

Environmental steroid hormones in aquatic systems: Biogeochemical dynamics, mixture risks, and sustainable mitigation

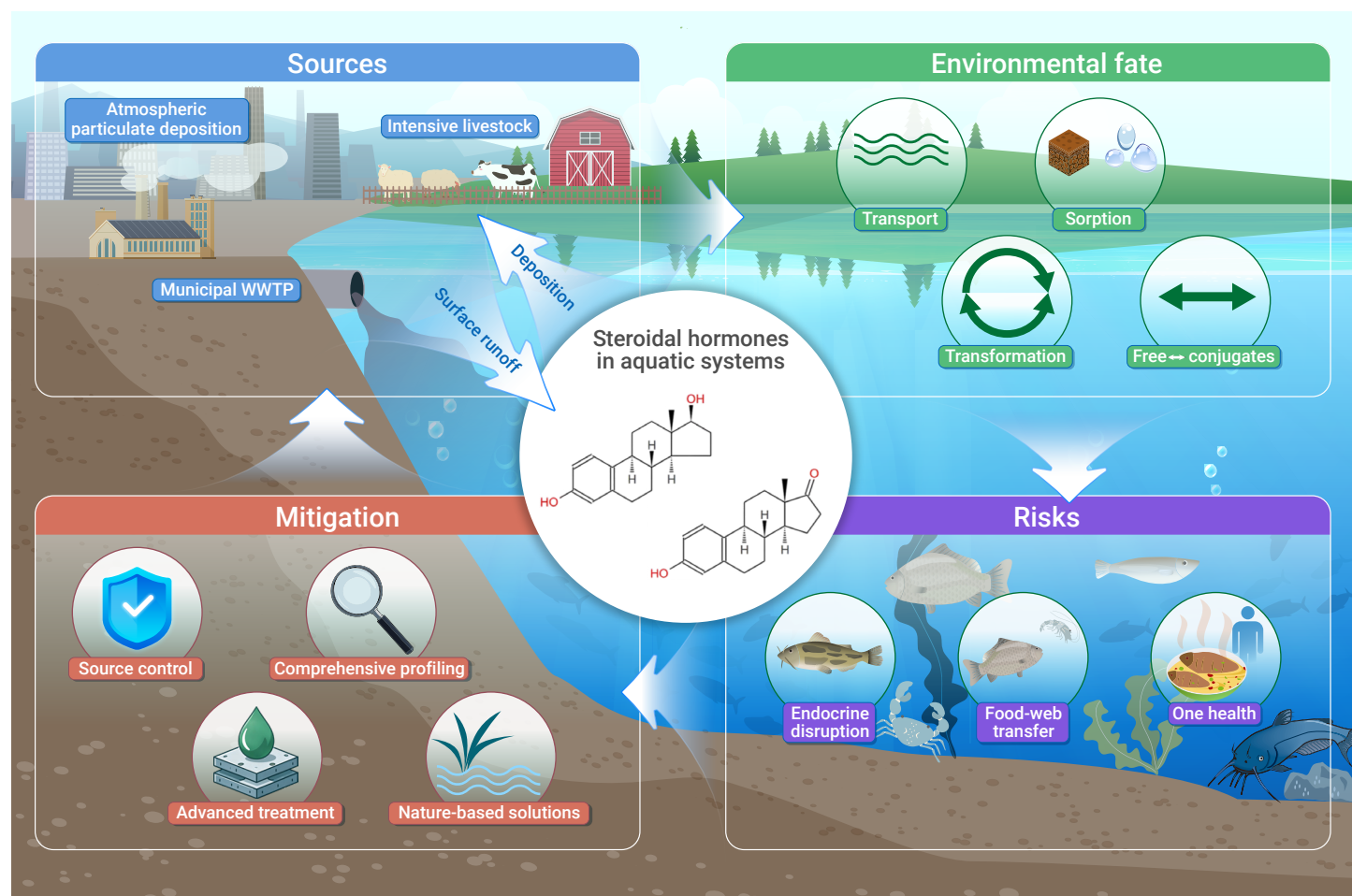
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GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- Steroid hormones enter and persist in waters via wastewater, manure runoff, and airborne particles.
- Conjugated forms and transformation products can be missed, while mixtures can amplify pollution risks.
- These pollutants can disrupt reproduction in aquatic life and may pose broader risks to human health.
- Monitoring, source control, and nature-based treatment provide a sustainable path forward.

Environmental steroid hormones in aquatic systems: Biogeochemical dynamics, mixture risks, and sustainable mitigation

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The pervasive occurrence of steroidal sex hormones in aquatic ecosystems poses a substantial challenge to aquatic ecosystem health and may also have implications for public health. Some of these potent endocrine-disrupting chemicals act at ultra-trace concentrations, challenging traditional single-substance toxicological paradigms, and are often incompletely removed by conventional wastewater treatment. This review critically synthesizes the biogeochemical dynamics of aquatic sex hormones, tracing their trajectories from anthropogenic sources through environmental transport, transformation, and persistence. It highlights the need to expand monitoring beyond targeted quantification toward comprehensive steroidal profiling, with particular attention to the potentially overlooked contributions of conjugated forms and uncharacterized transformation products. Furthermore, this review evaluates biological effects through the lens of adverse outcome pathways and the One Health framework, integrating well-established ecological effects in aquatic organisms with the more limited and emerging evidence relevant to human health under real-world mixture exposures. To address these threats, the efficacy and sustainability of advanced and emerging removal technologies are critically assessed, including catalytic oxidation, enzymatic biotransformation, hybrid systems, and nature-based solutions. Ultimately, safeguarding aquatic ecosystems requires an integrated, multi-barrier strategy that combines proactive source control, mixture-aware risk assessment, fit-for-purpose wastewater treatment, and sustainable mitigation aligned with circular economy principles.

INTRODUCTION

Steroidal sex hormones include natural estrogens (e.g., estrone [E1] and 17 β -estradiol [E2]), androgens (e.g., testosterone), and progestogens. These endogenous hormones are fundamental regulators of physiological homeostasis across vertebrates, whereas their synthetic analogues, such as 17 α -ethinylestradiol [EE2] and synthetic progestins, can also exhibit potent biological activity. Anthropogenic releases associated with pharmaceutical use, including hormonal contraceptives, and intensive livestock production have led to the continuous introduction of both endogenous hormones and synthetic analogues into aquatic environments.^{1,2} A recent global inventory documented the widespread occurrence of natural and synthetic estrogens across diverse aquatic systems, while also revealing substantial spatial variability in their concentrations and associated risks.³ The environmental behavior and receptor interactions of these compounds are influenced not only by their rigid cyclopentanoperhydrophenanthrene nucleus but also by their functional substituents, which collectively determine hydrophobicity, phase partitioning, receptor affinity, and susceptibility to biotransformation. Important gaps also remain in the comparative pharmac- and toxicokinetics of endogenous and exogenous estrogens.⁴

Steroidal endocrine-disrupting chemicals (EDCs) can elicit biological responses at concentrations in the nanogram-per-liter range or lower, and some may exhibit non-monotonic dose-response relationships. However, these patterns are compound-, endpoint-, and exposure-dependent rather than universal. These contaminants can interfere with hormone biosynthesis,

transport, metabolism, receptor binding, and downstream signaling, thereby disrupting physiological homeostasis.⁵ Their effects can extend across multiple levels of biological organization. Endogenous sex steroids regulate immune function and developmental processes, providing biological targets through which exogenous hormonal exposure may act.⁶ During development, endocrine-active exposures can disturb tissue organization, and estrogenic compounds can modulate teleost metamorphosis through interactions with the thyroid axis, although the direction and magnitude of the reported effects are context-dependent.^{7,8} Within a One Health framework, molecular- and organism-level effects in aquatic species may, when sustained, contribute to population-level changes and broader ecological consequences. The human gut microbiota can also modulate systemic steroid profiles and may thereby influence the composition of steroids ultimately excreted into wastewater, illustrating a plausible connection between host metabolism and environmental emissions.⁹ Although disruption of sex-hormone signaling has been implicated in metabolic and autoimmune diseases, direct evidence that environmentally relevant exposure to aquatic steroid hormones causes these outcomes in humans remains limited.^{10,11} Moreover, real-world aquatic environments contain complex mixtures of natural hormones, synthetic analogues, transformation products, and other EDCs. Their combined effects may be additive or non-additive, making single-substance assessments insufficient for characterizing environmental risk.^{2,12}

Accurate characterization and effective mitigation of these contaminants remain constrained by analytical and engineering limitations. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is one of the principal techniques for trace analysis of polar steroids in complex aquatic matrices.¹³ However, reliable determination of potent synthetic analogues such as EE2 at low-pg/L concentrations remains technically challenging, particularly in complex environmental matrices.¹⁴ A more fundamental limitation concerns monitoring scope. Conventional protocols often focus on a restricted set of parent compounds and therefore fail to characterize the complete steroidal profile, including conjugated forms and transformation products. Although many conjugates exhibit limited direct biological activity, they can undergo deconjugation to regenerate active parent hormones, while transformation products may retain or acquire biological activity.¹⁵

Conjugated and halogenated estrogens can account for 60%–70% of the total molar steroidal estrogen load in secondary effluents.¹⁶ Conventional municipal wastewater treatment plants (WWTPs) can substantially attenuate some steroidal hormones, but their performance is compound- and process-dependent, and residual hormones and endocrine activity may persist in final effluents. These limitations support the development of optimized, multi-barrier treatment strategies that integrate physicochemical and biological processes.

Previous reviews have documented different aspects of the occurrence of steroidal hormones in aquatic environments,¹⁷ their ecological risks,^{18,19} and their removal during wastewater treatment.^{20,21} Nevertheless, important gaps remain in integrating chemical speciation and comprehensive exposure profiles with real-world mixture effects and cross-system health implications. Many previous syntheses have emphasized targeted measurements of selected parent compounds and single-substance toxicological assess-

ments. Consequently, the environmental contributions of conjugated reservoirs and biologically active transformation products may be underestimated. Ecological effects and human health implications have also frequently been considered separately, limiting the ability of existing frameworks to evaluate the combined effects and uncertainties associated with complex environmental mixtures.

To bridge these fragmented disciplinary perspectives and address current bottlenecks, this review provides an integrative synthesis of the sources, transport, transformation, biological effects, analysis, and mitigation of steroidal sex hormones in aquatic systems. Using the One Health concept and adverse outcome pathways as organizing frameworks, we connect evidence across levels of biological organization while distinguishing well-established ecological effects in aquatic organisms from the more limited evidence concerning human health at environmentally relevant exposure levels. We also highlight the need to expand environmental monitoring beyond targeted quantification toward comprehensive steroidal profiling. Finally, we compare conventional and advanced physical, chemical, and biological treatments with hybrid systems and nature-based solutions, emphasizing fit-for-purpose, multi-barrier strategies that integrate source control with effective wastewater treatment. Through this framework, the review aims to support mixture-aware risk assessment and context-specific, sustainable mitigation consistent with circular economy principles.

SOURCES AND ENVIRONMENTAL FATE

The occurrence and environmental fate of steroidal sex hormones are shaped by interconnected source, transport, partitioning, and transformation processes. Anthropogenic inputs from municipal wastewater and livestock production are supplemented by diffuse pathways, including surface runoff, subsurface leaching, and dry and wet deposition of particle-bound steroids. Following release, their mobility and persistence are governed by phase partitioning among water, suspended particles, soils, sediments, and groundwater; microbial deconjugation and reversible interconversion; photochemical and biological transformation; and uptake and transfer through aquatic food webs. Understanding this source-to-sink continuum is essential for characterizing exposure, evaluating ecological risks, and identifying effective intervention points. Figure 1 summarizes the major emission sources, transport pathways, environmental compartments, and transformation processes.

Anthropogenic emissions

Human and livestock excretion represents a major primary source of steroidal hormones entering aquatic environments. Early inventories of livestock production systems indicated that annual hormone releases could reach tens to hundreds of tonnes, depending on the hormone class, animal population, production system, and geographical scope considered.²² Combined oral contraceptives have been widely used for decades and typically contain an estrogen and a progestin.²³ Following administration and metabolism, EE2, a widely used synthetic estrogen in these formulations, is excreted in free and conjugated forms and subsequently enters domestic wastewater.²⁴ Population-based mass-balance estimates indicate that EE2 accounts for approximately 1% of total human estrogen excretion in the Netherlands and probably less than 1% in the United States. Despite this small mass fraction, its environmental relevance should not be evaluated solely on a mass basis because mass contribution does not directly reflect estrogenic potency. Although naturally excreted human estrogens and livestock-derived hormones may dominate total estrogen loads by mass, pharmaceutical inputs such as EE2 can remain ecologically relevant because of their high biological potency.

The chemical form in which hormones are excreted further influences their mobility, transformation, and biological activity. Fecal excreta often contain higher proportions of free, biologically active hormones, whereas urinary excretion is generally dominated by water-soluble glucuronide and sulfate conjugates.^{25,26} These conjugates typically exhibit lower direct biological activity than their corresponding free hormones but can undergo deconjugation during waste treatment or after release into the environment, thereby acting as mobile reservoirs of active parent compounds. In a survey of fresh livestock excreta, conjugated estrogens accounted for more than 98% of the total estrogen excreted in swine urine.²⁷ Monitoring at two intensive swine

farms further detected 31 target androgens, progestogens, and glucocorticoids in wastewater, with concentrations reaching 11,200 ng/L for 5 α -dihydroprogesterone, illustrating the substantial and compound-specific steroid inputs associated with intensive livestock production.²⁸

Emission pathways can be broadly classified as point and non-point sources, although individual activities may contribute through both pathways. Municipal WWTPs are major point-source conduits because they receive human excreta and pharmaceutical residues and may discharge residual hormones and transformation products into receiving waters. A compilation of data from multiple countries reported steroidal estrogens at ng/L concentrations in treated effluents and demonstrated substantial geographical variability in their occurrence.²⁹ Where sewerage and treatment infrastructure are inadequate, the direct discharge of untreated sewage can become the dominant source. A nationwide source-apportionment study in China estimated that untreated sewage contributed more than 50% of the steroid-hormone burden in the investigated streams.³⁰ Treatment processes can also alter hormone speciation. During anaerobic digestion, E1 was observed to undergo reduction to the more potent E2, followed by subsequent E2 removal, illustrating the need to evaluate transformation pathways and residual estrogenic activity rather than relying only on individual parent-compound concentrations.³¹

Intensive animal feeding operations can generate both localized discharges from manure-storage and wastewater systems and diffuse emissions following land application. Whole primary swine-lagoon samples contained total free estrogen concentrations ranging from approximately 1,000 to 21,000 ng/L, demonstrating the high hormone loads that may occur in untreated animal waste.³² Subsequent application of manure or slurry to agricultural land can facilitate transport through surface runoff and groundwater leaching. In livestock-dominated catchments, the hydrological contribution and connectivity of individual agricultural areas to surface waters may be more important than local livestock density alone in determining estrogen concentrations in receiving waters.³³ The widespread detection of steroidal estrogens and estrogenic activity in dairy-farm watersheds with different effluent-management practices further indicates that management configuration alone may not eliminate diffuse releases.³⁴

Beyond aqueous runoff and groundwater leaching, atmospheric particulate deposition represents a plausible but still under-characterized non-point pathway for steroidal hormones and other emerging organic contaminants. Such contaminants can move among soil, atmospheric, and aquatic compartments through interacting wind-driven and hydrological processes.³⁵ Direct evidence of particle association was obtained from five commercial beef cattle feedyards, where airborne particulate matter contained detectable steroid growth promoters. The natural estrogens 17 α -estradiol and estrone were detected in more than 94% of samples at median concentrations of 20.6 and 10.8 ng/g, respectively, whereas melengestrol acetate and 17 α -trenbolone were detected in 31% and 39% of samples, respectively.³⁶ These findings demonstrate the atmospheric mobility of particle-bound steroids, although they do not alone establish their deposition into aquatic environments.

Complementary evidence was obtained from 35 hydrologically discrete playa wetlands located within 15 km of beef cattle feedyards. At least one veterinary pharmaceutical was detected in every wetland, and most analytes occurred at concentrations below 15 ng/g in sediments and below 70 ng/L in water. Given the limited hydrological connectivity of these wetlands, the observed distribution was consistent with aerial deposition of pharmaceutical-laden particulate matter as an additional input pathway in feedlot-intensive and semi-arid landscapes. Nevertheless, atmospheric deposition should presently be regarded as a plausible contributing route rather than a quantitatively established dominant source.³⁷

Transport dynamics

Once released into aquatic systems, steroidal sex hormones partition dynamically among the dissolved phase, colloids, suspended particles, soils, sediments, and biota. Their distribution is influenced not only by hydrophobicity but also by molecular structure, ionization state, organic matter, mineral composition, salinity, and particle properties. Androgens generally exhibit moderate hydrophobicity, with reported log K_{ow} values ranging from 2.75 to

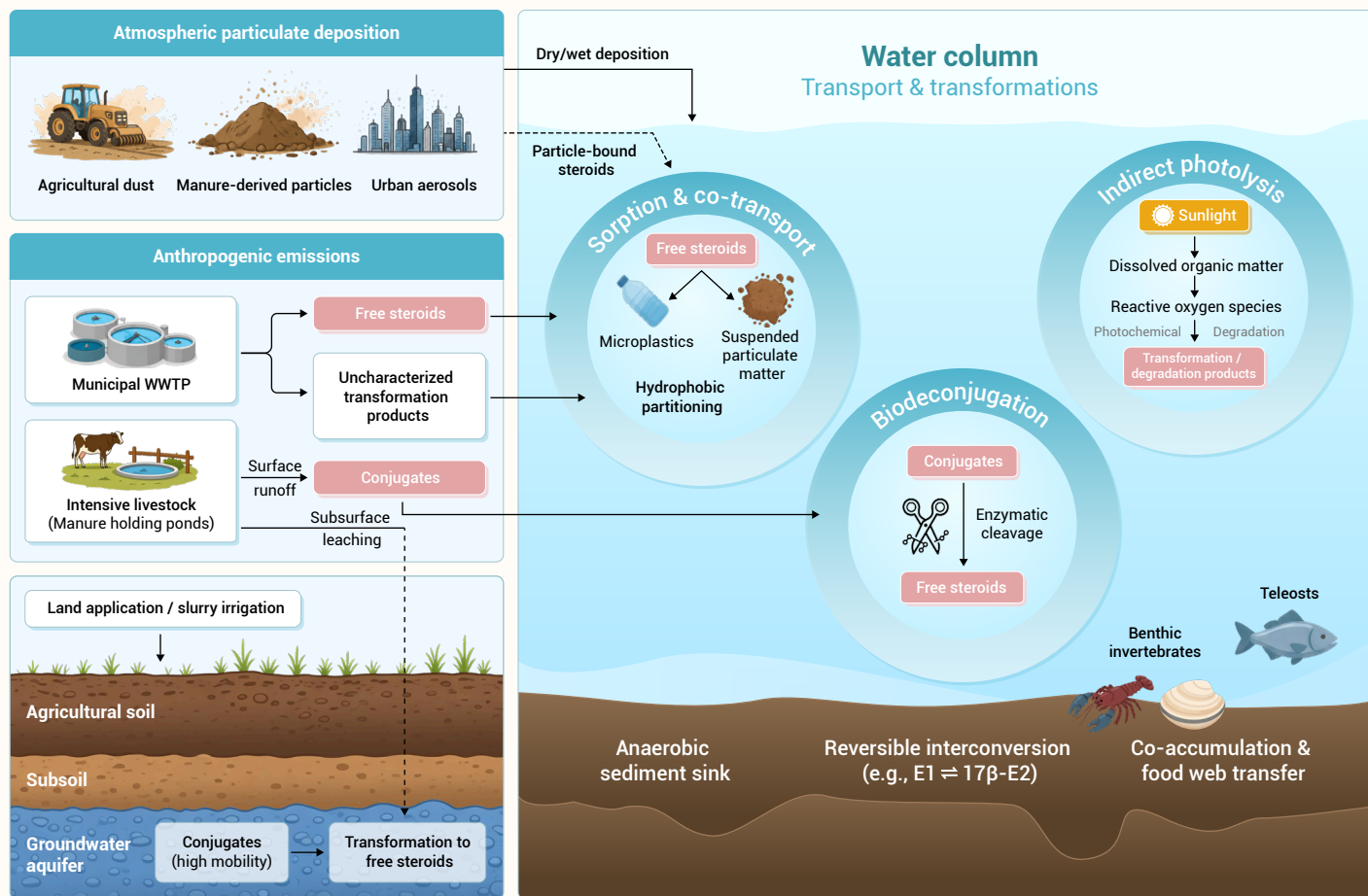


Figure 1. Conceptual framework of the sources, transport pathways, biogeochemical transformations, and environmental fate of steroidal hormones in aquatic systems.

4.40, although substantial differences exist among individual compounds.³⁸ Free steroid hormones can sorb to suspended particulate matter, soil organic matter, and benthic sediments, thereby reducing their dissolved-phase mobility while increasing their retention and potential exposure to sediment-associated organisms. In estuarine systems, organic carbon is an important determinant of partitioning among water, suspended particles, and sediments. However, transfer to solid phases does not necessarily represent permanent sequestration or a uniform increase in ecological risk.³⁹

Inorganic mineral surfaces, including iron oxides, can provide additional sorption sites, while changes in ionic strength and salinity may alter steroid partitioning through particle aggregation and flocculation.⁴⁰ The influence of particle size is not uniform. Experiments using natural sediments showed that steroid sorption capacity did not consistently increase with decreasing particle size, indicating that mineralogy, organic-matter distribution, and interactions among particle fractions must be considered together.⁴¹ In the Pearl River Estuary, a multimedia survey found that steroid metabolites predominated in the water phase, whereas parent steroids were more abundant in suspended particulate matter and sediments. This site-specific distribution suggests that relatively polar metabolites may have greater aqueous mobility, while parent compounds are more readily retained in particulate and sedimentary compartments.⁴²

Particulate- and sediment-associated steroids can enter aquatic food webs through benthic organisms, but their subsequent trophic transfer is strongly compound-, species-, and ecosystem-specific. Benthic invertebrates can take up particle-associated hormones and thereby connect sedimentary contamination with consumers at higher trophic levels.⁴³ Field evidence from an estuarine food web further showed that metabolites constituted substantial proportions of the steroid burden in aquatic organisms and that different synthetic steroids displayed contrasting trophic behaviors. Within the investigated food web, 17 α -methyltestosterone exhibited biomag-

nification, whereas 17 β -boldenone underwent trophic dilution.⁴⁴ Studies in the benthic fish *Pleuronectes yokohamae* also indicate that intestinal glucuronidation of 17 β -estradiol can promote metabolic transformation and excretion, thereby helping to limit biomagnification.⁴⁵ Food-web transport should therefore be evaluated using compound-specific profiles of parent compounds and metabolites rather than assumed to increase monotonically with trophic level.

In agricultural landscapes, manure or slurry application introduces free and conjugated estrogens into soils, where sorption, degradation, transformation, and vertical transport occur simultaneously.⁴⁶ Free estrogens generally sorb more strongly to surface soils, whereas their more polar conjugates can exhibit greater leaching mobility. In soil-column experiments, estrone-3-sulfate migrated more readily than E1 and underwent hydrolysis and abiotic transformation to products including estradiol and estriol.⁴⁷ These findings identify conjugated steroids as potential mobile precursors of free estrogens, although their transport into groundwater depends on soil properties, microbial activity, hydrological conditions, and wastewater composition. Batch and column experiments further showed that elevated total organic carbon and the presence of nonylphenol polyethoxylate surfactants were associated with reduced soil partitioning and earlier estrogen breakthrough.⁴⁸

Steroid transport also involves a continuum of suspended particles, colloids, and emerging anthropogenic carriers. In a river affected by feedlot wastewater, 12.7%–14.2% of the measured estrogens occurred in the colloidal phase, indicating that colloid-associated partitioning can influence their mobility, environmental persistence, and biological availability.⁴⁹ Microplastics can also adsorb free and conjugated estrogens under laboratory conditions and may therefore act as additional carriers. In specific polymer systems, environmental aging and decreasing particle size can enhance adsorption by modifying surface chemistry and increasing specific surface area.^{50,51} However, the quantitative importance of microplastic-mediated

transport relative to natural suspended particles in field environments remains unresolved. Overall, the mobility of steroidal hormones reflects a dynamic balance among sorption, desorption, transformation, and advective transport across dissolved, colloidal, particulate, sedimentary, and biotic compartments.

Transformation and persistence

The transformation and persistence of steroidal hormones result from interactions among microbial metabolism, abiotic reactions, and environmental conditions. Biotransformation is often a major attenuation mechanism, but its rate and extent vary with molecular structure, temperature, microbial community composition, electron-acceptor availability, and redox state. Many natural steroids are transformed more rapidly in oxygenated systems, whereas transformation is often slower under anoxic conditions. Anoxic environments, however, are not metabolically inactive. Denitrifying bacteria can degrade androgens through the oxygen-independent 2,3-seco pathway. In anoxic sewage, *Thauera* spp. were implicated in androgen degradation, and genes encoding the bifunctional enzyme 1-testosterone hydratase/dehydrogenase, together with characteristic pathway metabolites, were used as functional indicators of this process.⁵²

The synthetic estrogen EE2 generally shows greater resistance to microbial transformation than natural estrogens. In long-term anaerobic microcosms established with lake sediment or anaerobic digester sludge, no measurable loss of EE2 was observed at the tested initial concentration of 5 mg/L, even after 35–38 months.⁵³ This result demonstrates the potential for prolonged EE2 persistence under specific anaerobic conditions but should not be interpreted as a universal environmental half-life, particularly at trace concentrations. Aerobic transformation is generally more rapid, although reported rates vary substantially among microbial communities, treatment systems, and environmental matrices.

Natural estrogens can undergo reversible and redox-dependent interconversion. Under nitrate- and sulfate-reducing conditions, both E2 isomers were converted predominantly to E1, whereas E1 could be reduced back to E2, with preferential formation of the more potent 17 β -E2 in the investigated sediments.⁵⁴ Moreover, 17 α -E2 should not be considered solely an anaerobic transformation product. It was detected in all six human urine samples examined in one exploratory study and accounted for an average of 7.0% of the twelve measured natural estrogens. Although 17 α -E2 has relatively weak estrogenic potency, it can be biotransformed into 17 β -E2 under aerobic conditions.⁵⁵ Such reversible reactions can sustain temporal variations in estrogenic activity and demonstrate that the disappearance of an initial parent compound does not necessarily indicate complete detoxification.^{54,56}

A related transformation pathway has recently been demonstrated for synthetic progestogens. Across 18 phylogenetically diverse freshwater and marine phytoplankton species, norethindrone acetate was enzymatically deacetylated to the more potent product norethindrone. Chemoproteomic and functional analyses identified adenylosuccinate lyase as the enzyme responsible for this conversion, while metagenomic and metatranscriptomic analyses indicated that genes encoding this lyase are broadly distributed among aquatic eukaryotes.⁵⁷ These findings demonstrate that biotransformation can increase rather than reduce hormonal potency. However, the rates and ecological consequences of this pathway under field conditions remain to be quantified.

Deconjugation provides another pathway through which relatively polar steroid conjugates can generate free hormones. Microbial enzymes such as β -glucuronidase and arylsulfatase cleave glucuronide and sulfate moieties, respectively.^{58,59} Because conjugated steroids are generally more water-soluble and often sorb less strongly than their corresponding free forms, they may migrate through soils before undergoing hydrolysis. Nevertheless, their persistence and mobility are strongly matrix-dependent. In batch experiments using agricultural soils, dissolved E2-3-glucuronide persisted for 1–14 days depending on soil organic-matter content and was hydrolyzed to E2, which could subsequently be oxidized to E1.⁶⁰ Field monitoring beneath land receiving swine manure slurry found that glucuronide and sulfate conjugates of E2 and E1 were the only target estrogens detected in tile drains, although the overall detection frequency was only 5%. Both free and conjugated estrogens were detected in groundwater wells, with an overall detection frequency

of 11%.⁶¹ These findings support the role of conjugates as mobile precursors of free estrogens while also indicating that subsurface transport is episodic and site-specific rather than a universal bypass of root-zone attenuation.

In sunlit surface waters, direct and indirect photolysis can provide additional attenuation pathways. Dissolved organic matter (DOM) can promote indirect photolysis by generating reactive intermediates such as hydroxyl radicals and singlet oxygen, but it can also inhibit degradation through light screening and radical scavenging. Its net effect therefore depends on its concentration, composition, and optical properties, as well as the structure of the target hormone.^{62,63} Nitrate and ferric species may enhance photochemical transformation under some conditions, whereas bicarbonate and elevated turbidity can suppress it through radical scavenging and photon attenuation.⁶⁴ Photolytic rates measured in simplified solutions should therefore not be directly extrapolated to natural waters without accounting for water depth, sunlight intensity, suspended particles, and dissolved constituents.

Molecular structure also contributes to differences in persistence among steroidal hormones. Natural hormones are often more susceptible to microbial transformation, whereas the ethinyl substituent at the C17 position of EE2 contributes to its resistance to enzymatic attack. Nevertheless, persistence cannot be attributed to molecular architecture alone. Redox conditions, sorption and desorption, microbial adaptation, hydraulic residence time, and environmental matrix composition all influence transformation rates. Continuous inputs, incomplete treatment removal, and reversible formation of biologically active products can maintain steroidal hormones in aquatic systems even when individual molecules are degradable. They may therefore behave as pseudo-persistent contaminants in systems subject to sustained anthropogenic discharge.

ECOLOGICAL AND HEALTH RISKS

Steroidal sex hormones are widely detected in aquatic matrices, and some can elicit endocrine-mediated effects in sensitive organisms at environmentally relevant trace concentrations. Assessing these risks requires moving beyond isolated toxicological endpoints and single-compound tests to consider mixture composition, exposure pathways, and biological responses across multiple levels of organization. Figure 2 presents a conceptual framework linking molecular and cellular responses to organismal, population, and ecosystem-level effects, while distinguishing well-established ecological evidence from potential human exposure pathways and health concerns.

Aquatic ecotoxicity and cascades

The ecotoxicological risk posed by steroidal sex hormones arises from their capacity to perturb steroid-dependent developmental and reproductive pathways, with estrogen-mediated effects in teleost fish providing the strongest evidence base. In teleosts, activation of estrogen receptors constitutes a molecular initiating event that can alter estrogen-responsive transcription. Induction of vitellogenin (VTG), an egg-yolk precursor protein, in males and juveniles is among the most sensitive and widely used biomarkers of estrogenic exposure. However, VTG induction indicates exposure and biological activity and does not, by itself, demonstrate an adverse outcome. In a fathead minnow gonadal recrudescence assay, EE2 significantly elevated significantly plasma VTG significantly elevated plasma VT at 1 ng/L, whereas pronounced reductions in male secondary sex characteristics occurred at characteristics occurred higher exposure concentrations of 10–100 ng/L.⁶⁵ Long-term exposure can nevertheless produce adverse effects at similarly low concentrations: a two-year exposure of roach to a measured EE2 concentration of 1.3 ng/L feminized genetic males, shifted the genetic sex ratio toward females, and accelerated ovarian development.⁶⁶ Natural estrogens are also biologically active; E1 and E2 altered VTG concentrations and reproductive endpoints in mature fathead minnows, although their relative potency and effects depended on the compound, concentration, sex, and endpoint examined.⁶⁷ Molluscan VTG has also been proposed as a biomarker of estrogen exposure, but its endocrine regulation is less well resolved than that in teleosts and requires further species-specific validation.⁶⁸

Within an adverse outcome pathway framework, estrogen-receptor activation should be distinguished from downstream key events such as altered expression of *vtg*, *cyp19a1a*, and other genes associated with the hypothalamic

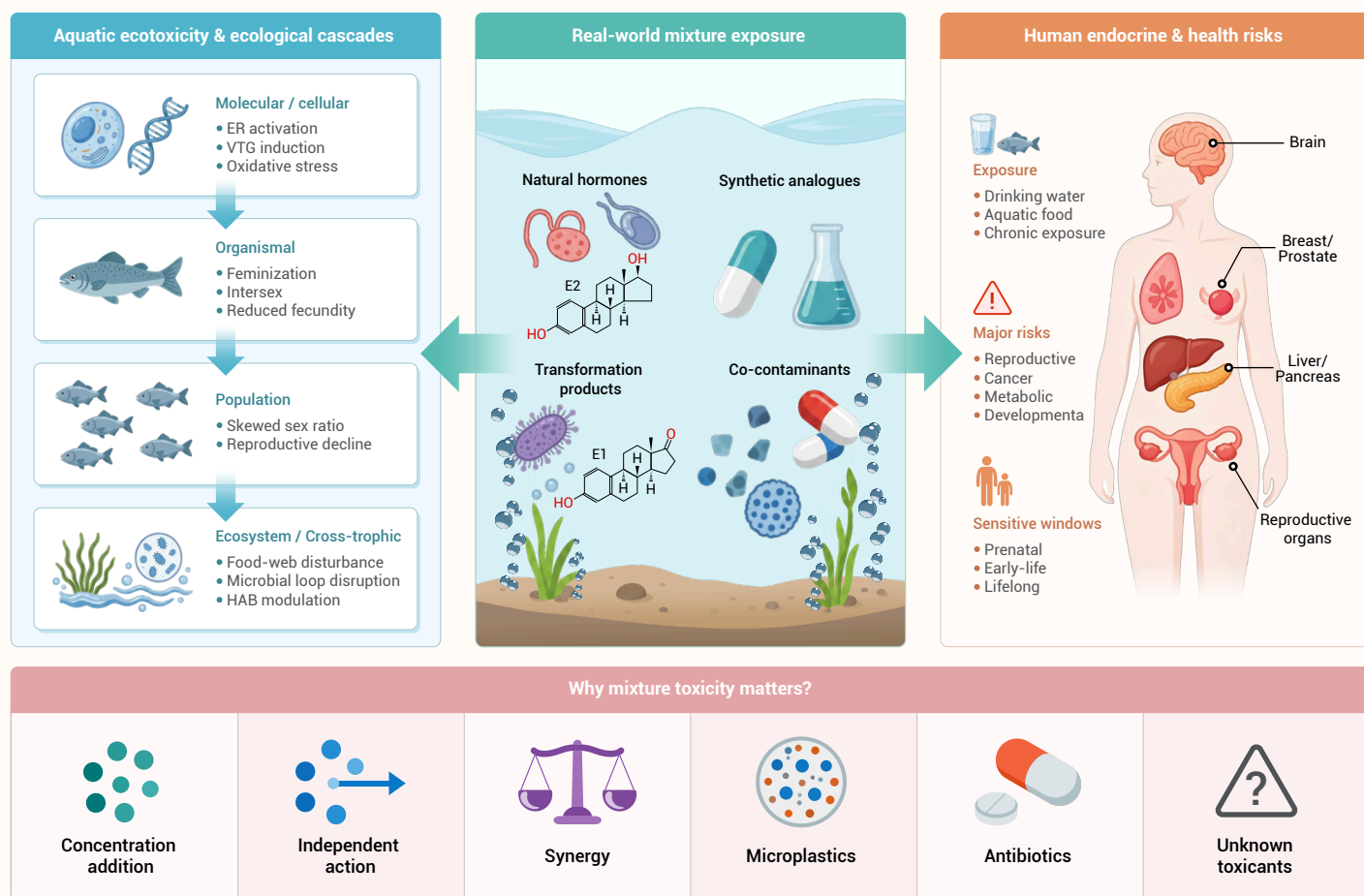


Figure 2. Conceptual framework for the ecological effects, mixture-toxicity determinants, and potential human health concerns associated with steroidal sex hormones in aquatic systems Molecular structures were drawn with reference to the Chemical Book database (<https://www.chemicalbook.com/ProductIndex.aspx>).

–pituitary–gonadal–liver axis, VTG protein induction, disruption of steroidogenesis, and altered gonadal differentiation. These key events may progress to intersex inter phenotypes, impaired gametogenesis, reduced fecundity or fertilization, and skewed sex ratios, but this progression is not inevitable and depends on exposure concentration, timing, duration, and species sensitivity. Table 1 summarizes the resulting spectrum of molecular, physiological, developmental, and reproductive responses across teleost models rather than implying a universal linear cascade. For example, early-life exposure to E1, E2, E3, or EE2 at nanogram-per-liter concentrations induced testis-ova or altered phenotypic sex in Japanese medaka.⁶⁹ Many fish species are particularly vulnerable during sexual differentiation because their comparatively labile sex-determination systems allow exogenous steroids to modify or override endogenous developmental cues.⁷⁰ Nevertheless, changes in gene expression or VTG concentrations should not automatically be interpreted as irreversible organism-level damage without supporting histological, reproductive, or population-level evidence. Moreover, because most available studies emphasize estrogenic responses, the effects of androgenic and progestogenic hormones should be evaluated using pathway-appropriate biomarkers rather than inferred directly from estrogen-responsive endpoints.

At the population level, ecological risk becomes particularly important when chronic exposure overlaps sensitive developmental or reproductive windows and impairs reproduction across successive cohorts. A study of a freeze–thaw basin found that estrogen occurrence and estimated ecological risk varied substantially among seasons, with runoff during thawing increasing contaminant inputs during a period that overlapped with aquatic-organism breeding. However, because the monitored compounds included both steroidal hormones and non-steroidal estrogenic chemicals, these seasonal effects cannot be attributed exclusively to steroidal sex hormones.⁷¹ Field evidence also indicates that improvements in wastewater treatment have reduced, but not eliminated, endocrine disruption. In a contemporary survey

of ten English river sites, intersex gonads were still detected at 60% of the sites, while average male plasma VTG concentrations exceeded natural baseline levels at 90% of the sites.⁷² For effect-based monitoring, Jarošová et al. proposed bioassay-specific safe estrogenic equivalent values ranging from 0.1 to 0.4 ng E2-EQ/L for municipal wastewater.⁷³ These values can serve as screening or trigger benchmarks for identifying samples that require further investigation, but they should not be treated as universal regulatory thresholds because their interpretation depends on the bioassay, wastewater dilution, mixture composition, and underlying compound-specific predicted no-effect concentrations.

Effects beyond fish reproduction are emerging but should not be overstated. In slow sand filters, exposure to natural estrogens altered coliform-grazing protozoan communities and impaired microbial pathogen-removal functions, while bioaugmentation with estrogen-degrading bacteria mitigated these effects.⁷⁴ This finding demonstrates that estrogens can disrupt microbial ecological functions in an engineered treatment ecosystem, but it cannot be directly generalized to natural aquatic food webs. Environmental estrogens may also affect microalgal growth, photosynthesis, oxidative status, and toxin production, with responses varying among compounds, concentrations, exposure durations, and algal species.⁷⁵ Although these interactions could modify primary-producer dynamics, current evidence does not establish steroidal hormones as general drivers of harmful algal blooms or food-web collapse. Broader ecological cascades are therefore biologically plausible but remain less firmly established than estrogen-mediated reproductive effects in teleost fish and require further community-level and field-based investigation.

Human endocrine disruption

While aquatic ecotoxicity is well documented, the human health implications of chronic, low-dose exposure to environmental steroid hormones

Table 1. Endocrine-related biomarker responses and observed effects of steroidal sex hormones in teleost models.

Model organism	Test compound	Exposure duration	Route of exposure	Exposure concentration /dose	Key biomarker responses	Observed effects	Reference
Gray mullet (<i>Mugil cephalus</i>)	EE2	28 days	Oral (dietary)	4–4000 ng/g body weight/day	Hepatic <i>vgtAb</i> : upregulated; Gonadal <i>zpb</i> : upregulated; Gonadal <i>zpc5</i> : downregulated	Testis-ova (moderate): induced at 40 and 400 ng/g/day. Fibrosis and germ cell loss (severe): induced at 4000 ng/g/day.	Ref. ⁷⁶
Zebrafish (<i>Danio rerio</i>)	EE2	0–64 days (post-fertilization), 95 days (recovery period)	Waterborne	3 and 10 ng/L	Hepatic <i>vgt</i> : altered; Estrogen receptor transcripts: altered; Thymocyte marker gene transcripts: reduced	Reproductive: altered gonadal differentiation. Immunological: retarded thymus development; thymic alterations were reversible in females but persisted in males after the recovery period.	Ref. ⁷⁷
Western mosquitofish (<i>Gambusia affinis</i>)	E1	119 days (full life-cycle)	Waterborne	25.4–4300 ng/L	Altered transcripts of genes related to the HPGL axis and sex differentiation in both adults and embryos.	Population: 100% female sex ratio at 4300 ng/L. Morphology: Feminization of male anal fins and skeletons. Histology: Inhibited spermatogenesis in males; increased oocyte degeneration in females.	Ref. ⁷⁸
Marine medaka (<i>Oryzias melastigma</i>)	EE2	Parental (45 days) and subsequent embryonic exposure	Waterborne	85 ng/L	Transcriptome Analysis: Revealed impairments controlled by the estrogen receptor, affecting pathways related to neuronal, synaptic, and eye development.	Growth retardation: reduced embryo heart rate and delayed eye pigmentation. Hatching and behavior: decreased hatching rate and impaired larval locomotion.	Ref. ⁷⁹
Largemouth bass (<i>Micropterus salmoides</i>)	EE2	4 weeks followed by a 10-day bacterial infection challenge	Waterborne	0.87 and 9.08 ng/L	Metabolic: reduced hepatic glycogen levels. Transcriptomic: altered gene expression related to immunometabolic processes in liver and spleen.	Post-challenge outcome: higher survival following bacterial infection at 9.08 ng/L, accompanied by altered hepatic and splenic immunometabolic responses.	Ref. ⁸⁰
Fathead minnow (<i>Pimephales promelas</i>)	EE2	32 days (embryo-larval stage)	Waterborne	4, 20, and 100 ng/L	Molecular/omics: increased vitellogenin abundance and disruption of visual-development pathways.	Histopathology: delayed or suppressed gonadal development and accumulation of proteinaceous material in the liver and kidney. Metabolic: evidence of energy depletion. Apical: reduced body size at 100 ng/L.	Ref. ⁸¹
Adult female western mosquitofish (<i>Gambusia affinis</i>)	E2, testosterone, and their binary mixtures	91 days	Waterborne	E2: 50 ng/L. testosterone: 50 and 200 ng/L	Gene Expression: altered transcripts of HPGL axis and steroidogenesis genes (e.g., <i>Cyp19a1a</i> , <i>Star</i>). Hormone Levels: ovarian E ₂ and testosterone concentrations decreased. Protein Levels: vitellogenin protein induced in ovaries and livers.	No significant histopathological changes in ovaries or livers; no masculinization of female anal fins observed.	Ref. ⁸²
European sea bass larvae (<i>Dicentrarchus labrax</i>)	EE2	8 days, followed by a 20-day recovery period.	Waterborne	150 ng/L and 5 µg/L	Upregulation of key neuroendocrine genes, including <i>cyp19a1b</i> (brain aromatase), <i>gnrh2</i> , and <i>kiss1</i> .	Growth: Reduced standard length at the high dose. Behavior: Increased locomotor activity and anxiety-like behaviors	Ref. ⁸³

Table 1. (Continued)

Model organism	Test compound	Exposure duration	Route of exposure	Exposure concentration /dose	Key biomarker responses	Observed effects	Reference
Zebrafish (<i>Danio rerio</i>) embryos/larvae	Estetrol (E4) vs. EE2, also with drospirenone (DRSP)	96–120 hours	Waterborne	FET assay: E4 up to 100 mg/L; EE2 at 1–2 mg/L. Behavioral/pr oteomic assays: EE2 at 0.3 and 30 ng/L and E4 up to 710 ng/L, alone or combined with DRSP.	E4: Minimal effects. EE2: Downregulated RNA/protein synthesis proteins at low conc.	E4: no acute effects up to 100 mg/L and no behavioral alterations at the tested concentrations. EE2: mortality and morphological abnormalities at 1–2 mg/L, hypoactivity at 30 ng/L, and altered protein abundance at 0.3 ng/L. DRSP reduced several EE2-induced responses, suggesting an antagonistic interaction.	Ref. ⁸⁴
Zebrafish (<i>Danio rerio</i>)	EE2	6 weeks	Oral (dietary)	10 µg/kg and 1000 µg/kg	Gene expression: altered reproductive gene expression (<i>vtg1</i> , <i>cyp19a1a</i> , etc.). Protein levels: increased vitellogenin. Biochemical: induced oxidative stress.	Histopathology: lesions in testes, including suppressed spermatogenesis and reduced sperm count at 1000 µg/kg. Mortality: significantly increased at 1000 µg/kg. Morphology: reduced body weight and increased body length.	Ref. ⁸⁵
Roach (<i>Rutilus rutilus</i>)	EE2	2 years	Waterborne	1.3 ng/L	Vitellogenin: plasma VTG concentrations were significantly elevated in genetic males.	Sex ratio: the proportion of genetic males was significantly reduced. Genetic males: 30% developed feminized gonads, and spermatogenesis was inhibited. Females: sexual maturation was accelerated. Population comparison: no significant difference in sensitivity was detected between populations with contrasting exposure histories.	Ref. ⁶⁶
Zebrafish (<i>Danio rerio</i>) larvae	E4 vs. EE2	Up to 30 days post-fertilization	Waterborne	EE2: up to 1,000 ng/L. E4: up to 320,000 ng/L	E4: minimal effects; did not affect metamorphosis-related pathways. EE2: Altered genes in pathways for macronutrient metabolism, estrogen response, and drug metabolism.	E4: No notable changes in metamorphic traits. EE2: Significant developmental delays in metamorphosis	Ref. ⁸⁶
Zebrafish (<i>Danio rerio</i>)	E1	49 days	Waterborne	20, 200, and 2000 ng/L	Hormones: Increased Testicular E2, decreased 11-KT. Signaling: Increased iNOS expression and nitrite production; increased IGF1R expression.	Histological: reduced gonadosomatic index; suppressed spermatogenesis; testicular inflammation. Functional: reduced sperm production and quality.	Ref. ⁸⁷

remain less certain. Potential exposure may occur through contaminated drinking water and the consumption of aquatic organisms in which these compounds have accumulated, making this an emerging public health concern within the One Health framework. Systematic evaluations of hormonal residues in edible fish confirm that steroid hormones can occur in multiple tissues and biological matrices, indicating the potential for dietary exposure.⁸⁸ Human consumers may therefore be exposed not only to individual parent hormones but also to co-accumulated mixtures of steroidal EDCs and their bioactive transformation products. Field investigations show that tissue-specific metabolism and accumulation in wild fish can govern food-web transfer and facilitate dietary exposure to complex mixtures of steroid

hormones and their metabolites.⁸⁹ Accordingly, cumulative dietary risk assessment is needed because exposure to multiple compounds through fish consumption may increase estimated health risks and, under some exposure scenarios, approach or exceed acceptable daily intake benchmarks.^{90,91} Because sex-steroid signaling is central to reproductive physiology and hormone-dependent cancers, chronic exposure to exogenous steroid hormones raises concerns about reproductive and neoplastic outcomes. However, direct epidemiological evidence linking environmentally relevant aquatic exposures to these diseases remains limited.^{92,93}

Evidence that endogenous sex-hormone dysregulation contributes to cardiometabolic disease raises the possibility that exogenous steroidal

signals may perturb the same pathways.^{94,95} However, direct evidence linking environmentally relevant exposure to steroidal EDCs with obesity, type 2 diabetes mellitus, and PCOS remains limited. PCOS is a common endocrinopathy affecting up to 10% of reproductive-age women and is characterized by follicular arrest, insulin resistance, and disturbances in the enteroinsular axis and pancreatic β -cell function.^{96,97} These features may increase susceptibility to exogenous hormonal perturbation, although this relationship requires further investigation. More broadly, chronic disruption of endocrine signaling may alter disease susceptibility across the human lifespan.¹¹ For instance, environmental estrogens upregulate inflammatory cytokines—particularly tumor necrosis factor—triggering oocyte apoptosis, destabilizing genomic integrity, and facilitating premature ovarian insufficiency.^{98,99} Chronic disruption of hormonal feedback has also been associated with gynecological disorders such as endometriosis.¹⁰⁰

These potential vulnerabilities may be particularly important during early life. Embryonic and fetal development represents a sensitive window during which maternally transferred endocrine-active contaminants may alter steroidogenesis and organogenesis, potentially increasing susceptibility to adult-onset chronic diseases and impairing fetal lung maturation.^{101,102} The established roles of sex steroids in hippocampal neuroplasticity and mood regulation provide a mechanistic basis for concern that exogenous perturbation of steroid signaling could affect cognitive and affective outcomes.^{103,104} Sex steroids also regulate cardiovascular signaling, immune responses, and endogenous gasotransmitter pathways involving nitric oxide and hydrogen sulfide.^{105–107} Together, these physiological roles identify potential targets of exogenous hormonal perturbation, although they do not by themselves establish adverse effects at environmentally relevant exposure levels. Environmental estrogens can also activate rapid non-genomic signaling through the G protein-coupled estrogen receptor, providing a plausible mechanism for biological responses to low-level exposure.^{108,109}

Real-world mixture toxicity

The traditional single-substance toxicological paradigm cannot fully capture the risks posed by real-world aquatic mixtures, supporting a shift toward mixture-based toxicological assessments.¹² Organisms are chronically exposed not to isolated compounds, but to complex cocktails comprising natural hormones, highly potent synthetic analogues, uncharacterized transformation products, and other co-occurring EDCs. Field monitoring further demonstrates the simultaneous occurrence of multiple steroid classes. In some wastewater-impacted urban rivers, the summed concentrations of androgens and progestogens can exceed those of estrogens, although this pattern is source- and site-dependent.^{110,111} Despite their often lower concentrations, EE2, E2, and E1 frequently emerge as major contributors to estrogenic activity and ecological risk because of their high relative potencies.^{112–114} For similarly acting estrogen receptor agonists, the concentration addition (CA) model predicts mixture effects by scaling individual components to equipotent concentrations and summing their contributions. The related estrogenic equivalency (EEQ) approach converts measured concentrations into E2-equivalent concentrations using compound-specific relative potency factors. Thus, EEQ is a potency-normalized metric rather than a separate mixture-toxicity model.

Within these mixtures, non-additive interactions can occur, producing deviations from predictions based on standard reference models. CA and independent action are commonly used for similarly and dissimilarly acting chemicals, respectively. However, their predictions may converge, and CA can provide a conservative approximation even for some mixtures containing components with different modes of action.¹¹⁵ For example, a seven-day assay with *Lemna minor* found that EE2 and methotrexate produced synergistic effects relative to CA predictions at concentrations in the low mg/L range.¹¹⁶ Because these experimental concentrations exceed those typically detected in aquatic environments, the findings demonstrate the potential for chemical interactions rather than directly establishing field-level risk. Even within steroid classes, complex interactions can arise. Full life-cycle exposure to binary mixtures of E2 and testosterone produced feminizing responses in male western mosquitofish, masculinizing responses in females, and interactive, sex-specific transcriptional changes along the hypothalamic-pituitary-gonadal axis.¹¹⁷ Emerging particulate contaminants,

particularly nanoplastics and microplastics, can further modify mixture toxicity. Co-exposure to nano-polystyrene and diethylstilbestrol, a nonsteroidal synthetic estrogen, exacerbated gonadal damage and reproductive impairment in zebrafish, while their combined inhibition of sex hormone and vitellogenin secretion was additive.¹¹⁸ Similarly, co-exposure to polyethylene microplastics and bisphenol A, another nonsteroidal EDC, produced more pronounced changes than single exposures for several steroidogenic and HPG-axis endpoints, with responses varying by sex and endpoint.¹¹⁹ Although diethylstilbestrol and bisphenol A are not steroid hormones, these findings illustrate how co-occurring endocrine-active chemicals and particulate contaminants can modify the effects of steroid-centered mixtures.

The complexity of environmental mixtures is further increased by *in situ* biotransformation, which can generate bioactive or potentially hazardous transformation products. During activated sludge treatment, some 19-nortestosterone-derived progestogens can undergo ring-A aromatization and form estrogen-like transformation products. For example, norethisterone acetate can be transformed into the potent synthetic estrogen EE2, whereas cyproterone acetate can yield 3 α -hydroxy cyproterone acetate, a transformation product reported to be genotoxic.¹²⁰ To resolve complex mixtures containing known, unknown, and transformed toxicants, researchers are increasingly applying effect-directed analysis (EDA). Recent EDA of municipal sewage sludge identified seven steroidal androgen-receptor agonists that collectively explained 52%–87% of the observed androgenic activity in three highly active sludge samples, demonstrating the utility of combining bioassay-guided fractionation with suspect and non-target screening.¹²¹ Consequently, bridging the gap between current regulatory frameworks and actual ecological threats requires the integration of predictive mixture-toxicity models with comprehensive chemical and effect-directed profiling.

ANALYTICAL METHODOLOGIES

Quantifying steroidal sex hormones in complex environmental matrices, including raw wastewater, activated sludge, sediments, and biological tissues, is analytically challenging because these compounds often occur at trace or ultra-trace concentrations and matrix effects can substantially influence analytical signals. Reliable determination therefore requires a multistage workflow integrating matrix-specific sample pretreatment, extraction and enrichment, chromatographic separation, instrumental detection, and data interpretation while balancing sensitivity, selectivity, accuracy, and throughput. Figure 3 summarizes this framework, spanning targeted quantification, non-targeted profiling, spatial imaging, and decentralized biosensing.

Extraction and enrichment

Direct instrumental analysis of environmental waters without preconcentration is often impractical because steroid hormones occur at trace or ultra-trace concentrations and coexisting matrix components can suppress or enhance analytical signals. Solid-phase extraction (SPE) therefore remains one of the most widely used approaches for concentrating target analytes and reducing matrix interference.¹²⁵ Careful sample pretreatment is critical to the reliability of the subsequent analysis. Filter selection should be validated for each analyte and study objective because filtration can reduce matrix interference but may also remove particle-bound hormones. For dissolved-phase measurements, glass-fiber filters have shown lower analyte losses than nylon filters in some estrogen-analysis workflows.^{126,127} When total concentrations are of interest, both aqueous and particulate fractions should be considered and the operationally defined fraction being measured should be clearly reported. When dealing with complex biological matrices, effective removal of lipid and protein interferences is often necessary, while excessive cleanup should be avoided to minimize analyte loss.¹²⁸ For food, tissue, and other complex solid or semi-solid matrices, the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method offers a relatively rapid and solvent-efficient sample-preparation strategy.¹²⁹ Extraction procedures should ultimately be tailored to the downstream analytical endpoint by balancing cleanup efficiency, analyte recovery, and sample throughput. Instrumental methods generally require more extensive purification, whereas screening methods such as enzyme-linked immunosorbent assays prioritize rapid and high-throughput processing.¹³⁰

Rather than replacing conventional SPE sorbents, advanced materials are

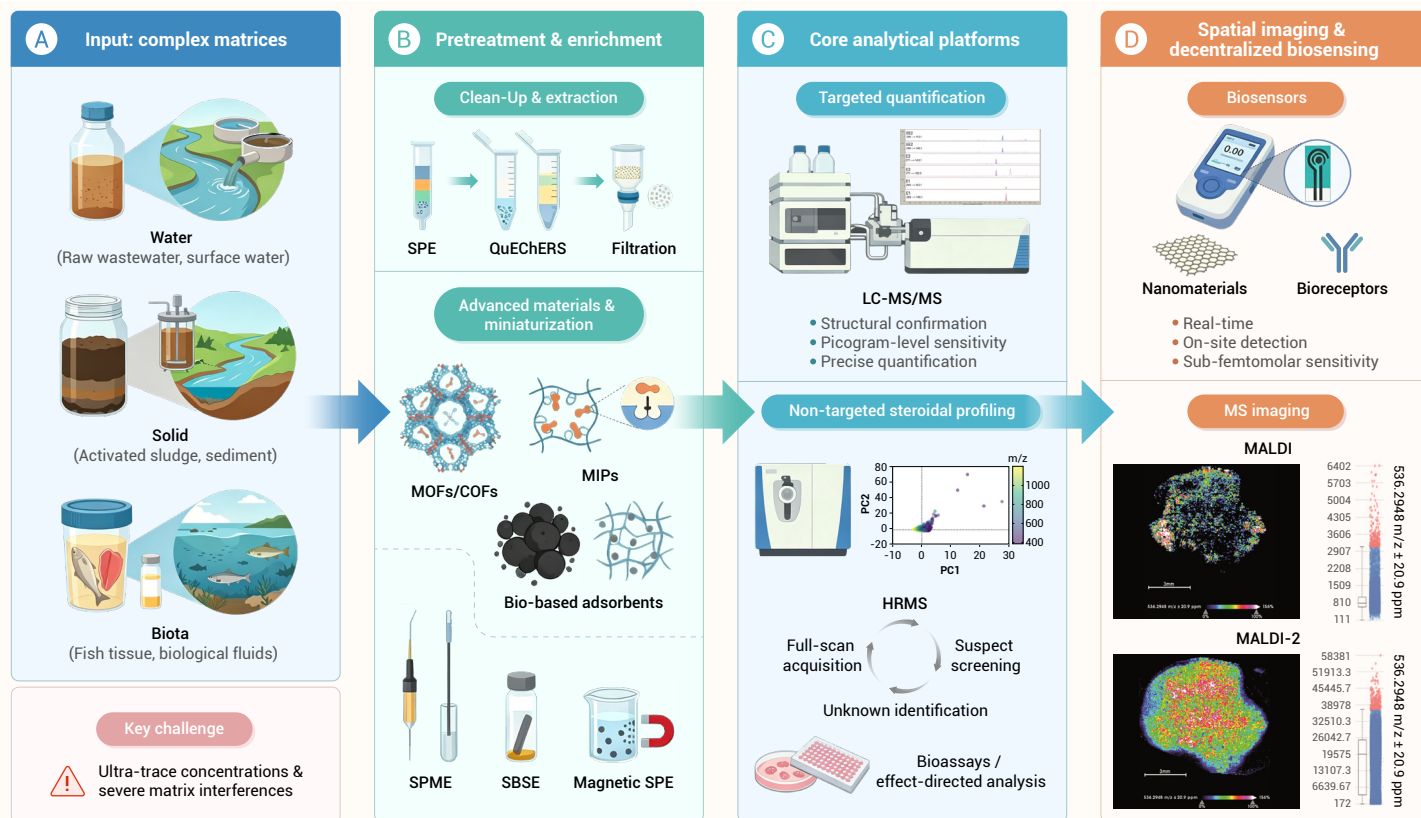


Figure 3. Integrated analytical workflow for steroid hormone analysis across environmental and biological matrices (A) Environmental and biological input matrices; (B) sample pretreatment and enrichment strategies; (C) targeted quantification and nontarget HRMS profiling; and (D) decentralized biosensing and spatial MS imaging. The LC-MS/MS MRM chromatogram, HRMS feature-analysis plot, and MALDI/MALDI-2 MSI panels were adapted from refs.¹²²⁻¹²⁴, respectively. Materials from refs.¹²²⁻¹²³ are licensed under the creative commons attribution 4.0 international license, whereas material from ref.¹²⁴ is licensed under the creative commons attribution 3.0 unported license. Modifications were made.

increasingly being developed to complement them by improving selectivity, adsorption capacity, or ease of phase separation. Magnetic metal-organic frameworks combine high surface areas, chemically tunable surfaces, and rapid magnetic separation and have been explored for pharmaceutical extraction from food, environmental, and biological matrices.¹³¹ As a steroid-relevant example, imidazoline-linked cationic covalent triazine frameworks prepared through the Debus-Radziszewski reaction can enrich environmental estrogens through the combined contributions of π - π interactions, hydrogen bonding, and electrostatic attraction.^{132,133} Poly(ionic liquid)s provide tunable polarity and multiple interaction mechanisms, expanding the range of sorbent designs available for microextraction, although their performance must be validated for individual steroid classes and sample matrices.¹³⁴ Aligning with green analytical chemistry principles, engineered bio-based sorbents are increasingly being investigated as potentially greener alternatives to conventional synthetic polymers. However, their selectivity, reusability, and batch-to-batch reproducibility should be evaluated alongside extraction recovery.^{135,136} Molecularly imprinted polymers provide template-defined recognition sites that are complementary to target molecules in shape and functional-group arrangement, thereby enhancing the selective extraction of phenolic estrogens from complex matrices.¹³⁷

Beyond material innovations, extraction formats are also evolving toward miniaturization, reduced solvent consumption, and automated operation. Sorptive microextraction techniques, including fiber-based and in-tube formats, can provide effective enrichment from small sample volumes, although their performance depends strongly on compatibility between the analytes and extraction coating.¹³⁸ Stir-bar sorptive extraction uses a larger volume of sorptive phase than conventional fiber-based SPME and can provide efficient extraction of relatively hydrophobic analytes. More polar steroids may, however, require derivatization or coatings with greater polarity.¹³⁹ A sol-gel-derived polydimethylsiloxane/ β -cyclodextrin/divinylbenzene coating has, for example, been applied to the stir-bar sorptive extraction of estrogens from animal-derived food matrices.¹⁴⁰ Concurrently, magnetic

solid-phase extraction simplifies phase separation and can reduce or eliminate the need for centrifugation.¹⁴¹ Porous monolithic capillary columns used for in-tube SPME can further facilitate automated online coupling with LC-MS/MS and effective estrogen enrichment.¹⁴² Table 2 summarizes representative extraction and analytical methods for steroid hormones and related endocrine-active compounds in complex matrices.

Ultra-trace instrumental analysis

Following extraction and enrichment, liquid chromatography coupled with tandem mass spectrometry is one of the principal analytical platforms for the sensitive and selective quantification of steroid hormones in environmental matrices, with method-dependent detection limits extending into the sub-ng/L range and, for some analytes, to tens of pg/L.¹⁵⁸ Although gas chromatography coupled with mass spectrometry remains valuable for volatile or derivatized steroids, LC-MS/MS is particularly advantageous for polar and nonvolatile analytes, including some estrogen conjugates, because it can often avoid prior derivatization.^{159,160}

Ionization conditions should be optimized for individual steroid classes. In a comparative study, Δ^4 steroids generally showed greater sensitivity with positive-mode heated electrospray ionization, whereas Δ^5 steroids and sterols generally responded better to positive-mode atmospheric pressure chemical ionization. However, exceptions occurred, and estrogens showed no consistent preference. Atmospheric pressure chemical ionization also produced artifactual 2n-Da losses, attributed mainly to the oxidation of hydroxyl groups, that may obscure precursor ions and complicate structural assignment.¹⁶¹

Beyond targeted quantification, high-resolution mass spectrometry provides accurate-mass precursor- and product-ion data that support suspect screening and non-target characterization. Accurate mass measurements, isotope patterns, retention behavior, and diagnostic fragments can support the annotation of previously uncharacterized steroid metabolites and transformation products. However, confident identification still requires

Table 2. Representative extraction and analytical methods for steroid hormones and related endocrine-active compounds in complex matrices.

Target contaminant	Sample matrix/source	Method	Results	Reference
Estrogens and xenoestrogens	Water and milk	Porous polymer functionalized magnetic composite used as an advanced adsorbent for MSPE	LODs: 0.0068–0.088 µg/L (water); LODs: 0.028–0.030 µg/L (milk); Recoveries: 80.7%–116%	Ref. ¹⁴³
Estrogens	Lake water and milk	Cyano-functionalized ionic porous organic polymer used as an adsorbent for SPE	LODs: 0.03–0.05 ng/mL (lake water); LODs: 0.30–0.70 ng/mL (milk); Recoveries: 85.0%–119.0%	Ref. ¹⁴⁴
Estrogens and xenoestrogens	Milk and yogurt	MIP fiber array for SPME using E2 as the template	LOD: 0.33 µg/L; Recoveries: 74.75%–119.41%	Ref. ¹⁴⁵
Estrogens	Serum, urine and pond water	A porous capillary monolithic column prepared via thiol-alkyne click polymerization used for in-tube SPME.	LODs: 5.3–9.6 ng/L; Recoveries: 80.3%–113%	Ref. ¹⁴²
Natural estrogens and bisphenol analogues	Source water and finished drinking water	Development of a sensitive GC-MS analytical method, coupled with SPE for pre-concentration.	LODs: 0.6–5.8 ng/L (source water); LODs: 0.3–4.6 ng/L (finished drinking water); Recoveries: 36.8%–129.4%	Ref. ¹⁴⁶
Estrogens and other endocrine disruptors	Water	An ultrasensitive plasmonic optical fiber biosensor based on a gold-coated tilted fiber bragg grating, using estrogen receptors as the recognition element.	LOD: 1.5 ng/L; Recoveries: 91%–112%	Ref. ¹⁴⁷
Glucocorticoid and progestogen metabolites	Bovine plasma, milk, and saliva	A UHPLC-HR-MS method using a simple liquid-liquid extraction for sample preparation and ESI with polarity switching for detection.	LOD: 10–200 pg/ml; Recoveries: 33%–114%	Ref. ¹⁴⁸
Natural estrogens	Poultry meat and blood	An analytical method combining ultrasonic-assisted extraction, SPE cleanup, and GC-MS for quantification.	LODs: 0.6–2.2 ng/L (blood); LODs: 0.6–1.4 ng/g (meat); Recoveries: 86%–119%	Ref. ¹⁴⁹
Estrogens and estrogen mimics	Milk and meat	A novel β-cyclodextrin-functionalized covalent organic framework-based magnetic nanocomposite used for MSPE.	LODs: 0.9–3.4 µg/kg; Recoveries: 89.0%–105.4%	Ref. ¹⁵⁰
Natural estrogens	Water	A novel Zn/Au-Ag/Ag sandwich-structured substrate for surface-enhanced raman spectroscopy detection.	LOD: 10 ng/L	Ref. ¹⁵¹
Estrogens	Water	A hybrid pre-concentration method combining MSPE using a novel multi-template MIP, followed by solidified floating organic drop microextraction	LODs: 0.29–1.11 µg/L; Recoveries: 90.5%–107.0%	Ref. ¹⁵²
E2	Milk	A highly sensitive immunochromatographic assay using novel 'universal' fluorescent nanoprobe (based on quantum dot nanobeads and Protein A/anti-IgG).	LOD: 0.93 ng/ml; Recoveries: 83.3%–104.5%	Ref. ¹⁵³
Estrogens and estrogen mimics	Water	An analytical method based on SPE coupled with LC-MS/MS.	LODs: 2.5–19.2 ng/L; Recoveries: 80%–130%	Ref. ¹⁵⁴
Estrogens	Milk	A novel magnetic graphene oxide adsorbent modified with a carboxyl-based deep eutectic solvent for MSPE.	LODs: 0.20–0.28 ng/ml; Recoveries: 90.3%–105.7%	Ref. ¹⁵⁵
Estrogens	Water and human urine	A novel hybrid monolithic coating for SBSE, consisting of a metal-organic framework (NH ₂ -MIL-101(Al)) covalently attached to a polymer monolith.	LODs: 0.015–0.58 µg/L; Recoveries: 70%–95%	Ref. ¹⁵⁶
Steroid hormones	Pork	A novel functionalized covalent organic framework/carbon nanotube composite used as an adsorbent for online SPE.	LOD: 0.02–0.30 µg/kg; Recoveries: 82.12%–116.49%	Ref. ¹⁵⁷

multiple lines of evidence and, where available, comparison with authentic standards.^{162,163}

However, the large and complex datasets generated by non-target HRMS create substantial data-processing challenges and have stimulated the application of artificial intelligence (AI) and machine learning (ML).^{164,165} These methods are increasingly being explored for feature prioritization and spectral deconvolution, as well as for predicting retention times and fragmentation patterns and ranking candidate structures. Nevertheless, definitive identification of unknown steroidal metabolites continues to require analytical validation.

In parallel, digital twins are being developed as dynamic virtual representations of wastewater treatment systems to support process monitoring, fore-

casting, and control.¹⁶⁶ Recent studies illustrate parallel progress in AI-assisted mixture-risk assessment and intelligent water-pollutant management, but these approaches have not yet established a validated digital-twin framework for steroid-hormone mixtures.^{167,168} Integrating real-time chemical monitoring, AI-assisted risk assessment, and digital twins may eventually improve the prediction of mixture effects and the optimization of treatment under changing conditions, but this capability remains a prospective research direction requiring validation with field-scale data.

Nontarget screening and comprehensive steroid profiling

A critical shift in environmental monitoring is the transition from targeted quantification of selected parent compounds to comprehensive profiling of

free steroids, conjugates, and transformation products. Early monitoring studies based on predefined analyte lists provided essential occurrence data but covered only a limited portion of the steroid chemical space.¹⁶⁹ More inclusive profiling is particularly important for estrogen conjugates, which can undergo biodeconjugation during wastewater treatment and cause parent-only mass balances to misrepresent removal performance.¹⁵ Recent modeling and on-site evidence further suggest that enzyme-mediated deconjugation, particularly of glucuronide conjugates, may coexist with reverse conjugation in WWTPs, thereby contributing to the incomplete removal of conjugated estrogens.¹⁷⁰

Characterizing this broader steroid profile requires integrated analytical workflows that distinguish intact conjugates from free steroids and track their transformation products. Where metabolically derived products are relevant, these workflows should encompass Phase I functionalization and Phase II conjugation products using chromatographic separation coupled with mass spectrometry.¹⁷¹ Two complementary strategies are commonly used for conjugate analysis. Direct analysis preserves information on the identities and concentrations of intact conjugates, but its coverage is constrained by the limited availability of authentic standards, variable ionization efficiencies, and matrix effects. Indirect analysis uses enzymatic hydrolysis or chemical deconjugation to estimate the combined burden of free and hydrolyzable conjugated steroids, but it does not retain information on individual conjugate species.

Acid-catalyzed solvolysis can be faster and less expensive than enzymatic hydrolysis under optimized conditions, particularly for sulfate conjugates. However, its efficiency depends on the conjugate class, and harsh acidic conditions may degrade acid-labile analytes.¹⁷² Enzymatic hydrolysis is generally more selective but may require 15–24 h of incubation and can be affected by enzyme specificity, matrix inhibition, and incomplete cleavage. Therefore, neither approach should be considered universally quantitative. Hydrolysis efficiency, analyte recovery, and potential artifact formation should be validated for each analyte set and sample matrix.¹⁷³

To expand analytical coverage beyond compounds for which authentic standards are available, direct and indirect workflows can be combined with HRMS-based suspect screening and nontarget analysis. Accurate-mass and tandem mass spectral data can support the annotation of previously uncharacterized steroid metabolites and environmental transformation products. However, identification confidence should be explicitly reported, and definitive identification generally requires comparison with authentic standards.^{162,163} Although HRMS provides broader chemical coverage, its quantitative sensitivity may be lower for some trace analytes, and its substantial data-processing requirements mean that it complements rather than replaces targeted LC–MS/MS.¹⁷⁴

Chemical occurrence data alone do not necessarily explain the integrated biological activity of complex samples.¹⁷⁵ EDA addresses this limitation by combining sample fractionation, bioassays, and chemical analysis to prioritize compounds responsible for observed effects. In surface waters, EDA has been used to isolate and identify previously unknown drivers of antiandrogenic activity, illustrating its value for resolving unexplained endocrine activity.¹⁷⁶ In a complementary human exposome study, detected target chemicals explained less than 1% of the measured estrogenic and (anti)androgenic activity in human amniotic fluid, demonstrating that substantial fractions of endocrine activity can remain unresolved in complex biological matrices.¹⁷⁷

Comprehensive chemical profiling should therefore be integrated with effect-based biological tools. Transcriptomic and proteomic approaches can identify molecular response pathways and candidate biomarkers of endocrine disruption, although they do not independently identify the chemicals responsible for these responses.¹⁷⁸ Highly sensitive reporter gene assays, such as the ER Isjaki assay, respond to E2, E1, and EE2 at sub-picomolar to low-picomolar concentrations and may strengthen effect-based monitoring. Nevertheless, matrix effects and sample-preconcentration requirements must still be evaluated when these assays are applied to environmental water samples.¹⁷⁹ Finally, new approach methodologies encompassing *in silico*, *in vitro*, and *eleutheroembryo* models can be organized within adverse outcome pathway frameworks as part of a weight-of-evidence assessment. These approaches support mechanism-informed endocrine hazard evaluation while reducing reliance on conventional animal

testing.¹⁸⁰ Together, targeted analysis, suspect screening, nontarget analysis, EDA, and complementary biological assays provide a more defensible basis for linking chemical occurrence with biological hazard.

Biosensors and *in situ* imaging

Beyond bulk extraction and tissue homogenization, mass spectrometry imaging (MSI) enables the spatially resolved analysis of steroid distributions directly within tissue sections. Conventional homogenization provides an average tissue concentration but removes information on anatomical localization. However, MSI analysis of steroid hormones remains challenging because of their low endogenous concentrations, poor ionization efficiencies, and spatially variable ion suppression. For carbonyl-containing androgens, on-tissue derivatization with Girard T reagent has been used to charge-tag testosterone and 5 α -dihydrotestosterone, enabling their spatial localization and quantification in mouse testis and rat prostate tissues.¹⁸¹ More recently, electrochemically generated thianthrene radical cations were applied to the on-tissue derivatization of estrogens, producing an approximately three-order-of-magnitude enhancement in MSI sensitivity and enabling their visualization in biological tissues.¹⁸² These studies demonstrate the potential of MSI to investigate localized steroid metabolism and accumulation. Applications to aquatic organisms could similarly help resolve tissue-specific uptake and biotransformation, although matrix-specific validation remains necessary. Quantitative MSI also requires careful control of derivatization uniformity, tissue-dependent matrix effects, internal-standard normalization, and comparison with validated bulk LC–MS/MS measurements.

At a different analytical scale, electrochemical, optical, and plasmonic biosensors offer potential routes toward rapid and portable screening of steroid hormones in environmental and food samples.¹⁸³ Screen-printed electrodes are particularly attractive because they can be mass-produced at relatively low cost and used as disposable sensing platforms. Nevertheless, many prototype sensors do not yet consistently match LC–MS/MS in selectivity, quantitative accuracy, or the detection of estrogens at environmentally relevant concentrations in complex water matrices.¹⁸⁴ Functional materials such as graphene, carbon nanotubes, metal-organic frameworks, and metallic nanoparticles can increase the electroactive surface area, accelerate electron transfer, and facilitate receptor immobilization, thereby improving analytical response under controlled conditions.¹⁸⁵ Molecular selectivity can be provided by biological recognition elements, including antibodies, aptamers, and estrogen receptors, or by synthetic recognition layers such as molecularly imprinted polymers. Although some laboratory platforms report nominal detection limits in the femtomolar range, such values should not be interpreted as field-applicable quantification limits without validation of recovery, selectivity, precision, and matrix effects in authentic samples.¹⁸⁶

A major obstacle to field deployment is maintaining reliable performance during prolonged exposure to complex aquatic matrices. DOM, proteins, suspended particles, and microbial biofilms can adsorb to sensor surfaces, causing signal drift, reduced sensitivity, poor reproducibility, and shortened operational lifetimes. Additional challenges include receptor cross-reactivity and degradation, variability among electrode batches, and calibration drift. Antifouling polymeric, zwitterionic, nanostructured, and hybrid coatings provide promising strategies for reducing nonspecific adsorption. Protective membranes, sample-conditioning or microfluidic components, and integrated calibration and regeneration procedures may further improve sensor robustness.^{187,188} However, these approaches must be evaluated under continuous exposure to authentic environmental waters rather than only in buffered or spiked laboratory samples. Field validation should report response time, analyte recovery, selectivity against relevant interferents, signal drift, operational lifetime, maintenance requirements, and agreement with reference LC–MS/MS measurements.

MSI, biosensors, bioassays, and conventional mass spectrometric methods answer different analytical questions and should therefore be viewed as complementary. Biosensors could provide high-frequency screening and event detection, whereas receptor-based bioassays measure integrated hormonal activity. Targeted LC–MS/MS and HRMS provide compound-specific confirmation and broader chemical characterization, while MSI resolves tissue-level spatial distributions. A tiered monitoring framework could use validated biosensor or bioassay thresholds to identify samples

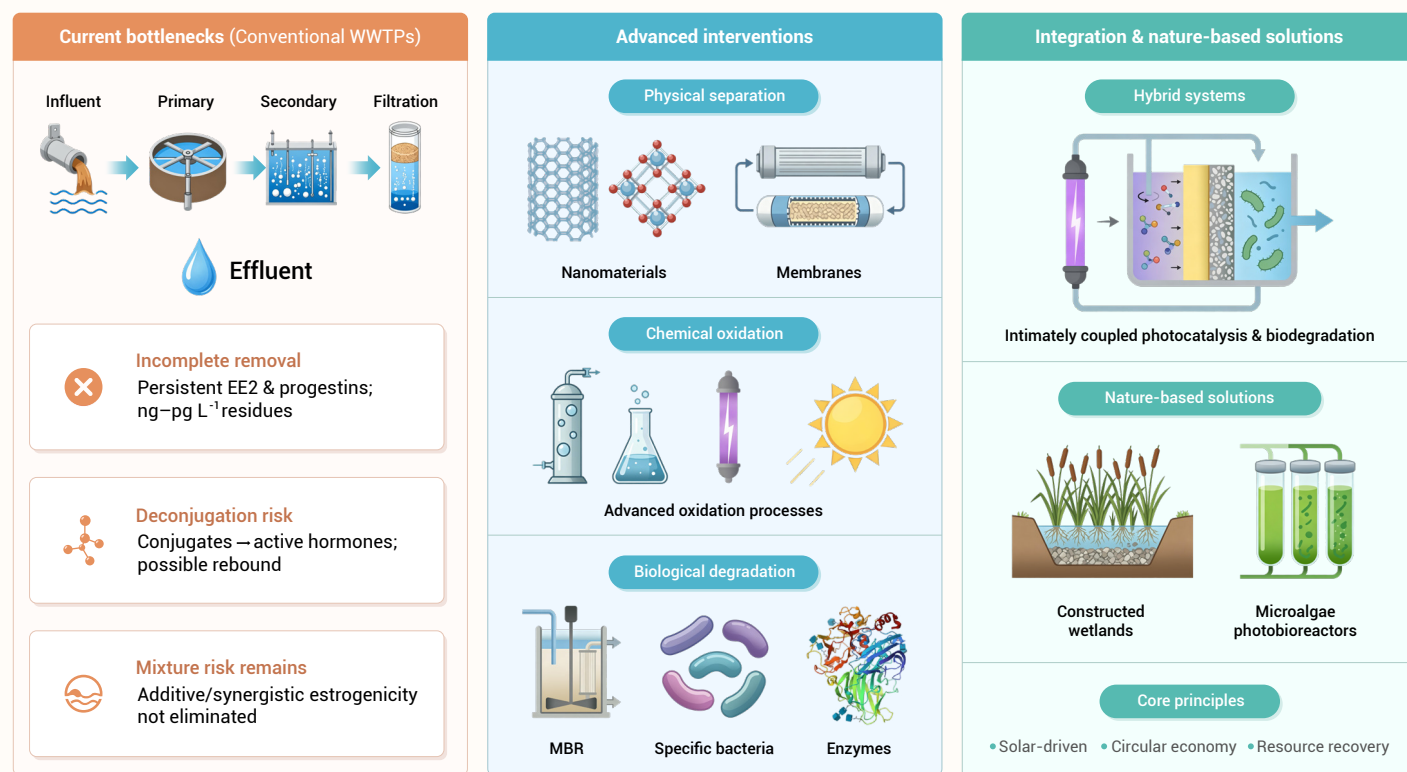


Figure 4. Current bottlenecks and emerging removal strategies for environmental steroid hormones The representative laccase structure was obtained from the RCSB Protein Data Bank (PDB ID: 1GYC).

requiring confirmatory LC–MS/MS or LC–HRMS analysis, thereby improving temporal coverage while limiting resource-intensive measurements. Nevertheless, fully autonomous sensor networks and automated triggering of confirmatory analyses remain prospective concepts that require field-scale validation. Bioassays can reveal aggregate receptor-mediated activity and mixture effects that are not explained by known target analytes, but they cannot independently identify the chemicals or transformation products responsible for those effects. Integrating biosensing and effect-based measurements with targeted and nontarget chemical analysis therefore provides a more defensible strategy for linking rapid surveillance with compound-specific identification and biological relevance.¹⁸⁹

REMOVAL TECHNOLOGIES

Conventional municipal WWTPs rely primarily on secondary biological processes such as activated sludge. Because these systems were not specifically designed to remove steroidal micropollutants, their performance is variable, and residual hormones and estrogenic activity can persist in final effluents. Primary clarification generally provides limited removal because many steroidal hormones remain in the dissolved or colloidal phases, making secondary biological treatment the principal attenuation stage.¹⁹⁰ Within secondary treatment, activated-sludge biotransformation, including processes associated with nitrifying microbial communities, can substantially reduce estrogenic loads, although performance depends on compound identity, redox conditions, temperature, hydraulic retention time, and sludge retention time. Field measurements at two tertiary WWTPs showed that most target estrogenic compounds declined primarily during secondary treatment and that natural estrogens were generally removed more efficiently than EE2 and the industrial xenoestrogens examined.¹⁹¹ Treatment can also alter chemical speciation. In one full-scale anaerobic/anoxic/oxic (A/A/O) WWTP, advanced flocculation promoted the conversion of residual estrogen conjugates to free estrogens, after which filtration further reduced natural estrogens but did not measurably remove EE2.¹⁹² Thus, decreases in parent-compound concentrations should be interpreted together with the occurrence of conjugates, transformation products, and residual biological activity.

Hormone elimination rates vary significantly across treatment plants.

Biotransformation is often the dominant removal pathway, whereas sorption to sludge can also contribute and should not be assumed to be uniformly minor because its relative importance varies among compounds and treatment processes.¹⁹³ Redox-dependent behavior is likewise compound-specific. In one full-scale A/A/O plant, many of the measured glucocorticoids, androgens, and progestogens were removed mainly in the anaerobic tank, whereas estrogens were degraded predominantly in the aerobic tank. Apparent mass increases of 21 α -hydroxyprogesterone and 6 α -methylhydroxyprogesterone in anaerobic or anoxic units were attributed to deconjugation.¹⁹⁴ Across 12 municipal WWTPs, four steroidal estrogens, namely 17 β -E2, EE2, E1, and E3, accounted for 79.92% $\pm$ 8.43% of the measured estrogenic activity in influent, secondary-effluent, and final-effluent samples. The secondary stage accounted for 88.59% $\pm$ 8.12% of the removal achieved across the secondary and tertiary treatment segments, and the A/A/O-MBR process showed the strongest performance among the surveyed secondary processes. Removal efficiency was also positively associated with sludge retention time.¹⁹⁵ These findings support process optimization and adequate biomass retention but do not imply uniform removal across treatment plants, compounds, or seasons.

Robust treatment strategies should combine complementary physical, chemical, and biological processes to reduce parent hormones, transformation products, and residual endocrine activity while accounting for cost, energy consumption, and byproduct formation.^{196,197} No single technology can be assumed to achieve absolute elimination under all wastewater conditions. Electrochemical advanced oxidation processes can degrade recalcitrant estrogens through direct electron transfer and/or electrogenerated oxidants, including hydroxyl radicals, whereas electrocoagulation primarily removes contaminants through coagulation, adsorption, and phase separation rather than complete mineralization.¹⁹⁸ Their performance should therefore be evaluated using not only parent-compound removal but also mineralization, transformation-product profiles, residual estrogenic activity, energy demand, and the management of resulting sludge or concentrate. Consequently, effective mitigation is best pursued through fit-for-purpose treatment trains that integrate physical separation, chemical transformation, and biological degradation, as summarized in Figure 4.

Table 3. Performance of advanced adsorbents and membrane processes for steroidal hormone removal.

Target Contaminant	Method	Results	Reference
E1, E2, E3	A composite material of nanoscale zero-valent iron supported on walnut shell-derived biochar.	Adsorption capacity: 26.315 mg/g (E1); Adsorption capacity: 27.778 mg/g (E2); Adsorption capacity: 25.779 mg/g (E3)	Ref. ²⁰⁵
E1, E2, E3	Walnut shell-derived biochar used as a high-performance adsorbent.	Adsorption capacity: 21.358 mg/g (E1); Adsorption capacity: 17.737 mg/g (E2); Adsorption capacity: 25.069 mg/g (E3)	Ref. ²⁰⁶
E1, E2, E3	A low-cost, sustainable biosorbent: in natura (unprocessed) soybean hulls.	Adsorption capacity: 2.560 mg/g (E1); Adsorption capacity: 1.978 mg/g (E2); Adsorption capacity: 0.835 mg/g (E3)	Ref. ²⁰⁷
E2, EE2	A novel magnetic mesoporous molecularly imprinted polymer, using E ₂ as the template, for selective adsorption.	Adsorption capacity: 372.7 mg/g (E2); Adsorption capacity: 291.4 mg/g (EE2)	Ref. ²⁰⁸
E1, E2	A novel magnesium-aluminum layered double hydroxide/molybdenum disulfide nanocomposite used as a high-performance adsorbent.	Adsorption capacity: 265.60 mg/g (E1); Adsorption capacity: 229.79 mg/g (E2)	Ref. ²⁰⁹
EE2	A low-cost biosorbent made from rice husk powder coated with copper oxide nanoparticles.	Adsorption capacity: 7.76 mg/g	Ref. ²¹⁰
Antibiotics and estrogens	Comparative analysis of four commercial nanofiltration and reverse osmosis membranes (XLE, NF90, NF270, DL).	Rejection efficiency ranked: XLE (94%–100%) ≈ NF90 (88%–100%) > NF270 (25%–85%) > DL (16%–75%)	Ref. ²⁰³

Physical separation processes

Physical separation processes remove steroidal hormones by transferring them from the aqueous phase to solid sorbents or by retaining them at membrane barriers, without necessarily destroying their molecular structures. Granular activated carbon and powdered activated carbon remain conventional benchmarks for adsorption-based wastewater polishing because of their large specific surface areas, developed pore structures, and affinity for hydrophobic steroidal molecules. Adsorption can involve pore filling, hydrophobic partitioning, π - π interactions, and hydrogen bonding, depending on the properties of the carbon surface and target compound. In complex wastewater matrices, however, background DOM can compete for adsorption sites and block accessible pores, thereby reducing adsorption capacity and shortening carbon service life. The magnitude of this interference depends on the characteristics of the DOM and activated carbon, as well as carbon dosage, contact time, and regeneration status. Activated-carbon adsorption should therefore not be regarded as intrinsically unsuitable for continuous polishing, but its performance requires matrix-specific optimization and appropriate regeneration or replacement strategies.¹⁹⁹

Engineered sorbents, including carbon nanotubes, metal-organic frameworks, molecularly imprinted polymers, and layered double hydroxide composites, are being investigated to increase accessible surface area, introduce selective binding sites, and facilitate sorbent recovery.^{200,201} As summarized in Table 3, reported adsorption capacities vary widely among material classes. Unprocessed soybean hulls, for example, exhibit maximum capacities of approximately 0.8–2.6 mg/g for natural estrogens, whereas some molecularly imprinted polymers and layered double hydroxide composites have shown capacities exceeding 200 mg/g under controlled laboratory conditions. However, these values should not be interpreted as a universal performance ranking because the studies differ substantially in initial contaminant concentrations, pH, sorbent dosage, water composition, equilibrium models, and experimental duration. High adsorption capacities measured in simplified solutions also do not demonstrate that a material can overcome competition from DOM or maintain comparable performance at environmentally relevant concentrations in real wastewater. Bio-based sorbents, including hydrogels, modified biomass, and biochars, may provide lower-impact alternatives to conventional synthetic materials. Although adsorption capacities of up to 233 mg/g for EE2 have been reported for specific biochars, these values were obtained under particular experimental conditions and require validation in authentic wastewater.²⁰² Regeneration efficiency, contaminant desorption, material leaching, life-cycle impacts, and performance under continuous-flow conditions should therefore be evaluated before large-scale implementation.

Pressure-driven membrane processes provide another important physical

separation route. Under controlled experimental conditions, the reverse osmosis membrane XLE and tight nanofiltration membrane NF90 achieved rejection efficiencies exceeding 95% for the tested estrogenic compounds, whereas the looser NF270 and DL membranes showed lower and more variable rejection. These results indicate that rejection performance is governed by membrane pore size and surface charge, together with the molecular size and ionization state of the target compounds and solution pH.²⁰³ For neutral and hydrophobic estrogens, size exclusion and solute–membrane hydrophobic interactions are generally important, while electrostatic interactions become more relevant when either the membrane surface or target compound is ionized. Membrane adsorption may also contribute to apparent removal during the initial filtration period but can lead to subsequent breakthrough as adsorption sites become saturated. Water-matrix effects are likewise membrane- and compound-specific. In the presence of humic acid, estrogen rejection decreased by 8%–16% for NF270 and 5%–14% for NF90, primarily because of weakened size exclusion. Subsequent addition of Ca²⁺ produced inconsistent changes in estrogen rejection because of competing effects on size exclusion, hydrophobic interactions, and membrane fouling, rather than causing a uniform decline.²⁰⁴ Performance measured in ultrapure or simplified test solutions should therefore not be directly extrapolated to real wastewater.

Membranes can provide high rejection but do not constitute absolute barriers or destroy the retained contaminants. Instead, they generate retentate, membrane-cleaning residuals, or other concentrated waste streams that require further treatment or controlled disposal. Adsorption similarly transfers contaminants to spent sorbents, which must be regenerated, treated, or disposed of safely. For engineered nanosorbents, additional uncertainties include particle recovery, material leaching, potential ecotoxicity, synthesis and regeneration costs, and long-term stability under continuous-flow conditions. Physical separation remains valuable for wastewater polishing and contaminant concentration, but its effectiveness should be evaluated using complete mass balances that include both aqueous and solid phases. Where destruction of steroidal contaminants and their biological activity is required, adsorption or membrane separation can be integrated with downstream oxidative or biological treatment processes to reduce contaminant transfer and residual environmental risks.

Chemical oxidation processes

Chemical oxidation processes transform steroidal hormones through direct reactions with oxidants or electrode surfaces, or through indirect reactions mediated by reactive species. Representative technologies include ozonation, UV/H₂O₂, photocatalysis, Fenton and Fenton-like processes, electrochemical oxidation, and ionizing radiation. As summarized in Table 4, many studies

Table 4. Performance of oxidation-based and hybrid processes for steroidal hormone removal.

Target Contaminant	Method	Results	Reference
EE2	Ozonation as an advanced oxidation process for drinking water or wastewater treatment.	Estrogenic activity reduction by >200-fold	Ref. ²¹⁸
Natural and synthetic progesterones	Comparison of five disinfection processes (Cl_2 , ClO_2 , O_3 , UV, and UV/ Cl_2) for hospital wastewater.	Removal efficiency ranked: UV/ Cl_2 > Cl_2 > O_3 > ClO_2 > UV. Natural progesterones were removed more easily than synthetic ones.	Ref. ²¹⁹
E2	A novel anode-electrodialysis system, where the feed solution first passes through the anode chamber for in-situ electrochemical oxidation.	Removal efficiency: 100%	Ref. ²²⁰
E1, E2, E3, EE2	A novel visible-light active photocatalyst: a ZnO/graphene oxide p-n heterostructure supported on clay, with a carbon interlayer from biomass.	Removal efficiency: 100%	Ref. ²²¹
E1, E2, E3, EE2	A hybrid system combining UV photolysis as a pre-treatment with a GAC sandwich biofiltration system.	Removal efficiency >96.2%	Ref. ²²²
E1, E2, E3, EE2	A novel visible-light active photocatalyst: a clay- Fe_2O_3 -graphene oxide composite with a carbon interlayer derived from biomass.	Removal efficiency >80%	Ref. ²²³
Diethylstilbestrol, E1, E2, E3, EE2	A novel 0.1% gold nanoparticles supported on titanium dioxide photocatalyst.	Removal efficiency: 99.5% (Diethylstilbestrol); Removal efficiency: 100% (E1); Removal efficiency: 100% (E2); Removal efficiency: 97.2% (E3); Removal efficiency: 100% (EE2)	Ref. ²²⁴
EE2	Activated carbon/titanium dioxide composite photocatalysts, prepared by a hydrothermal method.	Removal efficiency > 90%	Ref. ²²⁵
E1, E2, EE2, and levonorgestrel	Activated carbon/titanium dioxide composite photocatalyst, with synthesis and application optimized using response surface methodology	Removal efficiency: 99.8% (E1); Removal efficiency: 99.8% (E2); Removal efficiency: 99.0% (EE2); Removal efficiency: 92.1% (levonorgestrel)	Ref. ²²⁶
E2	A novel visible-light active photocatalytic nanocomposite consisting of gold nanoparticles, TiO_2 nanotubes, and graphene.	Removal efficiency: 100%	Ref. ²²⁷
αE2	A dual-function material of reduced graphene oxide-based iron nanoparticles, acting via a combined mechanism of adsorption and Fenton oxidation.	Removal efficiency: 99.9%	Ref. ²²⁸
E1, E2	Silver nanoparticles biosynthesized using orange extract, which act as a catalyst to activate persulfate in a Fenton-like oxidation process.	Removal efficiency: 100%	Ref. ²²⁹
EE2 and levonorgestrel	UVC Photolysis and UVC/ TiO_2 Photocatalysis.	Removal efficiency: 92% (photocatalysis, EE2); Removal efficiency: 97% (photocatalysis, levonorgestrel); Removal efficiency: 60% (photolysis, EE2); Removal efficiency: 94% (photolysis, levonorgestrel)	Ref. ²³⁰

report rapid removal of parent steroidal hormones, sometimes exceeding 90% under controlled experimental conditions. However, these removal efficiencies cannot be directly compared across studies because initial contaminant concentrations, water matrices, oxidant and catalyst doses, pH, irradiation intensity or absorbed dose, and reaction times differ substantially. Furthermore, disappearance of the parent compound does not necessarily indicate complete mineralization or detoxification. Ozonation can rapidly transform electron-rich estrogenic structures, whereas visible-light photocatalysis may utilize a broader fraction of the solar spectrum. Heterogeneous Fenton-like catalysts can facilitate catalyst recovery and reduce dissolved iron residues relative to homogeneous Fenton systems, but they generally still require consumable oxidants such as H_2O_2 or persulfate and may be constrained by catalyst leaching, surface inactivation, radical scavenging, and mass-transfer limitations in complex matrices.^{211,212} Ionizing radiation represents another emerging option. Electron-beam irradiation induces water radiolysis and generates reactive species, including hydroxyl radicals, hydrated electrons, and hydrogen atoms, allowing both oxidative and reductive transformation pathways. Its ability to degrade the androgen androstenedione in aqueous solution has been demonstrated, although treatment performance, energy requirements, transformation-product toxicity, and applicability to complex wastewater require further evaluation.²¹³

Reactive species generated during oxidation can attack electron-rich

regions of steroid molecules, including the phenolic A-ring of estrogens, unsaturated bonds, and carbonyl-containing substituents. These reactions may initiate hydroxylation, dealkylation, bond cleavage, ring opening, and subsequent molecular fragmentation. Under sufficiently intensive treatment conditions, further oxidation can ultimately convert organic carbon into carbon dioxide, water, and inorganic ions. In practice, however, parent-compound degradation generally occurs more rapidly than total organic carbon removal, and complete mineralization is rarely demonstrated. Oxidation can substantially reduce estrogen receptor-mediated activity, but the extent of detoxification depends on the identity and persistence of the resulting transformation products. Partial oxidation may generate intermediates with reduced, residual, or occasionally altered toxicity. For example, ozonation of EE2 produces multiple hydroxylated and ring-opened products whose predicted bioaccumulation, developmental toxicity, and mutagenic potential vary among transformation pathways.²¹⁴ Consequently, the performance of chemical oxidation should be evaluated using multiple endpoints, including parent-compound removal, total organic carbon or dissolved organic carbon reduction, transformation-product profiles, residual estrogenic activity, and organism-level or cell-based toxicity, rather than relying solely on the disappearance of the target compound.²¹⁵

The appropriate role of chemical oxidation depends on wastewater composition, treatment objectives, and process economics. Standalone

Table 5. Performance of representative biological and enzyme-based systems for steroidal hormone removal.

Target Contaminant	Method	Results	Reference
E2	A bio-nanocomposite based on reduced graphene oxide immobilizing the degrading strain <i>Ochrobactrum</i> sp. FJ1.	Removal efficiency: 99.76%	Ref. ²⁴²
E2, EE2	Immobilization of the enzyme laccase onto a custom 3D printed polylactide scaffold.	Removal efficiency > 30% (E2); Removal efficiency > 40% (EE2)	Ref. ²⁴³
Progesterone, norgestrel	Immobilized functional bacteria (<i>Rhodococcus</i> sp., <i>Citrobacter</i> sp.) in a bacterial gel containing bagasse-derived carbon aerogel, operated in a bacterial gels reactor.	Removal efficiency: 99.3% (progesterone); Removal efficiency: 97.1% (norgestrel)	Ref. ²⁴⁴
E2	Cultivation of the thermophilic cyanobacterium <i>Thermosynechococcus</i> sp. CL-1 in a photobioreactor.	Removal efficiency: 94.9%	Ref. ²⁴⁵
E1, E2, E3, EE2	An <i>in-situ</i> enhanced denitrification biofilter by adding slow-release nano calcium peroxide to the top filter layer.	Removal efficiency > 95%	Ref. ²⁴⁶
E2, EE2	A hybrid system that intimately couples UV irradiation with immobilized estrogen-degrading bacteria (<i>Sphingobium estronivorans</i>) in a single column reactor.	Removal efficiency: 94.6%–100%	Ref. ²⁴⁷
E2	Cultivation of the thermophilic cyanobacterium <i>Thermosynechococcus</i> sp. CL-1.	Removal efficiency: 69.4%	Ref. ²⁴⁸
EE2	An anaerobic electrochemical membrane bioreactor with an electric field	Removal efficiency: 83.1%	Ref. ²⁴⁹
E2, EE2	The enzyme laccase immobilized via covalent binding onto electrospun polyacrylonitrile/polyethersulfone fibers, using a polydopamine linker.	Removal efficiency: 92% (E2); Removal efficiency: 100% (EE2)	Ref. ²⁵⁰
E2, EE2	The enzyme laccase from <i>Trametes versicolor</i> , both in its free form and immobilized within calcium-alginate beads for use in a fluid-bed reactor.	Removal efficiency: 99.2% (E2); Removal efficiency: 98.5% (EE2)	Ref. ²⁵¹

oxidation may be suitable for polishing relatively clean effluents with low concentrations of radical-scavenging organic matter. In more complex matrices, however, high oxidant or energy demand, competition from background constituents, catalyst deactivation, and transformation-product formation can limit its efficiency. Integrating partial oxidation with downstream biological treatment provides a promising alternative because oxidation can convert recalcitrant contaminants into more biodegradable intermediates, while biological processes can subsequently remove residual organic carbon and oxidation products. Nevertheless, oxidation conditions must be optimized because insufficient treatment may leave biologically active products, whereas excessive oxidation can increase energy and chemical consumption without providing a proportional improvement in biodegradability. A continuous bench-scale study showed that UV/peracetic acid pretreatment followed by biological activated carbon filtration reduced organic micropollutants, estrogenic load, and several toxicity endpoints, although operational costs remained an important consideration.²¹⁶ Similar combinations of ozonation and biological filtration have also been investigated to balance rapid chemical transformation with subsequent biodegradation.²¹⁷ Chemical oxidation should therefore be implemented as part of a treatment train selected according to matrix characteristics, required risk reduction, resource consumption, and the fate of transformation products, rather than being universally prescribed as either a standalone treatment or a pretreatment step.

Biological degradation processes

Advanced biological processes can improve steroid-hormone attenuation by retaining high biomass concentrations and creating operating conditions that favor acclimated microbial communities. Membrane bioreactors (MBRs) offer an important operational advantage by decoupling hydraulic retention time from solids retention time, thereby allowing high biomass concentrations and extended solids retention. Longer solids retention times can favor slow-growing nitrifiers and other specialized degraders, although their effects are compound-specific. In bench-scale aerobic MBRs, E2 and E1 were effectively attenuated at a solids retention time of 8 days, whereas EE2 attenuation increased when the solids retention time was extended from 20 to 80 days and was partly associated with nitrification.²³¹ These findings indicate that extended biomass retention and nitrifying activity can promote EE2 transformation, but they do not guarantee complete removal, mineralization, or elimination of estrogenic activity.²³²

Aerobic granular sludge (AGS) provides another biological treatment configuration by combining high biomass retention with a compact, extracellular-polymeric-substance-rich granular matrix. In a long-term AGS study

conducted at an influent concentration of 10 µg/L, 93.5% of E2 and 84.8% of EE2 were removed. Batch experiments indicated that biodegradation was the dominant mechanism for E2 removal, whereas the more biorefractory EE2 was removed mainly through adsorption to the granules.²³³ Thus, decreases in aqueous concentrations within AGS systems should be interpreted using mass balances that distinguish biodegradation from temporary phase transfer. More broadly, steroid transformation in engineered biological systems may involve both growth-linked metabolism and cometabolic reactions performed by phylogenetically diverse microbial communities.²³⁴

Strain-level studies provide mechanistic insights into microbial estrogen degradation, although their performance cannot be directly extrapolated to full-scale wastewater treatment. Members of genera such as *Novosphingobium* and *Rhodococcus* have been reported to transform natural steroidal estrogens, but degradation rates, transformation products, and loss of estrogenic activity are strain- and condition-specific.²³⁵ In *Rhodococcus equi* DSSKP-R-001, the *hsd17b14*-encoded dehydrogenase transformed E2 into E1, whereas the flavin-binding monooxygenase encoded by *At1g12200* converted E1 into 4-hydroxyestrone. The *catA* and *hsaC* genes were associated with subsequent transformation and C–C bond cleavage at the C4 position of the estrogen A-ring.²³⁶ Low-temperature activity has also been demonstrated for *Rhodococcus* sp. RCBS9, which achieved 71.61% E2 degradation at 10°C under the reported experimental conditions, although its performance in authentic wastewater and continuous-flow systems remains to be established.²³⁷ Environmental organic matter can further modify biodegradation. Autochthonous, water-derived DOM promoted more extensive EE2 degradation by *Rhodobacter blasticus* than sediment-derived DOM, whereas humic acid produced the smallest stimulatory effect among the tested materials.²³⁸

Cell-free enzymatic biocatalysis provides a complementary approach for transforming estrogenic compounds under relatively mild reaction conditions. Oxidoreductases, particularly laccases and peroxidases, can transform natural and synthetic estrogens without requiring the maintenance of viable microbial cultures and may reduce biomass production compared with whole-cell treatment. Laboratory studies have reported substantial removal of parent estrogens and reductions in estrogenic activity, but enzymatic conversion does not necessarily imply complete mineralization, and products such as dimers and oligomers may be formed.^{239,240} Free enzymes are susceptible to denaturation, proteolytic degradation, washout, and inhibition by wastewater-matrix constituents. Immobilization can improve enzyme recovery, stability, and reuse, although support costs and mass-transfer limitations must also be considered.²⁴¹ Redox mediators such as 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and 1-hydroxybenzotriazole can expand

substrate accessibility in some laccase systems, but their necessity is system-specific, and their cost, potential toxicity, persistence, and recovery require evaluation before large-scale application. Representative biological and enzyme-based treatment systems and their reported performances are summarized in Table 5.

No biological treatment configuration can be considered universally optimal. MBRs, AGS systems, bioaugmentation, functional bacterial carriers, and immobilized enzymes each present distinct advantages and limitations related to wastewater composition, temperature, contaminant loading, biomass retention, operational stability, and cost. Targeted oxidation before biological treatment may improve the biodegradability of recalcitrant compounds such as EE2 in some treatment trains, but such coupling is not universally required and must be optimized to avoid inhibitory transformation products and disproportionate chemical or energy consumption. Integrated treatment systems should therefore be evaluated using parent-compound removal, mineralization, transformation-product profiles, residual endocrine activity, toxicity, resource consumption, and management of sludge or immobilization materials. The appropriate objective is robust and verifiable risk reduction rather than an assumed absolute elimination of steroidal contaminants.

Integrated processes and nature-based solutions

No single treatment process can reliably remove parent steroidal hormones, their conjugates, transformation products, and residual endocrine activity across all wastewater matrices. Hybrid treatment trains therefore combine processes with complementary functions, although their benefits depend on process sequence, operating conditions, and compatibility between chemical and biological stages. Visible-light-driven intimately coupled photocatalysis and biodegradation provides a representative example. In a system using N-doped TiO₂, 97.8%±0.4% of 17β-estradiol 3-sulfate was removed from synthetic wastewater, compared with 84.0%±2.2% by photocatalysis alone and 71.4%±1.8% by biodegradation alone. Photocatalysis promoted deconjugation, whereas biofilm co-metabolism contributed to subsequent transformation and mineralization.²⁵² These findings demonstrate process complementarity under controlled conditions but should not be extrapolated directly to full-scale wastewater treatment. Biofilm overgrowth may reduce light penetration or cover photocatalytic surfaces, while catalyst stability, mass transfer, microbial resilience, and transformation-product control require long-term evaluation.

Bioelectrochemical systems provide another integrated platform by coupling microbial metabolism with electrode-mediated electron transfer. Depending on reactor configuration, BESs may support estrogen transformation while generating electrical current or enabling other forms of resource recovery. Nevertheless, much of the available evidence remains laboratory-based, and contaminant removal should not automatically be equated with detoxification or net energy recovery. Low current density, limited extracellular electron transfer, electrode and membrane costs, fouling, and long-term operational stability remain important barriers to scale-up.²⁵³

Nature-based solutions, including constructed wetlands and microalgal or microalgal–bacterial photobioreactors, combine sorption, plant or algal uptake, photochemical transformation, and microbial degradation. The relative contributions of these processes vary with the organisms and substrates employed, hydraulic retention time, irradiance, temperature, nutrient availability, and wastewater composition. Laboratory studies reviewed by Sun et al. reported that microalgal removal efficiencies for environmental estrogens were mostly above 80% over treatment periods of approximately one week. However, interactions between microalgae and coexisting bacteria can be either synergistic or antagonistic and therefore require system-specific evaluation.²⁵⁴ In an outdoor study of constructed wetlands treating raw domestic sewage, the removal efficiencies of nine target steroid hormones ranged from 14.7%±3.04% to 100%, with artificial aeration and serial wetland configurations generally improving performance. Mass-balance analysis indicated that biodegradation was the dominant removal mechanism, whereas substrate sorption and plant uptake played smaller roles.²⁵⁵ In another laboratory-scale study, a gravel-based constructed wetland planted with *Ludwigia octovalvis* achieved 94.5%±2.47% removal of E2 under the tested conditions.²⁵⁶ These study-specific efficiencies should not be generalized without considering influent concentrations, hydraulic loading, retention time, climate, substrate

properties, and the distinction between degradation and phase transfer.

Microalgal biomass may provide opportunities for recovery as feedstock for bioenergy or other bioproducts, potentially linking wastewater treatment with resource valorization.²⁵⁷ However, the net benefit depends on harvesting and dewatering requirements, contaminant burdens in the recovered biomass, the safety of downstream applications, and overall life-cycle and economic performance. More generally, nature-based systems may reduce chemical and energy inputs relative to intensive treatment technologies, but they can require longer retention times and larger land areas and may be sensitive to seasonal changes in temperature and irradiance. The fate of hormones retained in wetland substrates, plant tissues, or algal biomass therefore requires explicit mass-balance assessment. Without evidence of degradation or mineralization, apparent aqueous-phase removal may represent contaminant transfer to a solid compartment that subsequently requires appropriate management.

Accordingly, these technologies should be selected and combined on a fit-for-purpose basis rather than assigned a universal hierarchy. Advanced oxidation and high-rate biological processes may be appropriate for concentrated point-source discharges, whereas constructed wetlands and algal systems may provide polishing, buffering, or interception functions where land, climate, and hydraulic conditions are suitable. The effectiveness and sustainability of a tiered, multi-barrier strategy should be evaluated using parent hormones, conjugates, transformation products, residual endocrine activity and toxicity, complete aqueous–solid mass balances, energy and chemical demand, greenhouse-gas emissions, cost, and long-term operational resilience. Such site-specific integration can improve overall risk reduction, but no fixed sequence of engineered and nature-based processes can be assumed to be optimal for all wastewater and agricultural-runoff scenarios.

PERSPECTIVES AND CONCLUSIONS

Mitigating the pervasive threat of steroidal hormones requires transcending incremental technological improvements. No single technological intervention cannot successfully safeguard global aquatic ecosystems from systemic endocrine disruption. To address current bottlenecks and align with global sustainability goals, the field must transition toward systemic, interdisciplinary paradigms.

Operationalizing the one health framework in future management

The One Health concept should function as an operational framework rather than merely as a general statement about the interconnectedness of different health systems. In environmental monitoring, this requires moving beyond the conventional measurement of a limited number of parent compounds in the water column. Coordinated surveillance should encompass municipal wastewater influents and effluents, livestock wastewater and manure, agricultural runoff, receiving waters, sediments, and sentinel aquatic organisms. Chemical monitoring should combine targeted quantification of priority steroid hormones with the analysis of conjugated compounds and transformation products, suspect and non-target screening, and effect-based methods. Repeated sampling under different hydrological and seasonal conditions is also necessary to distinguish persistent exposure patterns from transient emission events.

In risk assessment, the evolutionary conservation of many endocrine-signaling pathways among vertebrates provides a mechanistic basis for comparing biological responses across species. Nevertheless, reproductive impairment in fish should not be interpreted as direct evidence of human disease. Adverse outcome pathways can organize evidence from molecular initiating events to organism- and population-level outcomes, but cross-species extrapolation must account for differences in exposure routes, toxicokinetics, receptor sensitivity, metabolism, life stage, and physiological background. Human health assessments should likewise distinguish mechanistic plausibility from demonstrated environmental exposure and epidemiological evidence, particularly at environmentally relevant concentrations. From a management perspective, operationalizing One Health requires coordination among health-care systems, pharmaceutical manufacturers, livestock producers, agricultural authorities, wastewater utilities, and environmental agencies. Upstream measures may include pharmaceutical stewardship and appropriate disposal, control of manufacturing discharges, improved manure storage and treatment, optimized timing and rates of land application, and vegetated buffer zones that reduce surface runoff. Green-by-design

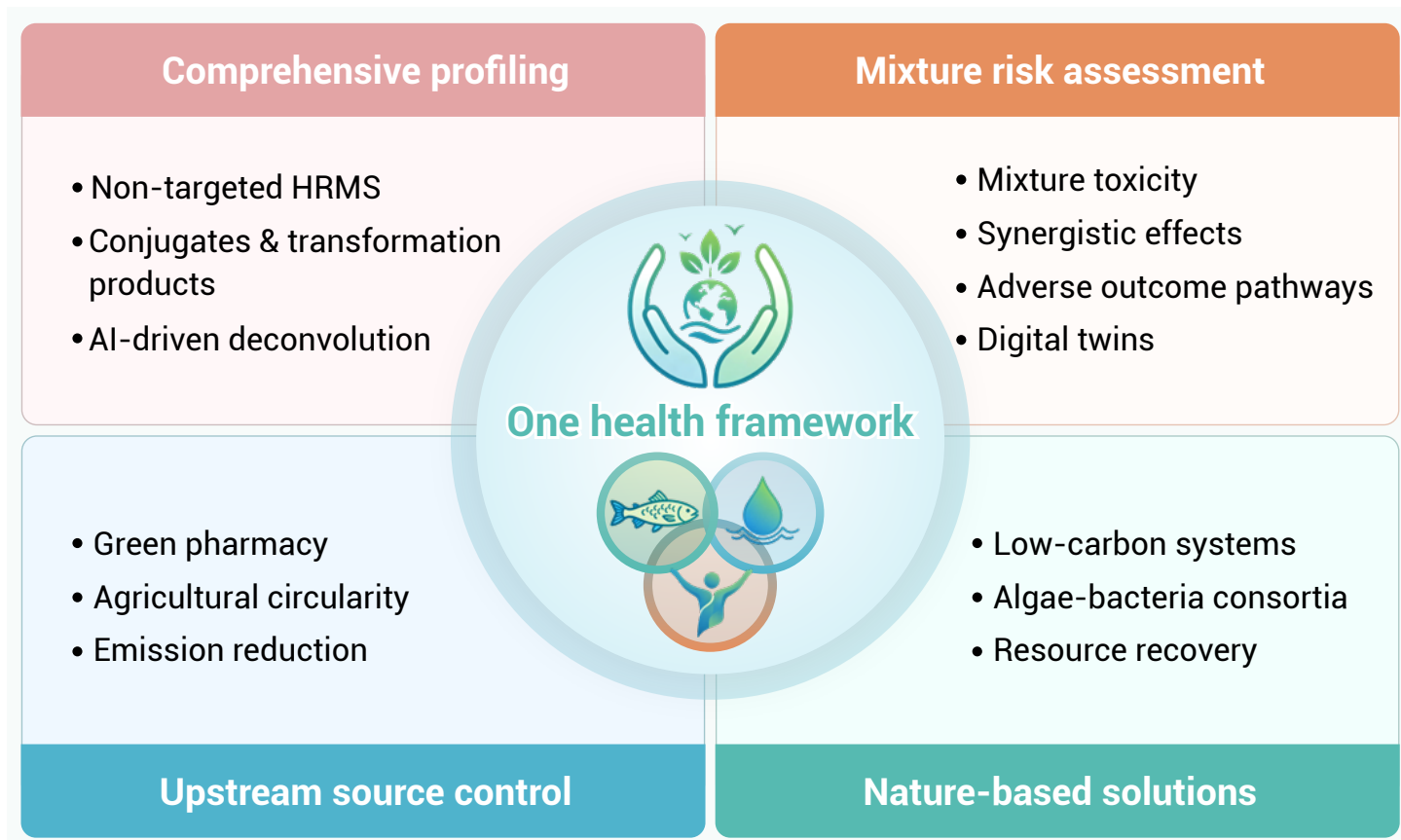


Figure 5. A one health-driven strategic roadmap for the sustainable mitigation of steroidal EDCs.

principles may also be explored for new pharmaceuticals, provided that therapeutic efficacy, patient safety, manufacturability, and life-cycle performance are not compromised. Because natural hormone excretion, existing pharmaceutical use, and diffuse agricultural emissions cannot be eliminated entirely through source control, upstream interventions should complement rather than replace wastewater treatment.

AI-driven innovations and digital twins in steroidal research

AI and machine learning can support multiple stages of steroid analysis, but they should be regarded as decision-support tools rather than substitutes for analytical confirmation. Within HRMS workflows, ML models can assist feature detection, spectral deconvolution, retention-time and fragmentation prediction, candidate-structure prioritization, and pattern recognition in large non-target datasets. Graph-based and deep-learning models may be particularly valuable for prioritizing previously uncharacterized transformation products and Phase II conjugates. Nevertheless, computational annotations should remain tentative until supported by authentic standards, diagnostic fragmentation evidence, or complementary structural techniques. AI-aided risk models may also help fill ecotoxicity data gaps and identify potential mixture-risk drivers, but they cannot automatically predict synergistic or antagonistic interactions or replace concentration-addition models, independent-action models, effect-based bioassays, or experimental toxicity testing. When combined with new approach methodologies, including reporter-gene assays, in vitro systems, fish-embryo models, and computational toxicology, AI may help prioritize chemicals and mixtures for further investigation and reduce unnecessary animal testing.

Digital twins provide an additional opportunity to integrate monitoring data with mechanistic and data-driven models. In wastewater treatment, these systems can combine sensor measurements, hydraulic information, process kinetics, and operational feedback to represent the evolving state of treatment plants or sewer networks. Extending digital twins to steroidal hormones would require validated descriptions of sorption, deconjugation, biodegradation, transformation-product formation, and residual biological activity. Such platforms could eventually compare operating scenarios, identify periods of elevated discharge, guide confirmatory sampling, and support treatment opti-

mization. However, existing digital twins have not been sufficiently validated to reproduce the complete environmental fate or interactive toxicity of complex steroid mixtures. Their practical deployment is also constrained by limited standardized datasets, methodological heterogeneity, censored or missing exposure data, incomplete transformation-product libraries, model domain shifts, and insufficient external validation. Progress will therefore require interoperable and quality-controlled databases, traceable data provenance, common benchmark datasets, prospective validation, and interpretable models that report both predictions and their associated uncertainties.

Priority actions and concluding remarks

Steroidal sex hormones should be regarded as continuously introduced, biologically active environmental contaminants whose risks are shaped by chemical speciation, environmental transformation, mixture composition, and exposure context. Evidence supports ecological concern for sensitive aquatic species, whereas the magnitude of human health risks arising specifically from environmental exposure remains less directly characterized. The following priority actions, summarized in Figure 5, would provide a more defensible basis for future monitoring and management:

Standardize comprehensive profiling. Develop tiered monitoring strategies that combine targeted quantification with suspect and non-target HRMS screening and effect-based methods. Priority parent hormones, major conjugates, and relevant transformation products should be included where suitable standards and validated methods are available. Harmonized quality assurance, reporting criteria, reference materials, and interlaboratory comparisons are needed to improve comparability among studies.

Implement mixture-based risk assessment. Supplement single-substance benchmarks with approaches that account for realistic co-exposure. Concentration-addition and independent-action models can provide initial baselines, while deviations from these predictions should be investigated experimentally where exposure or mechanistic evidence indicates possible interactions. Adverse outcome pathways can support mechanistic integration, but uncertainty in exposure, potency, and cross-species extrapo-

lation should be explicitly reported.

Strengthen upstream source control. Reduce avoidable emissions through pharmaceutical stewardship, control of manufacturing discharges, improved livestock-waste treatment, responsible manure application, and runoff-interception measures. Green pharmaceutical design should be encouraged where it can reduce environmental persistence without compromising clinical performance. Agricultural circularity should also be evaluated carefully to ensure that the reuse of manure or wastewater does not recirculate steroidal contaminants.

Deploy fit-for-purpose, multi-barrier mitigation. Combine optimized conventional treatment, advanced physicochemical or biological processes, and nature-based solutions according to source strength, wastewater composition, climate, land availability, and treatment objectives. Technology selection should consider transformation products, residual endocrine activity and toxicity, contaminant transfer to sludge or biomass, energy and chemical requirements, greenhouse-gas emissions, and life-cycle costs rather than relying solely on parent-compound removal.

Achieving these priorities will require sustained collaboration among environmental chemists, analytical scientists, mechanistic toxicologists, engineers, data scientists, health researchers, and policymakers. The objective should not be framed as the guaranteed or absolute elimination of all steroidal contaminants, but as a measurable reduction in exposure and biological activity without creating unacceptable contaminant transfers or resource burdens. A One Health-based strategy connecting comprehensive profiling, mixture-aware assessment, upstream source control, and context-specific mitigation provides a practical pathway for protecting aquatic ecosystems while supporting the long-term sustainability of water resources.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, the authors used ChatGPT 5.6 to improve the readability and language of the manuscript. After using these AI-assisted tools, the authors carefully checked and revised the content as needed and take full responsibility for the accuracy, integrity, and final presentation of the published work.

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AUTHOR CONTRIBUTIONS

H.Y.: Conceptualization, literature collection and analysis, writing – original draft, visualization. Q.F.: Literature collection, data curation, writing – review and editing. Y.T.: Literature collection, data curation, writing – review and editing. L.W.: Conceptualization, supervision, writing – review and editing. S.X.: Writing – review and editing. Z.W.: Supervision, writing – review and editing. M.Q.: Writing – review and editing. B.W.: Writing – review and editing. Y.Z.: Conceptualization, supervision, funding acquisition, writing – review and editing. All authors contributed to the manuscript and approved the final version.

DECLARATION OF INTERESTS

The authors declare no competing interests.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, the authors used ChatGPT 5.6 to improve the readability and language of the manuscript. After using these AI-assisted tools, the authors carefully checked and revised the content as needed and take full responsibility for the accuracy, integrity, and final presentation of the published work.

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