Finding of No Significant Impact

NDA 017031 S-041, Opill (norgestrel) tablet
0.075 mg, one tablet daily

Food and Drug Administration
Center for Drug Evaluation and Research

The National Environmental Policy Act of 1969 (NEPA) requires Federal agencies to assess the environmental impact of their actions. The Food and Drug Administration (FDA) is required under NEPA to consider the environmental impact of approving certain drug product applications and supplements to such applications as an integral part of its regulatory process.

HRA Pharma requests approval of the prior approval efficacy supplement for the complete prescription-to-nonprescription switch for Opill (norgestrel) tablet, 0.075 mg (NDA 017031 S-041, or sNDA). Opill is a progestin-only oral contraceptive. The active ingredient of Opill, norgestrel, is a racemic mixture of stereoisomers dextronorgestrel and levonorgestrel, the former of which is biologically inactive. Therefore, levonorgestrel—a potent agonist of the progesterone receptor, with weak androgenic activity—is the focus of the environmental analysis of Opill.

The applicant initially submitted a claim of categorical exclusion from an environmental assessment (EA) for this sNDA, per 21 CFR 25.31(b). FDA determined, however, that while the claim met the definition of this exclusion, the available data at the time established that, at the expected level of exposure, there was the potential for serious harm to the environment, per 21 CFR 25.21(a), and thus an EA was needed to evaluate the potential environmental impacts of Opill. After several rounds of communication with the applicant and progressively more refined analyses, the FDA EA team reviewed the final EA, as well as other information, and carefully considered the potential environmental impacts due to approval of the application. FDA concluded that the submitted information was sufficient to determine significance, and thus the EA was adequate for approval, per 21 CFR 25.15(a).

Based on the review of the entirety of this information, FDA has determined that while some adverse environmental impacts are possible, approval of this sNDA is not expected to have a significant impact on the environment. Therefore, FDA is issuing a finding of no significant impact (FONSI), per 21 CFR 25.15(b), with selected precautionary mitigations, and thus an environmental impact statement will not be prepared.

Attachment: ENVIRONMENTAL ASSESSMENT FOR NORGESTREL

Final report

Test Facility Study No. 20408937

ENVIRONMENTAL ASSESSMENT FOR NORGESTREL

(Version 3.0)

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AUTHENTICATION STATEMENT

Study title: Environmental Assessment of norgestrel
Study No.: 20408937

The report indicates that some of the data used in this environmental assessment were provided by the sponsor and we confirm that, when used in this report, these data are in accordance with the data provided. Furthermore, all applied data represent the current understanding and knowledge of the hazards and use of the substance. As required, assumptions were made and/or default values were used in the calculations. If more (detailed) information becomes available, the assessment would be subject to further review.

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TABLE OF CONTENTS

LIST OF TABLES

(b) (4)

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LIST OF ABBREVIATIONS

1/n	Freundlich regression exponent
API	Active Pharmaceutical Ingredient
BCF	BioConcentration Factor
CDER	Center for Drug Evaluation and Research
CEC	Cation Exchange Capacity
CF	Craniofacial
CFR	Code of Federal regulations
CV	Cardiovascular
DFOP	Double First-Order in Parallel
dpf	days post fertilization
DT50	Degradation half-life (time needed for 50% degradation
DT90	Time to reduce the initial concentration by 90%
EA	Environmental Assessment
ECHA	European Chemicals Agency
ECx	Concentration of the test item resulting in x% effect
EEC	Expected Environmental Concentration
EIC	Expected Introduction Concentration
EPA	Environmental Protection Agency
FDA	Food and Drug Administration
FOCUS	FOrum for the Co-ordination of pesticide fate models and their Use
FOMC	First-Order Multi-Compartment
FONSI	Finding Of No Significant Impact
FSH	Follicle Stimulating Hormone
GSI	GonadoSomatic Index
h	Hours
HPLC	High-performance liquid chromatography
HPLC-UV	High-performance liquid chromatography with UltraViolet detection
Kd	Distribution coefficient
Kf	Freundlich binding coefficient
Koc	Organic carbon normalized adsorption coefficient
Kow	Octanol-water partition coefficient
LH	Luteinizing Hormone
LNG	Levonorgestrel
LOQ	Limit of Quantification
MEEC	Maximum Expected Environmental Concentration
mgd	million gallons/day

MT	Metallothioneine
NDA	New Drug Application
NOEC	No Observed Effect Concentration
OC	Organic carbon
OECD	Organization for Economic Co-operation and Development
OECD (number)	OECD test guideline (number)
OPPTS	Office of Prevention, Pesticides and Toxic Substances
piGST	pi class glutathione S-transferase
pKa	Dissociation constant
POTW	Publicly Owned Treatment Works
ppb	parts per billion (µg/L)
S	Skeletal
SD	Standard deviation
SFO	Simple First-Order
SSC	Secondary Sexual Characteristics
TOC	Total Organic Carbon
U-HPLC	Ultra-High-Performance Liquid Chromatography
VTG	Vitellogenin
WWTP	WasteWater Treatment Plant

1. SUMMARY AND CONCLUSION

An environmental assessment was performed for norgestrel using data obtained from the public domain to support the authorization of the Rx-to- OTCswitch of Norgestrel 0.075 mg tablets (Opill), which contain norgestrel (0.075 mg/tablet). Norgestrel is a racemic mixture of two enantiomers (l- and d-norgestrel), with the d-form corresponding to levonorgestrel (LNG). Deracemization, if at all possible, can only occur under specific conditions such as under high temperature or specific light, and in most cases needs a catalyst. These are not naturally occurring environmental conditions and, therefore, breaking the chemical bonds that is necessary to convert one form into the other will not happen. Since levonorgestrel is the biologically active component, the EA focused on this enantiomer.

Levonorgestrel is degraded in wastewater treatment plants although rapid and complete degradation was not assumed based on currently available data. Further depletion due to adsorption to sludge and soil is limited and the aquatic environment is therefore expected to be the most relevant receiving compartment.

The Expected Introduction Concentration (EIC)-aquatic is 0.00064 µg/L which equals the Expected Environmental Concentration (EEC)-aquatic of levonorgestrel assuming zero dilution factor. The EEC was subsequently refined taking removal in the wastewater treatment plant (WWTP), dilution based on actual WWTP and river streams and degradation in surface water into consideration, as modelled in the iSTREEM model following the low flow and median flow scenario. The resulting median, 90th percentile and 99th percentile of the refined EEC are calculated in the assessment.

Data on the toxicity of levonorgestrel to activated sludge, aquatic invertebrates, fish and amphibians were found in the literature. Based on the data for activated sludge, it has been determined that levonorgestrel does not disrupt the wastewater treatment process. From the aquatic toxicity tests, the fish full life cycle test generated the most sensitive endpoint, confirming that this study is considered the most appropriate to assess the potential harmful effects of levonorgestrel on the environment at the expected level of exposure. A further endpoint from a fish reproduction assay was taken from FASS evaluations of drug products containing levonorgestrel. Predicted No Effect Concentrations (PNEC)-aquatic were therefore derived based on the No Observed Effect Concentration (NOEC) / EC10 value for fish. Comparing the refined EEC values with the PNECs show safe use (Risk Quotients ≤1) for the median EEC values calculated for both low and mean flow scenarios as well as for the 90th percentile values of the mean flow scenario. The 90th percentile of the low flow as well as higher percentiles of both low and mean flow scenario show Risk Quotients above 1. Public domain data comparing monitoring data with distributions of modelled concentrations of several substances concluded that the iSTREEM low flow model results yielded a conservative distribution of values, whereas the mean flow model results more closely resembled the concentration distribution of monitoring data. The authors concluded that a

conservative estimation can be obtained by choosing the mean flow 90th percentile which shows a no-risk situation for levonorgestrel. Moreover, the conservative approach in assuming all human excretion of levonorgestrel as active moiety, low-end depletion values, and high-end forecast sales figures likely overestimates the calculated Risk Quotients for levonorgestrel. Still, given the potential risk to fish, mitigation measures could be considered.

Due to the lack of exposure there is no concern for adverse effects on terrestrial organisms following use of Opill (Norgestrel, 0.075 mg tablets).

Based on the calculated whole-body concentration in animals at higher levels of the aquatic food chain to be more than three orders of magnitude lower than the lowest No Observed Effect Concentration, the risk to predators is expected to be negligible following use of Opill (Norgestrel, 0.075 mg tablets).

2. SCOPE AND GUIDELINES

An assessment of potential risks to the environment of medicinal products is a stepwise, phased procedure that may be terminated when sufficient information/data is available to either indicate that the medicinal product is unlikely to pose a risk to the environment or to identify and sufficiently characterize the potential risks.

The environmental assessment (EA) described in this expert report reflects the procedure outlined in the 1998 and 2016 FDA Guidance for Industry (FDA, 1998 and FDA, 2016). In line with the 1998 FDA Guidance, the estimated concentration of the drug substance at the point of entry into the aquatic environment would be below 1 part per billion (calculated in Section 7.1.4), and would normally qualify for a categorical exclusion under 21 CFR 25.31(b).

The 2016 FDA Guidance specifies, however, that drugs with estrogenic, androgenic, or thyroid hormone pathway activity (E, A, or T activity) have the potential to cause developmental or reproductive effects in the aquatic environment at concentrations below 1 part per billion (ppb), which can reflect “extraordinary circumstances”; i.e. at the expected level of exposure, there is potential for serious harm to the environment. Thus, the guidance suggests that Tier 3 chronic and life-cycle studies (e.g., fish reproduction assays) should be conducted on drugs with estrogenic, androgenic, or thyroid hormone activity. Since the mode of action of norgestrel, a progestin, suggests a potential to cause developmental or reproductive effects in the aquatic environment at low concentrations (Zeilinger, 2009), this EA also examines the potential for any effect of norgestrel exposure, even at levels below 1 ppb.

3. DATE/REPORT VERSION

This is the third version of the environmental assessment expert report. The report was updated to include Sponsor's responses to several FDA Information Requests (CMC IRs #2-5) with an emphasis on the refinement of the Expected Environmental Concentration.

Date	Reason for update	Reference
14 September 2021	N/A	de Roode and Vanwormhoudt 2021
7 February 2022	To update the literature search	de Roode and Vanwormhoudt 2022

4. SPONSOR

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5. DESCRIPTION OF PROPOSED ACTION

5.1. Requested approval

HRA Pharma has submitted an NDA pursuant to section 505(b) of the Federal Food, Drug, and Cosmetic Act for Opill (0.075 mg norgestrel) tablets, packaged in PVC/PVDC aluminium blister packs of 28 pills inserted in a carton box. An EA was submitted pursuant to 21 CFR Part 25.

5.2. Need for action

Norgestrel is a racemic mixture of two enantiomers (l- and d-norgestrel), consisting of equal shares of the biologically active levonorgestrel and dexonorgestrel. Dexonorgestrel is, confusingly, also referred to as norgestrel and is hormonally inactive (Kuhl, 2005). Progestin-only oral contraceptives such as Opill 0.075 mg tablets prevent conception by suppressing ovulation in approximately half of the cycles in users, thickening the cervical mucus to inhibit sperm penetration, lowering the midcycle Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) peaks, slowing the movement of the ovum through the fallopian tubes, and altering the endometrium. The proposed indication is for use by females of reproductive potential to prevent pregnancy.

5.3. Locations of use

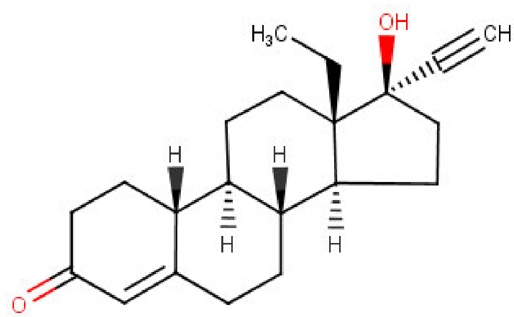
Opill 0.075 mg tablets will be used by patients in their homes. The use is not expected to be concentrated in a particular geographic region.

5.4. Disposal sites

Empty containers will typically be disposed of by a community's solid waste management system, which may include landfills, incineration, and recycling, although minimal quantities of the unused drug could be disposed of in the sewer system.

6. IDENTIFICATION OF SUBSTANCES THAT ARE THE SUBJECT OF THE PROPOSED ACTION

Nomenclature	
Established name:	Norgestrel, 0.075 mg tablets
Brand/Proprietary Name/Trade Name:	Opill
Chemical name	dl-13-beta-ethyl-17-alpha-ethinyl-17-beta-hydroxygon-4-en-3-one
CAS Registry No.	6533-00-2
Molecular Formula	$C_{21}H_{28}0_2$
Molecular Weight	312.446 g/mol
Molecular Structure	



7. ENVIRONMENTAL ISSUES

As indicated in the Guidance (FDA, 1998), an EA should normally focus on characterizing the fate and effects of the compound of interest in the environment when approval of the application increases the use of an active moiety and the estimated concentration of the active moiety at the point of entry into the aquatic environment is at least 1 ppb (µg/L), or, when based on available data, there is potential for serious harm to the environment at the expected level of exposure. The 2016 FDA Guidance (FDA, 2016) regarding drugs with estrogenic, androgenic, or thyroid activity specifies that Tier 3 chronic and life-cycle studies (e.g., fish reproduction assays) should be conducted in order to assess the potential harmful effects of the drug substance at the expected level of exposure. The 2016 FDA Guidance also states that the sponsor can evaluate existing information, including “ecological toxicity studies (fish and invertebrate short-term and life cycle studies, Endocrine Disruptor Screening Program (EDSP) studies, existing literature on the same or related compounds, modelling (including computational toxicology assessments reviewed with

the use of expert knowledge), structural elements, or other scientific data. Based on a thorough evaluation of the totality of this information, the sponsor should assess whether the data are adequate for a determination of E, A, or T activity or whether additional studies should be conducted to further characterize the drug's potential E, A, or T activity." (2016 FDA Guidance at Q6 (footnotes omitted)). Due to the mode of action of levonorgestrel as a progestin, it has the potential to cause developmental or reproductive effects in the aquatic environment at concentrations below 1 ppb (Zeilinger, 2009) and this EA has therefore been prepared. This EA is based on information that was available from the public literature including aquatic toxicity data from several trophic levels, and more specifically chronic and life-cycle studies in fish. The literature search was performed in April 2021 and again in December 202. Details on literature search are provided in Appendix 1. this EA has therefore been prepared. This EA further takes refinement of the Expected Environmental Concentration into account based on human excretion of levonorgestrel, environmental depletion mechanisms and dilution into U.S. river streams as modelled in 2023 by means of the iSTREEM calculation tool.
Although the EA will focus on the effects on the aquatic compartment due to chronic exposure, all other environmental fate and effect data currently available to the Sponsor on (levo)norgestrel are also presented in the EA.

7.1. Environmental Fate of Released Substances

7.1.1. Identification of Substances of Interest

Progestin-only oral contraceptives such as Opill 0.075 mg tablets prevent conception by suppressing ovulation in approximately half of the cycles in users, thickening the cervical mucus to inhibit sperm penetration, lowering the midcycle LH and FSH peaks, slowing the movement of the ovum through the fallopian tubes, and altering the endometrium.

Norgestrel is a racemic mixture of two enantiomers (l- and d-norgestrel), consisting of equal shares of the biologically active levonorgestrel and dexonorgestrel, the latter of which has been indicated to be hormonally inactive (Kuhl, 2005; Appendix 8). Based on *in vivo* data on fish, it can be concluded that levonorgestrel induces masculinization of female fish (refer to section 7.1.9). However, norgestrel (racemic mixture) did not lead to complete sex reversal at a concentration of 368 ng/L (Hou et al, 2018) while levonorgestrel exposure led to the complete absence of females at concentrations as low as 5.5 ng/L (Svensson et al, 2016) (both in partial life cycle tests). Since levonorgestrel is the active component, the EA focuses on this substance. The fate and effects of levonorgestrel are considered to cover the fate and effects of dexonorgestrel.

7.1.2. Physical/Chemical and Fate Characterization

Table 1 gives an overview of the physical-chemical and fate properties available for levonorgestrel. Levonorgestrel is a molecule with one ionizable group, but this group will only be ionized at very low pH values, which are not environmentally relevant (i.e. pKa -1.53, estimated using MarvinSketch 20.1, ChemAxon, 2020). Therefore, at environmentally relevant pH levels, levonorgestrel is present exclusively in its neutral form (see Appendix 1). Its log normalised octanol/water partition coefficient (logKow) was estimated to be 3.66 (ChemAxon, 2020) or 3.48 (US EPA, 2012; see Appendix 4 and Appendix 5); these values are comparable to values reported in online databases (DrugBank: https://www.drugbank.ca/drugs/DB00367; Hazardous Substances Databank: https://pubchem.ncbi.nlm.nih.gov/source/hsdb/6483), but lower than the value reported in the EA/Finding of No Significant Impact (FONSI) for Alesse® in 1996 (approx. 8 (FDA CDER, 1996)) and higher than the value determined by Backe Hansen in 2006 (2.6; HPLC method (Backe Hansen, 2006)). It should be noted that the value reported by FDA CDER was quoted as octanol/water partition coefficient (not logKow; for a 13now value of 8, the logKow would be 0.9), and the value reported by Backe-Hansen was determined using the HPLC method in 1989. The HPLC method (Organization for Economic Co-operation and Development (OECD) 117) is currently regarded as a screening method only, and definitive logKow values should be determined using better suitable test methods such as the shake flask method (OECD 107 (EMA, 2018)).

Studies on the adsorption behavior of levonorgestrel have been reported by Yang et al, 2020, Tang et al, 2012, and Hörsing et al, 2011. The studies by Yang et al, 2020 and Tang et al, 2012 were both conducted in soils collected in China, while the study by Hörsing et al, 2011 was conducted in sludge.

Yang et al, 2020 used five different agricultural soils and conducted an experiment that was in general agreement with the OECD 106 test guideline; a batch equilibrium test was conducted with 8 concentrations between 0.2 and 25 µg/L, 3 replicates per concentration, with a 24-hour contact time at 25 ºC. Soils were sterilized before use and included 3 sandy loam soils (pH 6.1, 5.78 and 5.17, organic carbon (OC) content 0.80, 1.65 and 2.19%) and 2 silty loam soils (pH 5.12 and 5.55, OC content 3.06 and 4.16%). Organic carbon normalized adsorption coefficient (Koc) and distribution coefficient (Kd) values were reported along with a correlation coefficient, but Freundlich regression exponent (1/n) were not reported, which was justified by the statement that "sorption isotherms collected over a range >2 orders of magnitude were best-fitted with the linear sorption model ($R^2 = 0.94–0.99$)". Kd values ranged between 5.13 and 33.2 L/kg; Koc values were 641, 642, 763, 798 and 1056 L/kg.

Tang et al, 2012 determined the Koc value of levonorgestrel in 5 soils following (part of the) Office of Prevention, Pesticides and Toxic Substances (OPPTS) test guideline 835.1230 (batch equilibrium). In addition, the degradation in soil was studied (see section 7.1.3). Soils were taken from the top 20 cm layer of four agricultural fields and from one grassland at different sites in China. They represented a range of soil characteristics, with pH values varying between 6.7 and 7.5, N content between 0.08 and 0.21%, cation exchange capacity (CEC) between 8.7 and 43.6 cmol/kg and total organic carbon (TOC) between 1.14 and 2.45%. The batch equilibrium study was conducted in sterilized soils and contained pre-tests to determine equilibration time, sorption to test vessels and stability of levonorgestrel under test conditions. Sorption isotherms were conducted at 5 concentrations ranging from 0.2 to 2 mg/L, three replicates per concentration, 24 hours contact time (at 25±2 °C in darkness). TOC normalized Freundlich binding coefficient (Kf) values (Koc values) ranged between 946 L/kg and 2586 L/kg (mean 1869 L/kg); 1/n values ranged between 0.69 and 1.23 (mean 1.01).

The authors of the Yang et al, 2020 study compared their Koc values to those reported by Tang et al, 2012, where Koc values were higher than in the 2020 study. This difference was attributed to the fact that the 1/n values in the study by Tang et al, 2012 were 0.69-1.23, indicating non-linear adsorption and concentration-dependence. Indeed, the concentrations in the study by Tang et al, 2012 were 100-1000 times higher than the concentrations used by Yang et al, 2020, leading to a potential overestimation of sorption (based on the low water solubility of levonorgestrel, precipitation of the substance may have occurred). Therefore, the Koc values reported by the latter authors are considered more reliable.

Hörsing et al, 2011 studied the adsorption of 75 active pharmaceutical ingredients (APIs), one of which was levonorgestrel, to sludge. All APIs were treated similarly, i.e. incubation time was 12 hours in darkness at 4°C, sludge concentration 1 g/L and test concentrations 0.08, 0.4, 2 and 10 µg/L. Sludge was collected from three wastewater treatment plants (WWTPs) which were representative of WWTPs for treatment of municipal wastewater in industrial countries. Incubations were performed with primary sludge, secondary sludge of short age and secondary sludge of long age (each taken from a separate WWTP). Information on the organic carbon content of the sludge was not reported. Levonorgestrel exhibited low sorption to the primary sludge and no sorption isotherm could be obtained. Isotherms were obtained in secondary sludge, with the linear fit being the best fit, followed by the Freundlich fit. In secondary sludge of long sludge age, the linear Kd value was 260 L/kg (R^2 66%) and the Freundlich Kd value was 18 L/kg (1/n = 5.3E-04, R^2 81%). These values were reported not to differ statistically. In secondary sludge of short sludge age, the linear Kd was 200 L/kg (R^2 86%) and the Freundlich Kd value was 0.40 L/kg (1/n = 0.91, R^2 83%). Again, these values were not statistically different. The effect of pH on adsorption was tested in secondary sludge with long sludge age at pH values of 6-8, 6, 7 and 8 at a concentration of 10 µg/L. The average Kd values of levonorgestrel at these pH values were $0.1 \pm 0.2 \times 10^3$, $2.5 \pm 0.6 \times 10^2$, 7 ± 193 and $0.2 \pm 0.1 \times 10^3$, respectively. These values were not statistically significantly different at the 5% level (i.e. $p>0.05$). The low Freundlich constant in secondary sludge of long age (<<0.1) indicates a potential for concentration dependence. Overall, it can be concluded that adsorption to sludge was low.

Information on bioconcentration potential of levonorgestrel have been reported by Contardo-Jara et al, 2011, Fent, 2015, Fick et al, 2010 and Miguel-Queralt and Hammond, 2008.

Contardo-Jara et al, 2011 showed that levonorgestrel bioconcentrates in mussels, using *Dreissena polymorpha* as a model of investigation. The bioconcentration potential was studied at three concentrations (0.312, 3.12 and 6.24 µg/L) in a flow-through system. The lowest concentration was 95-fold bioconcentrated within the first four days, after which the bioconcentration factor (BCF) further increased to 208 after 7 days of exposure. At 3.12 µg/L and 6.24 µg/L, the BCFs after 4 days were 30 and 56 and therefore lower than at the lowest concentration. After 7 days, the BCF at 3.12 µg/L had further increased to 53, while the BCF at 6.24 µg/L had decreased to 25. The authors stated that these data suggest enhanced metabolism and excretory processes at higher concentrations. Indeed, the immediate mRNA up-regulation of pi class glutathione S-transferase (piGST) proved that phase II biotransformation processes were induced, and mRNA up-regulation of P-glycoprotein (P-gp), superoxide dismutase and metallothioneine (MT) suggested enhanced elimination processes and ongoing oxidative stress.

It is noted that an alternative explanation may be the saturation of receptors involved in the uptake of levonorgestrel (see also Miguel-Queralt and Hammond, 2008 below).

The paper by Fent, 2015 is a review paper that refers to many other papers, among which some of the papers included in this document. The bioconcentration factor of levonorgestrel found by Fick et al, 2010 (see below) was discussed to be "not very probable, in particular as metabolism can be expected". This statement was supported by data on bioconcentration levels in roach, being 17-53 in different organs (blood plasma was not analyzed). The author stated that the very high BCF found by Fick and coworkers might be related to very high lipid content in the sampled fish plasma, or that it was caused by the exposure concentration being higher than 1 ng/L. The finding could however also be explained by the findings of Miguel-Queralt and Hammond, 2008, who found that levonorgestrel has high affinity to sex-steroid binding globulins in the gills (see below). BCF values for norgestrel were between 184 and 571 in shrimps, mussels and fish.

Fick et al, 2010 measured concentrations of pharmaceuticals, including levonorgestrel, in sewage effluents and fish plasma, following 2-week exposure of 100 g rainbow trout juveniles to undiluted effluents under flow-through conditions. The study was conducted in Sweden. Concentrations of levonorgestrel in fish blood plasma were 12 µg/L in Umeå, 8.5 µg/L in Stockholm and below the limit of quantification (LOQ) in Gothenborg. In comparison, effluent concentrations at these sites were 1 ng/L, <LOQ and <LOQ. The height of the LOQ was not reported in the main report (referral was made to the supplementary data). The ratio between fish plasma concentrations and effluent concentrations were calculated as 12000 for Umeå and >8500 for Stockholm. The predicted ratio (or BCF), based on lipophilicity (logPow taken to be 3.5) was 46. The much higher plasma concentrations were explained by referring to the paper by Miguel-Queralt and Hammond, 2008 (see below), who presented a mechanism for a higher-than-expected uptake of sex steroids from water into fish plasma, mediated via binding to sex-steroid binding globulins in the gills.

Miguel-Queralt and Hammond, 2008 investigated the role of the sex hormone-binding globulin (SHBG), which is the major transport protein for sex steroids in the blood of most vertebrate species including fish, in the uptake and excretion of steroids from water. In an *in vitro* saturation ligand-binding assay, the affinity of synthetic steroids (among which levonorgestrel) to recombinant zfSHBG was examined against that of the natural ligand dihydrotestosterone. The study also included an *in vivo* experiment in which the uptake and release of natural steroids was studied (i.e. levonorgestrel was not tested *in vivo*). The results indicate that levonorgestrel bound to zfSHBG with an affinity exceeding that of the fish androgen 11 ketotestosterone (i.e. 52% compared to 28%, when expressed as relative binding affinity compared to that of dihydrotestosterone). Further, SHBG ligands (natural steroids) were rapidly and specifically removed from water by the fish through their gills, whereas the accumulated sex steroids were released slowly.

In summary, reported BCF values vary between 17 and 571 L/kg in whole organisms, but much higher (up to 12000 L/kg) in fish plasma. The much higher value in fish plasma may be explained by the specific binding of levonorgestrel to sex-steroid binding globulins in the gills.

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Table 1: Physical-chemical and fate properties of levonorgestrel

Parameter	Result	Source
Water solubility	35.84 mg/L 1.73 mg/L	Estimated (US EPA, 2012) (Appendix 6) Database match (US EPA, 2012)
Dissociation constant (pKa)	-1.53	Estimated (ChemAxon, 2020) (Appendix 2)
Octanol/water partition coefficient (given as LogKow)	3.66 3.48 3.8 3.8 ~0.9 (from reported kow ~8) 2.6	Estimated (ChemAxon, 2020) (Appendix 4) Estimated (US EPA, 2012) (Appendix 5) Drugbank Hazardous Substances Databank FDA CDER, 1996 Backe Hansen, 2006
Vapor pressure	1E-009 mm Hg	Estimated (US EPA, 2012) (Appendix 7)
Sorption/desorption coefficient (Koc) ; Distribution coefficient (Kd)	Soil Kd (n=5): 5.13, 10.6, 16.7, 32.3, 33.2 L/kg Soil Koc (n=5): 641, 642, 763, 798 and 1056 L/kg (R^2 0.94-0.99)	Yang et al, 2020
	Soil K_Foc (n=5): 946, 1433, 2098, 2378 and 2486 L/kg (1/n 0.69-1.23, mean 1.01) [a]	Tang et al, 2012
	Secondary sludge Kd (n=1): Long age: Linear Kd 200 L/kg (R^2 86%) Freundlich K_Fd 18 L/kg (1/n 5.3E-04; R^2 81%) [a] Short age: Linear Kd 200 L/kg (R^2 86%) Freundlich K_Fd 0.40 L/kg (1/n 0.91; R^2 83%)	Hörsing et al, 2011
Bioaccumulation (BCF)	Whole body BCF (*Dreissenia*): 30-208 L/kg	Contardo-Jara et al, 2011
	Organ specific BCF (fish): 17-53 L/kg	Fent, 2015
	Whole body BCF (shrimp, mussel, fish): 184-571 L/kg	Fent, 2015
	Fish plasma BCF: >8500 – 12000 L/kg	Fick et al, 2010

[a] Low 1/n values (Freundlich regression exponents) indicate non-linear adsorption and concentration-dependence

7.1.3. Environmental depletion mechanisms

Data on degradation in wastewater treatment plants has been reported by Weizel et al, 2021, while Tang et al, 2019 and Tang et al, 2012 report on the degradation of levonorgestrel in soil.

Weizel et al, 2021 investigated the degradation of several progestogens by activated sludge. The study was not conducted according to OECD guidelines, but the methods used were reported in sufficient detail to allow an in-depth assessment.

To study degradation rate, activated sludge (200 mL) was obtained from the aeration tank of a conventional WWTP in Germany, characterized by a capacity of 320,000 inhabitants, a sludge retention time of 12 days, a hydraulic retention time of ~6 hours and a suspended solids concentration of 4 g/L. The sludge was diluted in 2 L effluent from the same WWTP to prevent sorption to suspended solids. The solutions, that contained 0.4 g suspended solids/L, were stirred and aerated for 12 hours prior to use. The initial concentration of levonorgestrel was 200 ng/L. A sterilized control was included to determine abiotic degradation. Samples were taken from the solution at different time points and analysed using mass spectrometry.

Based on comparison of the results from the sterile control and the non-sterile degradation, it was concluded that removal from the WWTP was predominantly driven by biotic degradation; abiotic degradation and sorption to suspended solids of levonorgestrel was negligible, while biotic degradation was fast and could be described by first-order kinetics. Complete removal was observed within 2 hours (estimated from graphical representation) and the degradation half-life of levonorgestrel was 0.48 ± 0.03 hours (n=3). Further, samples taken from the effluents of 8 different WWTPs showed that not a single sample contained quantifiable concentrations of levonorgestrel (LOQ 1.0 ng/L).

In an additional experiment, the authors identified the degradation products of three substances, but levonorgestrel was not included in this test. Therefore, experimental data on the degradation route of levonorgestrel are not available and it cannot be concluded if the degradation of levonorgestrel was complete.

In the study by Tang et al, 2019, the degradation of levonorgestrel was studied in 5 different soils in China. Farmland soil samples were taken from the top 20 cm layer in Heilongjiang Province (HLJ), Beijing (BJ), Yunnan Province (YN), and Guangxi Province (GX), while the grassland soil was taken from Inner Mongolia (NMG). Soils were dried, sieved (2 mm) and stored in plastic bags at 4 °C until use. A subsample of the soils was sterilized at 120 °C for 3 days.

Degradation tests were conducted in 150 g soil samples with water added at a 1:5 ratio (water: soil). The soil was pre-incubated at 25 °C for 14 days. Levonorgestrel was dissolved in methanol, added to 10 g of dry soil and mixed into the pre-incubated soil after evaporation of the methanol. The initial test concentration was 2 µg/g. Flasks were weighed during the 28-day incubation period and water loss was compensated for by addition of fresh water. Samples of 10 g of soil were removed at different time points and stored in a freezer (-21 °C) until analysis. The effect of temperature and of water-soil ratios on degradation were investigated in one type of soil (BJ). An abiotic control was also included. Further, the route of degradation was investigated in one soil, using an initial test concentration of 20 µg/g soil. All experiments were conducted in triplicate. Chemical analysis was conducted with HPLC-UV. The results from two soils with significantly different characteristics (BJ and NMG) were used to determine the best degradation model according to FOCUS Kinetics.

All three models (Simple First-Order (SFO), First-Order Multi-Compartment (FOMC) and Double First-Order in Parallel (DFOP)) gave acceptable fits (R^2 0.9679-0.9774, error percentage 4.27-6.06%), but the best fit was obtained with the DFOP model (R^2 ≥0.9879, error percentage 1.20-3.72%). Therefore, this model was used for all soils.

After 28 days of incubation, residual levonorgestrel was 90% in sterile soil and 8.5% in non-sterile soil. Resulting degradation half-life (DT50) and time to reduce the initial concentration by 90% (DT90) values are shown in Table 2. Degradation rates were not correlated to soil CEC, clay, N or TOC content.

In the temperature test, soil was incubated at 15, 25, 37 or 54 °C. Degradation rate was highest at 25 °C, followed by 37 °C, 15 °C and 54 °C. In the soil:water ratio test, fastest degradation was observed at a water:soil ratio of 1:5, followed by 1:10 and then 1:1 (flooded).

Three transformation products were found. Two products could be identified as 4,5-dihydro-levonorgestrel and 17-desacetylenyl-levonorgestrel; the third product was proposed to correspond to 6,7-dehydro-levonorgestrel.

Table 2. Endpoints for levonorgestrel in soil as fitted by the DFOP model

	BJ soil	NMG soil	HLJ soil	GX soil	YN soil
DT50 (days)	6.77	12.60	9.92	9.22	15.90
DT90 (days)	19.21	28.47	24.27	23.16	32.32

Tang et al, 2012 studied the degradation of levonorgestrel in 5 soils. As discussed in section 7.1.2, soils were taken from the top 20 cm layer of four agricultural fields and from one grassland at different sites in China and represented a range of soil characteristics.

Degradation of levonorgestrel was studied at 0.2, 2 and 20 µg/g. Vessels were incubated for 21 days for biotic degradation and for 120 days for abiotic degradation (at 25±2 °C in darkness, moisture content maintained at 9-35%). No significant degradation was found in any of the five sterilized soils, indicating that the degradation was dominated by microbial transformation. The biotic degradation of levonorgestrel was fitted by first-order kinetics. Degradation rate constants were 0.16, 0.13, 0.09, 0.08 and 0.06 day^{-1} (corresponding to half-lives were 4.3, 5.3, 7.7, 8.7 and 11.6 days, mean 7.5 days). The SFO fits were shown graphically but statistical significance was not reported; the visual fit was acceptable for all soils. It was stated that degradation of levonorgestrel was concentration dependent, with higher rates at lower concentrations. The authors hypothesized that this may have been caused by dose-related alteration of the bioactivity.

Experimental data on hydrolysis of levonorgestrel are not available. However, based on structural considerations, hydrolysis is not expected to be an environmental depletion mechanism, since the active moiety does not contain hydrolysable groups.

Experimental data on photolysis are not available. However, in accordance with ECHA (European Chemicals Agency) guidance on photodegradation (ECHA, 2017), the contribution of photodegradation in water to overall degradation is significant only for substances that reside in water to a considerable extent. As the water solubility of levonorgestrel is not very high and its microbial degradation is fast, levonorgestrel will not reside in water and therefore photodegradation is not considered a relevant depletion mechanism.

A study on transformation in aquatic/sediment systems according to test guideline OECD 308 was conducted and reported by Farmaceutiska Specialiteter i Sverige, (FASS, 2023) in which the transformation of [^{14}C] levonorgestrel in sediments and natural water was assessed in two different aerobic sediment/water systems over 100 days. The results of the study indicate that levonorgestrel is distributed to the sediment compartment, with relevant amounts remaining in the water phase (22 and 43% for the fine and coarse sediment, respectively). The degradation rate was 6-7% at the end of the incubation period. The DT_{50} (disappearance half-life from the water phase) for parent compound in water was estimated to be 2.5 and 3.2 days for the fine and coarse sediment, respectively. Levonorgestrel was concluded to be potentially persistent as the overall disappearance half-life from the system exceeded 120 days.

Based on the adsorption behaviour of levonorgestrel in sludge (Kd 0.4-18 L/kg; see section 7.1.2), binding of levonorgestrel to biosolids in wastewater facilities and sediments is not expected.

Based on the above considerations, even though levonorgestrel is expected to be rapidly degraded in the WWTP, it is not considered to undergo rapid and complete depletion. The aquatic environment is expected to be the most relevant receiving compartment.

Information on metabolites is not considered necessary, as the assessment is based entirely on levonorgestrel, which, based on its mode of action, should cover the risk from metabolites as well. Please refer to section 7.1.4 for more details.

7.1.4. Environmental concentrations

The main route of entry into the environment for levonorgestrel is due to consumption and excretion of the API by patients. Therefore, entry into the environment starts with release to publicly owned treatment works (POTWs). Due to its low Kd values in sludge (0.40-18 L/kg, Hörsing et al, 2011) and Koc values in soil (641-1056 L/kg (Yang et al, 2020) and 946-2486 L/kg (Tang et al, 2012)), (see section 7.1.2), levonorgestrel is not expected to bind to biosolids from wastewater facilities, and therefore exposure of the terrestrial compartment following application of biosolids to land is considered negligible. Based on the same data, exposure of sediment is also not expected. Since levonorgestrel is not released into the air and based on its low vapor pressure, exposure of the atmospheric compartment is expected to be negligible. Therefore, surface water is considered the most relevant receiving environmental compartment.

Considering the mode of action of levonorgestrel, there is no reason to expect metabolites to be more active than the parent. The assessment is entirely based on levonorgestrel, assuming that levonorgestrel is not metabolized/degraded in any way (either within or outside the patient). Therefore, this should represent a worst-case scenario that covers the risk from metabolites as well.

The EIC into the aquatic compartment is calculated as:

EIC-aquatic (ppb) = A x B x C x D where:
- A = kg/year produced for direct use (active moiety)
- B = 1/liters/day entering POTWs
- C = year/365 days
- D = 10^9 µg/kg (conversion factor)

The FDA states that the value for the volume of water entering POTWs may be found in the U.S. Environmental Protection Agency (EPA)'s *Needs Survey, Report to Congress*, and that the most recent survey data should be used. The most recent edition of this *Survey* is the *Clean Watersheds Needs Survey 2012 – Report to Congress* (US EPA, 2016). Detailed data from that survey are published online (http://www.epa.gov/cwns/clean-watersheds-needs-survey-cwns-2012-report-and-data) including the POTWs flow volumes to be used for calculating the EIC ('CWNS 2012 Number of Treatment Facilities by Flow Range'), where a value of 33,240 mgd (million gallons/day) is reported under Total Existing Flow. This translates to 1.26 x 10^{11} liters/day.
The calculation of the EIC-aquatic is shown in Table 3. The predicted use by the fifth year after marketing of Opill projected volumes within the U.S. were provided by the Sponsor (Appendix 3). The Sponsor estimated the maximum production volume to be 652,418,171 tablets each containing 0.075 mg, i.e. 49 kg norgestrel,which is equivalent to 24.5 kg levonorgestrel. In CMC Information Request # 3 dated November 02, 2022, FDA questioned this estimate and as a result, the Sponsor applied a 20% conservatism factor and 29.4 kg/year was used in the calculations . This volume of production is highly unlikely considering that the production volume without variance was already a high-end estimation based on high end assumptions. It should be noted that the EIC is considered a worst-case estimation of the environmental exposure, as the calculation assumes no metabolism of the active moiety (or metabolites activities are equal to that of the active moiety).

From a worst-case scenario, the EEC-Aquatic may be calculated from the EIC, using a dilution factor of zero as suggested by FDA (CMC Information Request #2 dated September 26, 2022).

Table 3: Calculation of the EIC-and EEC aquatic of levonorgestrel

Parameter	Value	Source
A, production of active moiety for direct use [kg/year]	29.4	Information from applicant (Appendix 3)
Liters of wastewater entering the POTWs per day [L/day]	1.26×10^{11}	US EPA, 2016
B, 1 / liters of wastewater entering the POTWs per day [day/L]	7.94×10^{-12}	Calculation
C, year/365 days [year/day]	0.0027	Default
D, conversion factor [µg/kg]	1×10^{9}	Default
EIC-Aquatic [ppb or µg/L]	0.00064	Calculation
EEC-Aquatic [ppb or µg/L]	0.00064	Calculation

The EEC-Aquatic may be further refined based on human excretion of the levonorgestrel, degradation in the WWTP, dilution in U.S. water streams and degradation in surface water.

Human excretion of levonorgestrel

Metabolism in women has been studied by Sisenwine and co-workers. Following administration of ^{14}C-levonorgestrel to women, unchanged norgestrel (levonorgestrel and/or dextronorgestrel) accounted for maximum 15% of the total radioactivity in plasma (Sisenwine et al, 1975a). 3α, 5β-tetrahydro-d-norgestrel was identified as the main metabolite; it was found mainly as glucuronide (max 2.1%) and sulphate (max 7.3%) conjugates (Sisenwine et al, 1975a). Information on excretion was reported in studies by Sisenwine et al, 1975b where women received ^{14}C labelled norgestrel (racemic mixture) and by Stanczyk and Roy, 1990 where women received ^{14}C labelled levonorgestrel. Sisenwine et al, 1975b report that approximately 45% of administered radioactivity was excreted in urine and 32% in feces. The radioactivity in urine was characterized and found to contain <4% levonorgestrel and ~10% of the main metabolite 3α, 5β-tetrahydro-d-norgestrel. Characterization of radioactivity excreted via feces was not performed. Stanczyk and Roy, 1990 reported similar levels of levonorgestrel in urine (sum of unconjugated, sulphated and glucuronidated: <0.7%) and ~11% 3α, 5β-tetrahydro-d-norgestrel (0.1% unconjugated, 1.5% sulphated and 9.5% glucuronidated). In general, glucuronide and glucoside conjugates of a drug substance should be considered as the parent compound, as these conjugates may undergo deconjugation in the environment. However, in the absence of pharmacological activity data of any of the metabolites, all excreted radioactivity was taken to be levonorgestrel. This is in line with the Guidance for Industry (FDA, 1998) but represents a conservative overestimation as several sources claim that metabolites of levonorgestrel do not display any activity. According to Drugbank, no active metabolites of levonorgestrel have been identified (https://go.drugbank.com/drugs/DB00367). From the literature, information on activity of norgestrel metabolites is limited. A paper by Uniyal et al, 1977 stated that “Since the 3α-hydroxy-5βH-tetrahydronorgestrel and 3β-hydroxy-5βH-tetrahydronorgestrel are far less biologically active as compared to norgestrel (*Russell*, personal communication), the biotransformation of norgestel into these metabolites may represent the mechanism involved by which the level of norgestrel in the uterus is regulated”. Modelling metabolite activity relative to the activity of levonorgestrel was investigated but concluded not feasible (for rationale, see Appendix 9).

Therefore, the calculation of the EEC may be refined based on the total fraction of unchanged plus metabolized norgestrel excreted in urine (0.45) and feces (0.32) as reported by Sisenwine et al, 1975b. This 0.77 fraction is used in the iSTREEM modelling (“loading factor”) as presented in Appendix 11.

This is still considered an overestimation since dextronorgestrel is not pharmacologically active (see Appendix 8) and a distinction between dextronorgestrel and levonorgestrel could not be made with respect to excretion in the studies by Sisenwine and co-workers in 1975. Furthermore, all metabolites are assumed to display the same pharmacological activity as unchanged levonorgestrel and the excretion via urine and feces is assumed to consist entirely of unchanged levonorgestrel.

<u>Degradation in WWTP</u>
The available activated sludge degradation study from Weizel et al, 2021 showed complete removal of levonorgestrel within 2 hours and a corresponding degradation half-life of 0.48 ± 0.03 hours. However, the use of this removal rate was questioned by FDA (CMC IR#3 dated November 02, 2022) as both reported and modelled (see below) degradation and removal[1] rates were variable.

In the absence of experimental data, modelling can be applied to estimate the removal rate of levonorgestrel. By running EPI Suite (US EPA, 2012), using Biowin output and the EPA draft method for assigning half-lives, a removal rate based on biodegradation and adsorption to sludge of 36.85% can be estimated (Appendix 10) and the resulting fraction entering the water bodies would be 63.15%.

WWTP removal rates can also be taken from environmental monitoring data on influent and effluent concentrations from different WWTPs. Readily available data are mainly from outside the U.S. as summarized in Appendix 12. Removal rates in four plants in the Netherlands and three plants in Canada employing primary (conventional) treatment were in the range 48% to >88% (as detailed in Table A1 of Appendix 11). Although these plants were not located in the U.S., based on treatment procedures applied as well as climatic conditions, the obtained removal rates for a number of these plants could be considered representative for U.S. conditions (see Appendix 11) calculating a mean removal rate of 73.5%. The data indicate that the removal rate of 36.85%

[1] For levonorgestrel, removal from the WWTP is predominantly degradation

estimated from the EPI Suite calculation tool is conservative and, therefore, is also presented in separate iSTREEM modelling in Appendix 11.

Degradation in surface water

The reported half-life (DT50) of levonorgestrel from two water sediment-systems is >120 days based on which levonorgestrel is considered potentially persistent (FASS, 2023). The DT50 of 120 days, representing potential persistency, and the resulting in-stream first order loss constant (k=0.006 day^{-1}; calculated as ln(2)/120) is used in the iSTREEM model as presented in Appendix 11.

Dilution in U.S. waters

A default dilution factor of 10 for calculating the EEC from the EIC is generally considered appropriate (FDA, 1998). However, a zero dilution factor is currently recommended by FDA given the increases over time of water scarcity and water reuse and thus decreases in dilution (CMC Information Request #2 dated September 26, 2022). Dilution in the U.S. water streams can, however, also be modelled to better represent actual dilutions. To accommodate this, the iSTREEM® model predicting environmental concentrations of chemicals in river segments in the continental U.S. receiving municipal wastewater discharges as a result of down-the-drain disposal of consumer products is used. The rationale for using the iSTREEM® model as well as details of the calculations is provided in Appendix 11; key results are summarized in the Table below.

Table 4: In-stream concentrations of levonorgestrel estimated using iSTREEM

	EEC-aquatic concentration levonorgestrel[a] (ng/L)	
	Low flow	**Mean flow**
Median	0.0065	0.00045
90th percentile	0.062	0.0047
99th percentile	0.16	0.043
Maximum	0.42	0.38

[a] Taking the following refinements into consideration:
- fraction levonorgestrel excreted by humans: 0.77
- removal rate in STP: 73.5%
- degradation in surface water: DT50 = 120 days
- dilution in the U.S water streams: predicted by the iSTREEM model

The 90th percentile concentration may be used to calculate risk quotients (e.g., Kapo et al, 2016; Burns et al, 2022), but risk quotients calculated using the median, 90th percentile, 99th percentile and maximum concentrations have also been reported (e.g., Laurentson et al, 2014).

According to Ferrer and DeLeo 2017, above the 99th percentile, the iSTREEM® model (particularly at low flow) highly overestimates the predicted concentrations. This is expected, particularly in low flow scenarios, due to the model's overestimation of the high-end values, as detailed in Kapo et al, 2016. The model estimated maximum concentration is therefore considered to be insufficiently reliable for risk assessment.

The median, 90th percentile and 99th percentile refined EEC-Aquatic are presented in the environmental assessment in section 7.2.

7.1.5. Environmental Effects of Released Substances

As no rapid, complete depletion mechanism was identified, levonorgestrel is evaluated for its toxicity to environmental organisms. Table 5 presents an overview of the currently available environmental toxicity data from the applicant (data obtained from literature); the underlying studies have been summarised in sections 7.1.6, 7.1.7, 7.1.8, 7.1.9 and 7.1.10 below.

It is acknowledged that the Guidance for industry (FDA, 1998) indicates that EC50 values should be used in the risk assessment. However, EC50 values are typically derived from acute tests, such as performed in Tier 1 and 2. For long-term tests (Tier 3), results are expressed as no effect concentrations (NOEC values). Per definition, NOEC values are lower than EC50 values and therefore represent a more conservative approach for risk assessment. Therefore, the EA was based on NOEC values.

Table 5: Overview of environmental effects of levonorgestrel

Organism	NOEC	Effects on which endpoint was based	Reference
Activated sludge bacteria	NOEC 200 ng/L	General toxicity	Weizel et al, 2021
Chlorella vulgaris	NOEC 1 mg/L	Growth rate	Czarny et al., 2019a
Scenedesmus armatus	NOEC 50 mg/L	Growth rate	Czarny et al., 2019a
Mycrocystis aeruginosa	NOEC 75 mg/L	Growth rate	Czarny et al., 2019b
Anabaena variabilis	NOEC 100 mg/L	Growth rate	Czarny et al., 2019c
Amphipods (*Gammarus locusta*)	NOEC <10 ng/L	Reproduction	Cardoso et al, 2018
Inland silverside (*Menidia beryllina*)	NOEC <10 ng/L	Reproduction success and survival in generations produced by fish exposed during early life stages	DeCourten et al, 2020
Zebrafish (*Danio rerio*)	NOEC 0.06 ng/L	Sex ratio (masculinization)	Teigeler et al, 2021
Fathead minnow (*Pimephales promelas*)	NOEC <0.7 ng/L	Sex ratio (masculinization), hatching success, post-hatch survival, reproduction (number of larvae/day surviving to day 30 post hatch)	Zeilinger, 2009
Fathead minnow (*Pimephales promelas*)	NOEC <10 ng/L	Reproductive behavior in male fish, sperm motion characteristics	Frankel et al, 2018
Fathead minnow (*Pimephales promelas*)	NOEC <10 ng/L	Secondary sexual characteristics in female fish, oogenesis, gonadosomatic index (GSI) and vitellogenin (VTG)	Frankel et al, 2017
Mosquitofish (*Gambusia holbrooki*)	NOEC <10 ng/L	Masculinization of female fish, reproductive behavior of untreated male fish mated with treated females	Frankel et al, 2016
Medaka (*Oryzias latipes*)	NOEC 7.3 ng/L	Secondary sexual characteristics in female fish, number of fertile eggs	Onishi et al, 2020
Fathead minnow (*Pimephales promelas*)	NOEC 0.5 ng/L	Egg production, secondary sexual characteristics in female fish	Runnalls et al, 2015
Zebrafish (*Danio rerio*)	NOEC < 5.5 ng/L	Sex ratio (masculinization), advanced maturity in male gonads	Svensson et al, 2016
Fathead minnow (*Pimephales promelas*)	NOEC <0.8 ng/L	Fecundity, reproductive behavior, secondary sexual characteristics in female fish, androgenic effect on male and female reproductive tissues	Zeilinger et al, 2009
Xenopus laevis	NOEC <31.2 ng/l	Reproductive behavior (advertisement calling and rasping)	Hoffmann and Kloas, 2012
Xenopus laevis	NOEC <156 µg/L	Testicular morphology, ovarian tissue, delayed metamorphosis	Kloas et al, 2009
Xenopus tropicalis	NOEC < 18.7 ng/L	Reproductive organ development, oogenesis and fecundity	Kvarnryd et al, 2011
Xenopus laevis	NOEC <3120 ng/L	Inhibited metamorphosis	Lorenz et al, 2011a, Lorenz et al, 2011b, Lorenz et al, 2018
Xenopus tropicalis	NOEC <1.3 ng/L	Oocyte development	Säfholm et al, 2012
Xenopus tropicalis	NOEC 30 ng/L	Reproductive organ development, delayed metamorphosis	Säfholm et al, 2016
Fathead minnow (*Pimephales promelas*)	EC10 0.1 ng/L	Fecundity, sexual reversal	FASS, 2023, Rigevidocont assessment

From the data in Table 5, it can be concluded that fish are the most sensitive species for levonorgestrel. The overall lowest NOEC was 0.06 ng/L. Only one study is available for norgestrel (racemic mixture of dexo- and levonorgestrel) with a NOEC of 3.6 ng/L (Hou et al, 2018). The most prominent effect of levonorgestrel was masculinization of female fish, as demonstrated by histopathological assessment of the gonads. This effect was also induced by norgestrel (racemic mixture), albeit at much higher concentrations (i.e. at and above 36 ng/L). The difference in potency cannot be attributed to test duration, as in partial life cycle tests with levonorgestrel, complete masculinization of female fish was observed at and above 5.5 ng/L, while norgestrel induced the presence of spermatogonia (at and above 35.8 ng/L) and early spermatocytes (368 ng/L) in ovaries but did not lead to complete sex reversal. These data indicate that levonorgestrel is active in fish while dexonorgestrel is not – leading to higher effective concentrations of the racemic mixture.

7.1.6. Microbial Inhibition Testing

Data from a microbial toxicity test with levonorgestrel are not available. However, in the degradation study reported by Weizel et al, 2021, levonorgestrel (initial concentration 200 ng/L) was completely removed by microbial degradation within a few hours (suspended solid concentration 0.4 g/L), indicating that the substance was not toxic to wastewater bacteria.

7.1.7. Algae

The toxicity of levonorgestrel was studied in 4 species of algae (Czarny et al 2019a-c). In the algal toxicity studies conducted by Czarny and coworkers, exponentially growing algae (5 replicates per test group, initial cell density $2x10^5$ – $1x10^7$ cells/mL) were exposed to nominal levonorgestrel concentrations of 0.1, 1, 10, 25, 50, 75 and 100 mg/L for 14 days. Growth was determined as dry weight of the biomass of the algal cells and by measuring chlorophyll *a* content. Reported EC50[2] values were based on chlorophyll *a* content after 7, 10 and 14 days. According to the OECD 201 guideline, the study aims at determining the reduction of growth rate, calculated over a 72-hour period after onset of exposure. Further, a test is considered valid if the control cultures demonstrated an exponential increase in biomass (at least 16 times over 72 hours) and if the variation between replicates in the control is lower than 35% (section-by-section growth rate) or 7% (average growth rate). Details on growth in individual replicates were not reported, but the supplemental information presented mean ± standard deviation (SD) chlorophyll *a* contents in the different treatments for days 0, 1, 2, 3, 7, 8, 10, 13 and 14. The data for the first 72 hours are shown in Table 6, Table 7, Table 8 and Table 9. These were used to calculate growth rate, assuming that there was a correlation between biomass and chlorophyll.

[2] Concentration displaying 50% effect, i.e. 50% growth rate reduction

From these data, it can be concluded that the validity criterion for a 16 times-increase in algal biomass was not met (i.e. factor 1.4 for *Chlorella vulgaris* and *Scenedesmus armatus*, factor 1.3 for *Mycrocystis aeruginosa* and factor 1.1 for *Anabaena variabilis*). Variation in chlorophyll *a* levels between control replicates on days 1-3 was however very low (i.e. 0.11-0.23%, 0.12-0.24%, 1.7-2.2% and 0.29-0.82% for *Chlorella vulgaris*, *Scenedesmus armatus*, *Mycrocystis aeruginosa* and *Anabaena variabilis*, respectively). From the relatively low increases in biomass, it may be concluded that the algal cells were no longer in their exponential phase. This may have been caused by two deviations from the OECD 201: 1) the initial cell densities were much higher than the recommended initial cell densities for slower growing species (up to 2-3 orders of magnitude), and 2) a photoperiod of 12/12 – 14/10 h light/dark was reported to have been used for culturing. Therefore, the test is not entirely in agreement with OECD 201. However, the data indicate that the effect of levonorgestrel on green algae and cyanobacteria was limited: based on mean measured chlorophyll *a* contents per concentration per day, growth rate reduction (calculated according to OECD 201) was negligible up to a concentration of 50 mg/L, 75 mg/L and 100 mg/L for *Scenedesmus armatus*, *Mycrocystis aeruginosa* and *Anabaena variabilis*, respectively (empirically derived NOEC). For *Chlorella vulgaris*, growth rate reduction was certainly not affected at concentrations up to 1 mg/L but may have been slightly affected at higher concentrations (empirical NOEC 1 mg/L). The EC50 for all species can be concluded to exceed 100 mg/L.

Table 6: Chlorophyll *a* contents during the algal growth inhibition test with *C. vulgaris* and calculated growth rate reduction (data from Czarny et al., 2019a)

Nominal concentration levonorgestrel [mg/L]	Chlorophyll *a* ± SD [10^2µg L^{-1}]		0-72 h Growth rate [a]	
	0 hours	72 hours	day^{-1}	% reduction [a]
control sample	176.98 ± 0.33	239.13 ± 0.36	0.100	-
0.1	176.98 ± 0.33	238.32 ± 0.14	0.099	1
1	176.98 ± 0.33	239.92 ± 0.32	0.101	-1
10	176.98 ± 0.33	229.68 ± 0.64	0.087	13
25	176.98 ± 0.33	639.82 ± 0.15	0.428	-327 [b]
50	176.98 ± 0.33	214.14 ± 0.47	0.064	37
75	176.98 ± 0.33	227.37 ± 0.29	0.084	17
100	176.98 ± 0.33	211.26 ± 0.21	0.059	41

[a] Calculated according to OECD 201
[b] Anticipated to be due to a typographical error in chlorophyll *a* content after 72 hours

Table 7: Chlorophyll *a* contents during the algal growth inhibition test with *S. armatus* and calculated growth rate reduction (data from Czarny et al., 2019a)

Nominal concentration levonorgestrel [mg/L]	Chlorophyll a ± SD [10^2µg L^{-1}]		0-72 h Growth rate [a]	
	0 hours	72 hours	day^{-1}	% reduction [a]
control sample	221.65 ± 0.40	311.67 ± 0.65	0.114	-
0.1	221.65 ± 0.40	313.41 ± 1.10	0.115	-2
1	221.65 ± 0.40	305.46 ± 0.69	0.107	6
10	221.65 ± 0.40	292.37 ± 0.91	0.092	19
25	221.65 ± 0.40	324.04 ± 0.29	0.127	-11
50	221.65 ± 0.40	318.00 ± 0.38	0.120	-6
75	221.65 ± 0.40	300.75 ± 0.45	0.102	10
100	221.65 ± 0.40	273.29 ± 0.25	0.070	39

[a] Calculated according to OECD 201

Table 8: Chlorophyll *a* contents during the algal growth inhibition test with *M. aeruginosa* and calculated growth rate reduction (data from Czarny et al., 2019b)

Nominal concentration levonorgestrel [mg/L]	Chlorophyll *a* ± SD [mg L^{-1}]		0-72 h Growth rate [a]	
	0 hours	72 hours	day^{-1}	% reduction [a]
control sample	25.75± 0.56	32.95± 0.57	0.082	-
0.1	25.75± 0.56	33.39± 0.59	0.087	-5
1	25.75± 0.56	34.78± 0.58	0.100	-22
10	25.75± 0.56	34.31± 0.62	0.096	-16
25	25.75± 0.56	34.49± 0.60	0.097	-19
50	25.75± 0.56	34.87± 0.61	0.101	-23
75	25.75± 0.56	33.69± 0.60	0.090	-9
100	25.75± 0.56	29.71± 0.61	0.048	42

[a] Calculated according to OECD 201

Table 9: Chlorophyll *a* contents during the algal growth inhibition test with *A. variabilis* and calculated growth rate reduction (data from Czarny et al., 2019c)

Nominal concentration levonorgestrel [mg/L]	Chlorophyll *a* ± SD [mg L^{-1}]		0-72 h Growth rate [a]	
	0 hours	72 hours	day^{-1}	% reduction [a]
control sample	404850 ± 2584	465350 ± 3815	0.046	-
0.1	404850 ± 2584	444050 ± 2308	0.031	34
1	404850 ± 2584	473150 ± 895	0.052	-12
10	404850 ± 2584	480050 ± 5507	0.057	-22
25	404850 ± 2584	499850 ± 652	0.070	-51
50	404850 ± 2584	484350 ± 962	0.060	-29
75	404850 ± 2584	508850 ± 1782	0.076	-64
100	404850 ± 2584	520450 ± 2080	0.084	-80

[a] Calculated according to OECD 201

7.1.8. Invertebrates

Cardoso et al, 2018, report on the *in vivo* effects of levonorgestrel on amphipods. The authors studied different combinations of temperature (18° and 22 °C), pH (7.6 and 8.1) and levonorgestrel concentrations (10 and 1000 ng/L) in a full factorial design. There were 16 different treatments with 6 replicates per treatment; test vessels were 650 mL glass cups containing a thin layer of sand (1 cm) and *Ulva* spp. Each replicate contained 14 individuals of *Gammarus locusta* from a laboratory culture. Per replicate, 4 organisms were individually confined in small containers within the glass cup to allow evaluation of individual survival, growth, condition index and consumption rates. The remaining 10 organisms/replicate were separated by sex, 3 replicates containing males and 3 replicates containing females; these organisms were used to assess post-exposure reproduction. The test was conducted under flow-through conditions of test medium. The study contained a pre-mating and a post-mating phase. During the pre-mating phase, organisms were exposed to levonorgestrel 3 times a day for 21 days, by direct addition of levonorgestrel to the test medium. Survival was recorded at days 7, 15 and 21; growth and condition of individually kept organisms was determined at day 1 and day 7 (2/replicate) or at day 1 and day 21 (2/replicate). Consumption rates were assessed over 24 hours during the first and the last week of exposure, after starving the organisms for 24 hours.

The post-mating phase started at the end of the 21-day exposure period. Pairs of males and females were assembled and maintained in separate units (1 pair/unit). Embryonic development time was measured as the period from oviposition to release of juveniles from the brood pouch; fecundity was expressed as the number of neonates released per female.

Levonorgestrel concentrations at 30 and 90 minutes after dosing were analytically verified by U-HPLC.

Measured levonorgestrel concentrations were close to nominal 30 minutes after dosing but decreased considerably (more than 60-90%) during the following 60 minutes.

Results were not reported for all different scenarios, but the overall effect of levonorgestrel appeared to be as follows:

- Growth was unaffected as compared to the untreated control but increased compared to the solvent control (ethanol 0.01%), which showed significantly lower growth than the untreated control.
- Food consumption rates were not equivocally affected by levonorgestrel, with an apparent increase at the lowest concentration compared to the solvent control.
- Reproduction was reduced by levonorgestrel at both tested concentrations when compared to the solvent control but not when compared to the untreated control, which showed a statistically significantly lower fecundity than the solvent control. Furthermore, there was not a dose response relation.
- Survival was not affected by levonorgestrel.

The NOEC may be concluded to be lower than the lowest tested concentration (10 ng/L).

7.1.9. Fish

Several *in vivo* studies in fish are available from the public literature. The most relevant studies are those that describe the complete life cycle, or even multiple generations. Such studies have been reported by DeCourten et al, 2020, Teigeler et al, 2021 and Zeilinger, 2009. Further partial life cycle studies have been reported by Frankel et al, 2016-2018, Onishi et al, 2020, Runnalls et al, 2015, Svensson et al, 2016 and Zeilinger et al, 2009 (levonorgestrel) and Hou et al, 2018 (norgestrel). Since these only cover a part of the life cycle, these studies have only been described briefly.

Fish life cycle studies

DeCourten et al, 2020 describe a multigeneration study, where only the first generation was exposed to levonorgestrel. Embryos of an estuarine fish species (Inland silverside, *Menidia beryllina*) were exposed to a single concentration of levonorgestrel (nominal 10 ng/L) from 8 hours post-fertilization to 21 days post hatch, and subsequently reared through to the early life stage of the F_2 generation in clean water. Embryos were kept in 25 mL beakers (90/replicate) until day 7-10, after which they were transferred to 1.4 L jars for the remainder of the exposure period. At that time, fish were transferred to 13 L tanks. There were 5 replicates per treatment. The analytically verified concentration of levonorgestrel was 9.27±0.59 ng/L.

Embryos (51 per replicate) were examined for craniofacial (CF), cardiovascular (CV) and skeletal (S) deformities and malformities. Spawning of adult fish (~225 days post hatch) was assessed over 48 hours after introducing a spawning substrate. Fecundity and fertilization were recorded, and adult fish were euthanized for sex determination. Gonads from adult fish were subjected to histological examination. In addition, immune response assays were conducted in adult fish (4/replicate) by measuring inflammation after injection of a phytoheamagglutinin solution into the caudal peduncle. Molecular endpoints were also assessed by measuring the expression and methylation of 20 genes involved in multiple pathways, including steroidogenesis, reproduction, growth, immune function, DNA methylation, thyroid function, cell proliferation, stress and metabolism, in 21-day old larvae (2/replicate).

Levonorgestrel exposed F_0 larvae were larger than controls, while the expression of a gene for growth (*igf2*) was reduced. These contradictory results were attributed to feedback mechanisms and timing of sampling for gene expression. Although gene expression of two genes from the reproductive pathway was reduced (*esr1* and *gnrhr*), reproduction was not affected in the F_0 generation. Hatching success of the F_1 generation was higher than that in the control. However, fecundity of the F_1 generation was reduced. CF deformities appeared to be increased in the F_1 and F_2 generations, although this was only statistically significant in the F_2 generation. These malformations were fatal, leading to death within hours after hatch. As a result, survival among F_2 larvae was reduced. Other population-relevant effects were not observed.

It is concluded that levonorgestrel reduced reproduction success and survival in generations that were produced by fish that were exposed only during their early life stages. The NOEC is <10 ng/l.

In the full life cycle test reported by Teigeler et al, 2021, zebrafish were exposed to levonorgestrel under flow-through conditions for 126 days, spanning life stages from egg stage until after first reproduction. The test was conducted based on OECD guidelines 210, 229 and 234. Zebrafish fertilized eggs (4-stage early blastula stage, < 3 hours post fertilization, pf) were used to start the test, which contained 5 concentrations and a control, each tested in 4 replicates of 50 eggs (2 x 25). Eggs and larvae were maintained in hatching trays in the aquaria until day 21, when the fish were transferred into the main aquaria. Hatching was assessed from day 3 until day 6 pf, survival was assessed on day 21 and 28, and length and survival were assessed on day 35 and 63. On day 35 and 63, fish were thinned to 30 and 20 individuals (if possible), respectively. On day 64, spawning trays were introduced, and egg numbers and fertilization rates were evaluated daily from day 84 until test end. At the end of the test, length, weight and sex of the fish was determined and blood samples were taken for subsequent VTG and 11-ketotestosterone measurements. Fish were processed for histopathological analysis.

Test conditions were stable and within the ranges indicated in the test guidelines. Mean measured concentrations of levonorgestrel were 0.06, 0.16, 0.47, 1.64 and 5.45 ng/L, representing 94.2-118.8% of nominals. Validity criteria of the different guidelines were met, indicating all phases of the study were valid. Hatching success and growth of larval fish were not affected by levonorgestrel. Mortality of larvae was reduced at and above 0.47 ng/L, although a clear dose response was not observed. At day 35, length of juvenile fish appeared to be increased compared to the control at all test concentrations, but this was not statistically significant, nor dose related. Survival of juvenile fish was not affected. The first spawning events were observed on day 64 pf, with a mean time to first spawning being 66 days for the control and 69, 69, 67 and 69 days for 0.06, 0.16, 0.47 and 1.64 ng/L, respectively. Spawning was not observed at the highest test concentration. Mean fecundity in control fish was 21 eggs/day/female; at 0.06, 0.16, 0.47 and 1.64 ng/L, fecundity was increased to 38, 34, 36 and 33 eggs/day/female, respectively. Fertilization success was 91% in the control and 90, 84, 85 and 76% at 0.06, 0.16, 0.47 and 1.64 ng/L, respectively. The reduction was statistically significant at and above 0.16 ng/L, even though the fertilization success at 0.16 and 0.47 ng/L was above the guideline quality criterion for the control (80%). Growth of male fish was statistically significantly affected at and above 0.16 ng/L, while growth of female fish was not affected at any concentration where females could be determined. At the highest tested concentration, females were not found. Sex ratio expressed as % females, determined by macroscopic inspection and histological examination of the gonads, was 60% in the control and 69, 53, 61, 29 and 0% at 0.06, 0.16, 0.47, 1.64 and 5.45 ng/L, respectively. The reduction in female numbers was statistically significant at and above 1.64 ng/L. Histological examination of male gonads revealed no mixed gonads, increased proportions of spermatogonia or interstitial cell hypertrophy/hyperplasia, although there was a (statistically non-significant) increase in less mature gonads with increasing concentration. 11-ketotestosterone concentrations in male plasma decreased with increasing test concentration, and the reduction was statistically significant at 5.45 ng/L. Levels of VTG in male plasma were close to the LOQ at all concentrations. Female gonads displayed the same level of maturation, although an increase in enhanced histological abnormalities (oocyte atresia and egg debris) was observed with increasing concentration. VTG levels in blood was unaffected at all concentrations where female fish were encountered.

Overall, the most prominent population relevant effect induced by levonorgestrel was the shift toward an increased number of male fish at 1.64 ng/L and especially at 5.45 ng/L, where all fish were males. Reproduction was affected due to a reduced fertilization rate that was statistically significant at and above 0.16 ng/L. The NOEC from this study is 0.06 ng/L.

In her doctoral thesis, Zeilinger, 2009 describes a fish full life cycle test with fathead minnow. The study started with freshly fertilized eggs (<24 hours old, 2 replicate petri dishes per group, each containing 60 eggs) and lasted through sexual maturation and production of F_1 fish. Nominal levonorgestrel concentrations were 5, 50 and 100 ng/L. Eggs were maintained under continuous stirring until hatching. Larvae (50 per replicate) were transferred to 10 L vessels on day 7 post hatch and maintained until day 56 post hatch, when they were thinned to 30 fish per replicate and transferred into 20 L vessels and maintained until the development of secondary sexual characteristics (day 130 post hatch), after which 8 pairs per group were formed for reproduction. The reproduction phase lasted 3 weeks, the eggs were allowed to hatch, and the larvae were maintained until 28 days post hatch. During the study, length and weight of the fish was determined on days 30, 56 and 160 post hatch of the F_0 generation. At the end of the reproduction phase of the F_0 generation, fish were subjected to histological analysis. Mean measured levonorgestrel concentrations represented 14%, 44% and 29.7% of nominal at 5, 50 and 100 ng/L, respectively. The results were based on the mean measured concentrations of 0.7, 22.0 and 29.7 ng/L. The author stated that in all exposure groups, the recovery rate increased from the first sampling until the end of the study, but that stock solutions showed an adequate recovery rate and were stable during exposure time.

Hatching of the F_0 generation was completed within 5 days, and hatching success was 78% in the control and 70-82% in the levonorgestrel groups. Mortality until day 30 post hatch was 13% in the control and 12-16% in the levonorgestrel groups. Length and weight were not statistically significantly affected at any of the tested concentrations at 30 and 56 days post hatch. At 160 days post hatch, length and weight was statistically significantly increased at 22.0 and 29.7 ng/L, but not at 0.7 ng/L. At 160 days post hatch, the male:female sex ratio (as determined by histology) had shifted from 11:11 in the control to 17:9 at 0.7 ng/L and to 22:0 and 17:0 at 22.0 and 29.7 ng/L. Secondary sexual characteristics showed a dose dependent masculinization of the female fish after exposure to levonorgestrel. Histological examination revealed degenerative changes in the ovary (atretic follicles, interstitial edema). Ovaries were not found at the two highest concentrations. Instead, testes ova were found in one fish each at the two highest concentrations, and, some males had immature testes, pointing to masculinization of females. During the reproduction phase, control fish laid an average of 22 eggs per day. At 0.7 ng/L, only 3 of the 4 pairs produced a small number of eggs (2.2 eggs/day). No eggs were produced at 22.0 and 29.7 ng/L. In the F_1 generation, mean hatching success in the control was 90% and survival until day 28 post hatch was 87%. At 0.7 ng/L, hatching success was reduced to 38%, and survival until day 28 post hatch was reduced to 17%.

It can be concluded that levonorgestrel shifted the sex ratio towards a male dominated population at concentrations as low as 0.7 ng/L. At this concentration, hatchability and post-hatch survival were reduced. The overall effect on reproductive success, expressed as the number of larvae/day surviving until day 30 post hatch (i.e. mean number of eggs/day × hatchability × post-hatch survival) was 17 in the control and 0.14 at 0.7 ng/L (i.e. reduction of 99.2%). The NOEC from this study is <0.7 ng/L.

Fish partial life cycle studies

Frankel et al, 2018 describe an *in vivo* study in male fathead minnows (*Pimephales promelas*). Male fish were exposed to nominal levonorgestrel concentrations of 10 and 100 ng/L and a control for 14 days. 48 hours after completing the exposure phase, treated and control males were paired

(1:1 control:treated or control:control) and introduced in a competitive nesting assay, and the time spent on exhibiting aggressive behavior and holding the test were analysed. In addition, samples of semen were analysed for sperm motion parameters. Evaluations were based on nominal concentrations, which is acceptable as mean measured concentrations were within or slightly above 80-120% of nominal (i.e. 11.6 at 10 ng/L and 126.5 ng/L at 100 ng/L). At both concentrations, nest acquisition success was increased while aggression was reduced and sperm motion characteristics were decreased. Therefore, the NOEC was lower than 10 ng/L.

In the study reported by Frankel et al, 2017, adult female fathead minnows (*Pimephales promelas*) were exposed to nominal levonorgestrel concentrations of 10 and 100 ng/L and a control for 14 days under flow-through conditions. Following exposure, ovaries were weighed and examined for oogenesis, and, in a repeated test, blood plasma was analysed for VTG levels. Evaluations were based on nominal concentrations, which is acceptable as mean measured concentrations were within 80-120% of nominal (i.e. 8.8 and 9.8 ng/L at 10 ng/L and 91.6 and 91.6 ng/L at 100 ng/L). At both tested concentrations, fish displayed male secondary sexual characteristics, and ovaries contained a significantly lower fraction of late-stage oocytes. At 100 ng/L, GSI and VTG levels were reduced. The NOEC may be concluded to be lower than 10 ng/L.

Frankel et al, 2016 conducted an *in vivo* study in adult mosquitofish (*Gambusia holbrooki*), a viviparous species. A specific trait of viviparous fish is internal fertilization, which is enabled by an androgen driven elongation of the anal fin into the male gonopodium. Fish were exposed to nominal levonorgestrel concentrations of 10 and 100 ng/L and a control for 8 days under semi-static conditions with daily renewal of test concentrations. After completion of the exposure phase, fish were examined for differences in the 4:6 anal fin ratio and paired social interaction assays were conducted between control males and control or treated females. At both tested concentrations, levonorgestrel lead to masculinization of female fish, displayed by a significant increase in 4:6 anal fin ratios. In addition, 4:6 anal fin ratios of males were significantly increased at 100 ng/L. Reproductive behavior of control males was reduced when paired with 100 ng/L treated females. The NOEC may be concluded to be lower than 10 ng/L.

In the fish short-term reproduction test conducted according to OECD 229 and reported by Onishi et al, 2020, adult medaka were exposed to mean measured levonorgestrel concentrations of 7.3, 42 and 226 ng/L under flow-through conditions for 21 days. Fish were observed daily for mortality, abnormal behavior and appearance, and spawned eggs were collected daily. At the end of the test, length and weight of the surviving fish were recorded, liver samples were stored for VTG analysis, and the anal fin was imaged, or the posterior region of the fish including the anal fin was collected and stored in fixative for determination of secondary sexual characteristics (SSCs), expressed as the number of joint plates with visible papillary processes.

No male fish died, while mortality among females was 8% in the control and at 42 ng/L. VTG levels were unaffected in males but statistically significantly reduced by 60% at the highest concentration in females. SSCs were unaffected in males but statistically significantly increased at the highest two concentrations in females (from 0 to 44 and 83 at 42 and 226 ng/L, respectively). Fecundity, expressed as the total number of eggs, showed a concentration related reduction (3, 30 and 80% reduction at 7.3, 42 and 226 ng/L, respectively), but only the reduction at the highest concentration was statistically significant. The absolute number of fertilized eggs was statistically significantly reduced at the two highest test concentrations (reduction 11, 39 and 82% at 7.3, 42 and 226 ng/L, respectively), but fertilization rate (%) was not statistically significantly affected at any concentration (reduction of 5, 11 and 11% at 7.3, 42 and 226 ng/L, respectively). Length, weight, hepatosomatic index and gonadosomatic index were not affected in either males or females at any test concentration. Overall, the NOEC from this study was 7.3 ng/L.

In another fish short-term reproduction assay, reported by Runnalls et al, 2015, adult fathead minnows were exposed to nominal levonorgestrel at 0.5, 5 and 25 ng/L for 21 days (mean measured concentrations 0.42, 5.25 and 15.8 ng/L, respectively). Endpoints assessed included egg production, secondary sexual characteristics and plasma VTG and hormone levels. Egg production was reduced in a concentration-related manner, with a 20%, 40% reduction at 5 and 25 ng/L, respectively; it was further reported that in an earlier study by the same authors, egg production was completely inhibited at 100 ng/L. Male sexual characteristic were unaffected, while females developed dorsal fin spots and fatpads in all concentrations as well as facial tubercles at the two highest concentrations. At the highest tested concentration, weight, length and condition factor and abdominal girth were increased. The NOEC from this study was 0.42 ng/L.

Svensson et al, 2016 exposed juvenile zebrafish from 20 to 80 days post-fertilization (dpf), i.e. during maturation, to levonorgestrel concentrations of 5.5, 79 and 834 ng/L. Fish were sampled at several time points during exposure to determine gonadal gene transcript levels. Gonads were subjected to histopathological examination on 50 dpf and at the end of the exposure period.
At all concentrations, all adult fish were males. Transcript levels of gonadal genes were statistically significantly increased at 44 dpf. Histological assessment revealed advanced maturity in male gonads at all concentrations.

Zeilinger et al, 2009 studied the effects of levonorgestrel in fathead minnow in a fish short term reproduction assay according to the draft OECD 229 guideline[3]. Fish were exposed to mean measured concentrations of 0.8, 3.3 and 29.6 ng/L.

Fecundity was reduced at the two lowest concentrations (i.e. reduction of ~67-75%, estimated from graphical representation of the data) and the number of eggs decreased to almost zero in weeks 2

[3] The final OECD 229 test guideline contains several modifications compared to the draft version, aimed at improving reproduction success.

and 3. At the highest concentration, the number of eggs was very low during the first two weeks of exposure (i.e. reduction of >90%) and zero during the last week. Behavior of male fish was also affected, as displayed by the lack of interest for their spawning tiles and aggressive behavior. All treated females showed dose-related morphologic changes, such as an increase of abdominal girth above 0.8 ng/L and stronger pigmentation of fins and skin at 29.6 ng/L. Histopathological evaluation of the gonads revealed an androgenic effect on male and female reproductive tissues. At the highest concentration, an increase in the frequency of atretic follicles and pre-vitellogenic and vitellogenic oocytes was accompanied by a decrease in oogonia. The ovary was overfilled with mature oocytes with an increased span of the abdomen. In males, levonorgestrel caused an increase of mature spermatids and testes size and at the highest concentration, tubuli appeared enlarged and the lumen was filled with mature spermatids. The germinal epithelium was thickened, and therefore the interstitial tissue appeared compressed; the pressure seemed to cause atrophy of interstitial Leydig cells and Sertoli cells. The NOEC from this study appears to be lower than 0.8 ng/L.

Hou et al, 2018 exposed female mosquitofish (obtained from a local pet store, age not specified) to nominal norgestrel (not indicated, but presumably the racemic mixture of dexo- and levonorgestrel) concentrations of 0, 5, 50 and 500 ng/L for 42 days, with daily renewal of test solutions (mean measured concentrations 0, 3.6, 35.8 and 368 ng/L). After the 42-day exposure period, male control fish were paired with control or treated female fish for behavior assessment. At the end of the test (after the behavior test), fish were anesthetized, and standard length and biomass were recorded. The width of the third ray of the anal fin, the number of segments on the identical ray and the length ratio of ray 4/ray 6 were determined. Ovaries were subjected to histopathological examination.

Standard length and weight were not affected by norgestrel at any concentration. Morphology of the anal fin was not affected at the lowest tested concentration. However, the length ratio of ray 4/ray 6 was significantly increased by 28% and 44% at 35.8 and 368 ng/L, respectively, while the width of ray 3 was significantly increased by 200% and 280%, and the number of segments observed in ray 3 was increased 3.0 and 4.6-fold (derived from tabulated data; text indicated 2.0-fold and 3.9-fold), respectively. Furthermore, ovarian cell development was adversely affected at the two highest test concentrations. Control ovaries contained several developmental stages of follicular cells from perinuclear oocyte, early vitellogenic oocyte, vitellogenic oocyte and atretic oocyte. After exposure to norgestrel, ovaries contained spermatogonia (at 35.8 and 368 ng/L) and early spermatocytes (368 ng/L). Norgestrel caused a significant increase in the relative frequency of atretic follicular cells at all concentrations, and a significant reduction of late vitellogenic oocytes at 35.8 and 368 ng/L.

In the behavior test, male fish spent significantly less time on attending behavior to females exposed to 368 ng/L. Time spent on following behavior and close following behavior were significantly reduced at all concentrations.

It is concluded that norgestrel induced masculinization of female mosquitofish at and above 35.8 ng/L.

FASS, 2023 reports a proprietary owned short-term reproduction assay with levonorgestrel on the fathead minnow (*Pimephales promelas*) following OECD 229 Test guideline covering the endpoints fecundity and sexual reversal. As no details of the study are available a detailed evaluation is not possible. The EC10[4] of 0.1 ng/L was used by FASS for the PNEC derivation of levonorgestrel.

7.1.10. Amphibians

Several studies describing effects of levonorgestrel in amphibians are available. From the reported data, it is clear that fish are more sensitive than amphibians. Therefore, the fish studies (see section 7.1.9) are considered more relevant and the studies in amphibians are only briefly described below.

Hoffmann and Kloas, 2012 report on an *in vivo* study in male frogs (*Xenopus laevis*). Levonorgestrel increased the proportion of advertisement calling, indicating a sexually aroused state of the animals, at all tested concentrations (10^{-7} M, 10^{-8} M and 10^{-10} M; equivalent to 31.2, 3.12 and 0.0312 µg/L). The highest tested concentration increased the relative proportion of rasping, a call type indicating a sexually unaroused state of the male.

Kloas et al, 2009 published a review paper, containing preliminary results from an *in vivo* study in *Xenopus laevis* larvae. It was reported that levonorgestrel did not affect sex ratio at $5x10^{-7}$ M (equivalent to 156 µg/L), while it did significantly disrupt testicular morphology and ovarian tissue. In addition, gonadotropin expression was suppressed and a delay of metamorphosis was observed.

Kvarnryd et al, 2011 investigated the effects of levonorgestrel on sex differentiation, reproductive organ development and fertility in the frog *Xenopus tropicalis*. Tadpoles were exposed to 0.06 or 0.5 nM (i.e. 18.7 or 156 ng/L) levonorgestrel via the water until metamorphosis, followed by a 9 months post-exposure phase. Underdeveloped oviducts were observed in the only surviving female at 18.7 ng/L while all females completely lacked oviducts at 156 ng/L; they also displayed a larger fraction of immature oocytes than control females. Upon mating with unexposed males, the only surviving female at 18.7 ng/L produced 53 eggs, which was significantly lower than control egg production (mean 586 eggs). No eggs were produced at 156 ng/L.

Lorenz et al, 2011a and Lorenz et al, 2011b describe an *in vivo* study in *Xenopus laevis* tadpoles, which were exposed to levonorgestrel concentrations of 10^{-11}, 10^{-10}, 10^{-9} and 10^{-8} M (equivalent to

[4] Concentration displaying 10% effect

3.12, 31.2, 312 and 3120 ng/L) during metamorphosis. At the highest tested concentration, metamorphosis was inhibited, resulting in developmental arrest at early climax stages as giant tadpoles or tailed frogs. These results were reproduced in a similar study by the same authors and reported in 2018 (Lorenz et al, 2018).

Säfholm et al, 2012 exposed sexually mature *Xenopus tropicalis* females to levonogestrel concentrations of 51 and 307 ng/L for 7 days or to concentrations of 1.3, 18, 160 and 1240 ng/L for 28 days. In the 7-day exposure test, proportions of previtellogenic and immature oocytes were increased at 307 ng/L. After 28 days of exposure, the proportion of immature oocytes was statistically significantly reduced at the three lowest test concentrations; the proportions of vitellogenic and mature oocytes were generally reduced at all concentrations, while the proportion of previtellogenic oocytes was increased. These data indicate vitellogenesis was inhibited. At 1240 ng/L, gonadosomatic index was significantly reduced, while ovaries had transparent regions devoid of mature oocytes.

In a more recent study by the same authors (Säfholm et al, 2016), *Xenopus tropicalis* larvae were exposed to levonorgestrel at nominal concentrations of 3, 30 and 300 ng/L during the first three weeks of development. Analytical verification of the test concentrations revealed concentrations of <LOQ – 3 ng/L, 5-66 ng/L and 32-337 ng/L, respectively; mean measured concentration calculated from reported values 1, 22 and 175 ng/L.

Development of gonads or Müllerian ducts (assessed histologically) was not affected, while mRNA levels of hormone receptors were affected. In juveniles, folliculogenesis had initiated and the Müllerian ducts were larger in females than in males. There were no effects on survival, but the time to metamorphosis was significantly longer at 300 ng/L (nominal). The NOEC for population relevant effects (i.e. time to metamorphosis) appears to be 30 ng/L (nominal; mean measured concentration 22 ng/L).

7.2. Environmental Assessment

7.2.1. Aquatic compartment

As discussed in section 7.1.6, it is expected that levonorgestrel does not affect microbial activity at a concentration of 200 ng/L. This concentration is 313 times higher than the EIC-aquatic of 0.64 ng/L. Therefore, it is concluded that levonorgestrel does not disrupt the waste treatment process.

Information on environmental effects of levonorgestrel is available from chronic aquatic toxicity tests in algae, invertebrates and fish. As discussed in section 7.1.5, the current EA is based on NOEC values, even though the Guidance for industry (FDA, 1998) indicates that EC50 values should be used in the risk assessment. EC50 values are typically derived from acute tests, such as performed in Tier 1 and 2, while results from long-term tests (Tier 3) are expressed as NOEC values. Since NOEC values per definition are lower than EC50 values, they represent a conservative approach for risk assessment. For the most sensitive species (fish) a Predicted No Effect Concentration (PNEC) of 0.006 ng/L can be derived based on the lowest NOEC of 0.06 ng/L and applying the default assessment factor of 10 for chronic aquatic toxicity when data on at least three trophic levels are available. The available EC10 of 0.1 ng/L from an OECD 229 compliant short-term reproduction assay used by FASS in the risk assessment on levonorgestrel (drug product: Rigevidocont), is considered trustworthy and therefore the FASS derived PNEC of 0.01 ng/L is also taken further in the risk assessment.

These endpoints are compared to the refined EEC (see section 7.1.4) for risk assessment, which is shown in Table 10. Furthermore, the refined EEC is still considered a worst-case approximation of environmental exposure as indicated in section 7.1.4.

Table 10: Risk assessment for the aquatic compartment for levonorgestrel

Study	**NOEC (ng/L)**	**Flow**	**Percentile**	**EEC[a] (ng/L)**	**Risk quotient**	**Observed effects at EEC?**
					NOEC/EEC	
Algal growth inhibition test (4 species)	1,000,000	Low	50	0.0060	1.6 x 10^{8}	No
			90	0.057	1.8 x 10^{7}	No
			99	0.15	6.7 x 10^{6}	No
		Mean	50	0.00041	2.0 x 10^{9}	No
			90	0.0043	2.3 x 10^{8}	No
			99	0.039	2.6 x 10^{7}	No
Invertebrate reproduction (*Gammarus locusta*)	<10	Low	50	0.0060	<1,667	Unknown
			90	0.057	<175	Unknown
			99	0.15	<67	Unknown
		Mean	50	0.00041	<24,390	Unknown
			90	0.0043	<2,326	Unknown
			99	0.039	<256	Unknown
	PNEC				**EEC/PNEC[b]**	
Fish Full Life Cycle (*Danio rerio*)	0.006	Low	50	0.0065	1.1	No
			90	0.062	10	Yes
			99	0.16	27	Yes
		Mean	50	0.00045	0.08	No
			90	0.0047	0.78	No
			99	0.043	7.2	Yes
Short-term reproduction assay (*Pimephales promelas*)	0.01	Low	50	0.0065	0.65	No
			90	0.062	6.2	Yes
			99	0.16	16	Yes
		Mean	50	0.00045	0.05	No
			90	0.0047	0.47	No
			99	0.043	4.3	Yes

[a] See Table 4

[b] Calculated using rounded up values

As can be seen from Table 10, the NOEC/EEC ratio for algae is much higher than the trigger values of 10-1000 for Tier 1, Tier 2 and Tier 3 tests (FDA, 1998). For invertebrates, the unbound NOEC leads to the NOEC/EEC ratio being unbound, and it is unknown if effects would be observed at the level of the EEC. However, considering that the lowest tested concentration of 10 ng/L is 32 to 20,000 times higher than the EEC and that fish are expected to be driving the overall risk assessment due to the mode of action of levonorgestrel, the risk to aquatic invertebrates is expected to be acceptable.

For fish as the most sensitive species, safe use (Risk Quotients ≤1) is calculated for the median EEC values for both low and mean flow scenarios show Risk Quotients above 1.

The iSTREEM modelling shows that the results are highly variable based on the input assumptions, and there are uncertainties that cannot be resolved based upon available data because data are lacking regarding the exact level of excretion and bioactivities of the metabolites. Moreover, several of the input assumptions may still be very conservative, including the estimation of API production volume, the excretion factor, and the WWTP removal efficiency. Furthermore, the Risk Quotients vary significantly by the flow scenario, the in-stream concentration percentile, and the PNEC that is used in the calculation.

Regarding the conservatism of the iSTREEM output, Kapo et al (2016) compared national distributions of modelled concentrations of two substances (one fragrance and one insect repellent) to available monitoring data at comparable flow conditions and concluded that the iSTREEM low flow model results yielded a conservative distribution of values, whereas the mean flow model results more closely resembled the concentration distribution of monitoring data. These conservative values in the low flow results are due to the use of low river flows and hence WWTP flows greatly exceeding these flows. As a consequence, in an adjacent modelled downstream segment, the concentration is inflated as the WWTP flow is disregarded and the concentration load is attributed to the low river flow only. The authors concluded that a conservative estimation can be obtained by choosing the 90th percentile. Burns et al (2022) also concluded that iSTREEM mean flow predicted environmental concentrations were reasonable and conservative when compared with global surface water octinoxate concentrations in U.S. rivers. Still, based on currently available data and following the conservative scenarios, Risk Quotients above 1 are calculated at the high-end percentiles and especially at the low flow scenario and hence given the potential risk to fish risk mitigation measures could be considered.

7.2.2. Terrestrial compartment

Since the adsorption of levonorgestrel to sludge is low (Kd 0.4-18 L/kg; see section 7.1.2), binding of levonorgestrel to biosolids in wastewater facilities and sediments is not expected. Therefore, testing for fate and effects in the terrestrial environment was not considered required and due to the lack of exposure there is no concern for adverse effects on terrestrial organisms following use of Opill (Norgestrel, 0.075 mg tablets).

7.2.3. Animals at higher levels of the aquatic food chain

Following CMC IR#2 dated September 26, 2022, a further assessment was made on the occurrence and potential impacts of levonorgestrel on animals at higher levels of the aquatic food chain, which are taken to be birds and mammals feeding on fish and lower organisms. Data on birds and mammals directly impacted by exposure to environmental concentrations of levonorgestrel are not available. Nevertheless, several studies providing information of the use of quinestrol and levonorgestrel isolated or in combination as a sterilization agent of harmful rodent populations are available. These studies can give valuable information on the impacts levonorgestrel might have on small mammals' reproduction.

Shi et al, 2020 investigated the effects on reproductive behavior and changes in the reproductive system, reproductive hormone levels, and toxicity in adult Brandt's voles (*Lasiopodomys brandtii*) exposed to 2 mg/kg dose of levonorgestrel (L group) and quinestrol (Q group) and a 1:1 mixture of the two (EP-1 group). Levonorgestrel was shown to significantly inhibit the reproductive process, without affecting the normal development of the animals.

A study conducted by Zhao et al, 2007 also evaluated the effects of levonorgestrel (P; 0.67 mg/kg bw), quinestrol (E; 0.33 mg/kg bw) and its combination (EP-1, 6:3 ratio) on the fertility of males and females Brandt's voles. Levonorgestrel did not affect testes parameters in males or fertility status in females.

Lv et al, 2012 examined the effects of quinestrol (QE) and levonorgestrel (LNG) serum prolactin level in lactating Mongolian gerbils (*Meriones unguiculatus*) and reproductive parameters of their offspring. The reproduction of female pups from LNG-treated gerbils was not significantly affected by the treatment (0.6–16.2 µg/g body weight). No obvious effects on reproductive hormones or organs were observed in female pups from LNG-treated gerbils, which suggests that the amount of LNG received through the maternal milk was not sufficient to impact offspring reproduction. A similar conclusion can be drawn with regards to the male pups from the LNG-treated mothers.

Massawe et al, 2018 performed a laboratory study to investigate the effects of using fertility compounds on an outbreaking rodent pest species found throughout sub-Saharan Africa. *Mastomys natalensis* were fed bait containing the synthetic steroid hormones quinestrol and levonorgestrel, both singly and in combination, at three concentrations (10, 50, 100 ppm) for 7 days. Levonorgestrel reduced the weight of the seminal vesicle in males at 10 and 50 ppm but not at 100 ppm and reduced sperm count and motility at all doses. Time to delivery, pregnancy and litter size were all unaffected by levonorgestrel. Hence, the NOEC for levonorgestrel related to population relevant endpoints (number of offspring) can be concluded to be 100 ppm.

In summary, the lowest NOEC for environmentally relevant effects of levonorgestrel in mammals is 0.67 mg/kg bw. Data on birds were not found. Whole body concentrations in animals at higher levels of the aquatic food chain can be calculated from the EEC (ng/L), the bioconcentration factor (BFC) (L/kg) and a default Biomagnification Factor (BMF). The highest BCF for levonorgestrel is that in fish (571 L/kg). For substances with a logPow value <4.5, the BMF is 1 (ECHA, 2016). For levonorgestrel, the logPow is 3.8 (high end value, see Table 1) and therefore the BMF is 1. As a conservative approach, the non-refined EEC is taken (0.64 ng/L; Table 3). Thus, the whole-body concentration in predators is calculated as

Cpredator = EEC [ng/L] × BCF [L/kg] × BMF [-] = 0.64 [ng/L] × 571 [L/kg] × 1 [-] = 365 [ng/kg]

This concentration is more than three orders of magnitude lower than the lowest NOEC (670,000 / 365 = 1836).

Therefore, the risk to animals higher in the aquatic food chain is expected to be negligible following use of Opill (Norgestrel, 0.075 mg tablets).

8. DATA SUMMARY TABLE

The data discussed in sections 7.1 and 7.1.5 are summarized in Table 11.

Table 11: Data summary for the environmental assessment of levonorgestrel

Physical/chemical characterization	
Water Solubility	1.73 mg/L (database match) 35.84 mg/L (estimated)
Dissociation Constant(s) (pKa)	-1.53 (estimated)
Log Octanol/water Partition Coefficient (LogKow)	3.48 - 3.66 (estimated) 3.8 (database)
Vapor Pressure or Henry's Law Constant	1E-009 mm Hg (estimated)
Distribution coefficient (Kd)	Soil (n=5): 5.13-33.2 L/kg Sludge (n=1): 0.40-18 L/kg
Sorption/Desorption coefficient (Koc)	Soil (n=10): 641 – 2486 L/kg
Bioconcentration Factor BCF	Mussel: 30-208 L/kg Fish (whole body): 184-571 L/kg Fish (organ): 17-53 L/kg Fish plasma: >8500-12000 L/kg
Depletion mechanisms	
Hydrolysis	Not expected relevant
Photolysis	Not expected relevant
Aerobic Biodegradation by activated sludge (wastewater)	DT50: 0.48 hours (n=3)
Soil Biodegradation	DT50: 4.3-15.9 days (n=10)
Degradation in water/sediment system	DT50: 2.5-3.2 days (n=2) disappearance half-life from the water phase
Sediment Biodegradation	No data
Metabolism	Metabolites are covered by parent
Environmental effects	
Microbial Inhibition	NOEC 200 ng/L EC50 >200 ng/L
Acute Toxicity to invertebrates or fish	Not assessed
Algal growth inhibition	NOEC 1,000,000 ng/L
Gammarus reproduction	NOEC <10 ng/L
Fish full life cycle test	NOEC 0.06 ng/L
Fish, short-term reproduction assay	EC10 0.1 ng/L

9. MITIGATION MEASURES

Given the potential risk to fish, risk mitigation measures could be considered.

10. ALTERNATIVES TO THE PROPOSED ACTION

No potential adverse effects have been identified which would necessitate alternative actions to that proposed in the sNDA. If the sNDA is not approved, the result would be the continued use of currently marketed prescription and non-prescription hormonal birth control products and other forms of birth control that the proposed product would replace. Such action would have no significant environmental impact.

APPEARS THIS WAY ON ORIGINAL

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From 1986 onwards, many study reports and advices in the area of physico-chemistry, (eco)toxicology and risk assessment were prepared for clients of NOTOX BV, ARCADIS BV, WIL Research and Charles River Den Bosch. As this data is confidential information, it is not added to the above list.

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From 1999 onwards, many study reports and advices in the area of ecotoxicology and risk assessment were prepared for clients of NOTOX BV, WIL Research Europe and Charles River Den Bosch. As this data is confidential information, it is not added to the above list.

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