, Acute Toxicity Test), to ensure comparability and regulatory relevance. In addition, chronic exposure assessments could employ OECD Test Guideline 210 (Fish, Early-Life Stage Toxicity Test) or OECD Test Guideline 211 (*Daphnia magna* Reproduction Test) to evaluate long-term sublethal impacts under environmentally relevant conditions.

6. Summary

Pharmaceutical residues are increasingly recognized as environmental contaminants due to their continuous release into aquatic systems. Isotretinoin is a vitamin A derivative used in dermatology to treat severe acne. It is a biologically active micropollutant, yet its ecological effects and potential impact on aquatic organisms remain poorly understood. This thesis aimed to investigate the acute ecotoxicological effects of isotretinoin on the green alga *Desmodesmus subspicatus*. The study aimed to evaluate whether isotretinoin exerts inhibitory or stimulatory effects on algal growth and photosynthetic activity under standardized laboratory conditions.

The algal growth inhibition tests were conducted in accordance with OECD Guideline 201 (Freshwater Alga and Cyanobacteria, Growth Inhibition Test). Each experiment was performed three times independently to ensure reproducibility and reliability of the results. In this method, exponentially growing algal cultures are exposed to a range of test-substance concentrations for 72 hours. The test evaluates the substance's ability to inhibit algal growth relative to untreated controls. Algal growth inhibition was assessed by optical density at 750 nm, and chlorophyll-a content was additionally determined following ethanol extraction. Additionally, the photosynthetic activity was evaluated using the FluoroMeter Module, which was applied as a complementary analysis outside the standard OECD 201 protocol to evaluate photosynthetic performance and stress responses.

The optical density results showed no inhibition of algal growth at any of the tested isotretinoin concentrations. A moderate growth stimulation was observed in the solvent control, indicating that ethanol contributed to increased biomass. Statistical analysis confirmed that the observed differences among treatment groups were primarily due to solvent effects rather than isotretinoin exposure. Therefore, isotretinoin did not cause measurable growth inhibition in *D. subspicatus* under the acute exposure conditions applied. Chlorophyll-a measurements revealed a more pronounced increase in pigment concentration, particularly at higher isotretinoin levels. This response suggests a physiological adjustment involving enhanced pigment synthesis rather than a toxic effect on biomass production. The observed pattern may indicate a mild stimulatory, hormetic response, characterized by low-dose enhancement of metabolic activity.

During the complementary photosynthetic activity measurements changes in key chlorophyll-a fluorescence parameters indicated slower excitation kinetics and reduced electron transfer efficiency at the highest tested concentration (10 mg/L). These results suggest that isotretinoin can influence the photosystem II (PSII) electron transport chain, reflecting a mild physiological

stress rather than direct toxicity. As very limited ecotoxicological and environmental analytical data are available about isotretinoin, this study fills a gap in the environmental risk assessment of the active substance.

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9. List of Figures and Tables

10. Declarations

MATE Organizational and Operational Regulations
III. Requirements for Students
III.1. Study and Examination Regulations
Appendix 6.13: The MATE Uniform Thesis /thesis / final thesis / portfolio guidelines
Annex 4.2: Declaration of public access and authenticity of the thesis/thesis/dissertation/portfolio

DECLARATION

the public access and authenticity of the thesis

Student's name:	Jázmin Jakab
Student's Neptun code:	CO8FPU
Title of thesis: Substance Isotretinoin	Ecotoxicological Study of the Pharmaceutical Active
Year of publication:	2025
Name of the consultant's institute:	Institute of Environmental Sciences
Name of consultant's deparment:	Department of Ecotoxicology

I declare that the thesis submitted by me is an individual, original work of my own intellectual creation. I have clearly indicated the parts of my thesis or dissertation which I have taken from other authors' work and have included them in the bibliography. Furthermore, I declare that the artificial intelligence tools (e.g. text generation, linguistic correction, translation, data analysis) used during the preparation of the thesis did not substitute my own research and creative work; their use was indicated either in the list of sources or in the methodology section, and I acted in accordance with professional and ethical expectations.

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Date: 2025.11.05.

Student's signature

DECLARATION

Dr. Eszter Takács (student Neptun code: CO8FPU) as a consultant, I declare that I have reviewed the thesis[1] and that I have informed the student of the requirements, legal and ethical rules for the correct handling of literary sources.

I recommend / do not recommend[2] the thesis / the final thesis / dissertation / portfolio to be defended in the final examination.

The thesis contains a state or official secret: yes no*[3]

Date: 2025. November 05.

Takács Eszter

insider consultant

[1] The other types should be deleted while retaining the corresponding thesis type.
[2] The appropriate one should be underlined.
[3] The appropriate one should be underlined.

Declaration of Students and Doctoral Candidates on the Use of Artificial Intelligence (AI)"

1. general information:

Name of the student:	**Jázmin Jakab**
Neptun ID:	**CO8FPU**
Level of program (mark with X):	☒ BSc/BA ☐ MSc/MA ☐ Doctoral School (PhD) ☐ Other: .....
Name and code of the subject*:	**Thesis Work, KORTU132N**
Title of the work:	**Ecotoxicological Study of the Pharmaceutical Active Substance Isotretinoin**

* Not required to be completed in the case of a doctoral dissertation.

2. Declaration on the Use of AI

I, the undersigned, fully aware of my ethical responsibility, make the following declaration:

(Please choose one of the options below!)

☐ A) I have not used any artificial intelligence system or service.

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(Please fill in the relevant tables!)

3. Details of Artificial Intelligence Usage

TABLE I: Assistant or Minor Usage (e.g., translation, language proofreading, brainstorming, etc.)

(For these uses, attaching the specific prompts and responses is not required.)

Purpose of Use	Name and Version of the AI Tool Used	Affected Section (if not applicable to the entire text)
Proofreading, grammar correction, translation, Matlab code bug identification	ChatGPT 5, MATLAB Copilot	

TABLE II: Significant Content Contribution (e.g., generating an entire figure or a longer text section)

(In these cases, documenting the key prompts used and the raw responses provided by the AI, and attaching them as an appendix to the work, is required.)

Purpose of Use	Name, Version, and Access Information of the AI Tool Used	Exact Number of the Affected Chapter / Figure / Table	Entry Number of the Appendix Containing the Prompt Log

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If the instructor or supervisor of the course has established specific rules or expectations regarding the use of AI tools, please summarize them in the field below:

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Rules Prescribed by the Lecturer or Supervisor

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