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Environmental Designer Drugs: When Transformation May Not Eliminate Risk

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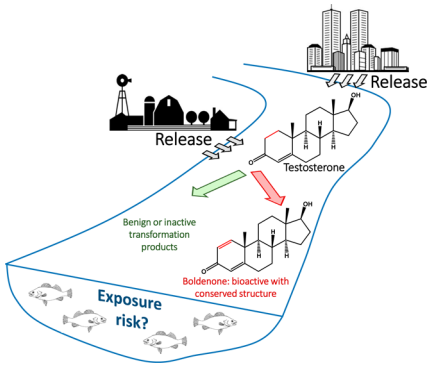

Environmental transformation processes, including those occurring in natural and engineered systems, do not necessarily drastically alter molecular structures of bioactive organic contaminants. While the majority of generated transformation products are likely benign, substantial conservation of structure in transformation products can imply conservation or even creation of bioactivity across multiple biological end points and thus incomplete mitigation of ecological risk. Therefore, focusing solely on parent compound removal for contaminants of higher relative risk, the most common approach to fate characterization, provides no mechanistic relationship to potential biological effects and is inadequate as a comprehensive metric for reduction of ecological risks. Here, we explore these phenomena for endocrine-active steroid hormones, focusing on examples of conserved bioactivity and related implications for fate assessment, regulatory approaches, and research opportunities.

INTRODUCTION

One critical lesson learned from many decades of research is that organic contaminants do not magically disappear when discharged to soil, air, or water, even with engineered treatment before discharge. Unfortunately, contaminants often remain with us, either as the original parent compound or converted into transformation products by processes like biodegradation, chemical oxidation, photolysis, or hydrolysis. Persistent characteristics are especially likely for some synthetic chemicals where implicit resistances to transformation are exploited in molecules to increase their function. Indeed, many of our foundational examples of unintended consequences arising from our use of synthetic organic chemicals like pesticides (e.g., DDT) or dielectric fluids (e.g., PCBs) result because we did not accurately define the relationship between their release, environmental persistence, and potential effects on exposed organisms until it was too late. Over time, through many lessons hard won, we now appreciate that characterizing environmental fates for the millions of chemicals produced, used, and inevitably discharged to the environment is important, even though we often have limited, or even no, insight into potential biological effects arising from exposure.

So where might unintended consequences still exist? What are the critical exceptions to our generalizations and assumptions about contaminant fate? While early research efforts usually examined contaminants like pesticides with demonstrable adverse effects, recent efforts have explored a wider variety of organic contaminants. Examples include pharmaceuticals, personal care products, perfluorinated compounds, and plasticizers (so-called contaminants of emerging concern or CECs) present in municipal wastewater, urban stormwater, and/or agricultural runoff. Research on these trace organics is driven by factors such as their widespread detection by improved analytical instrumentation, their incomplete removal in traditional treatment systems, and growing interest in water reuse, implying potential exposure for recalcitrant contaminants. Observations of sublethal effects in aquatic organisms arising from exposure to wastewater-derived steroid hormones and other endocrine-active contaminants also has raised concerns, especially with respect to estrogenic endocrine disruption in fish. This issue in particular exemplifies our struggle to incorporate the potential for bioactive contaminants that induce chronic or sublethal effects into risk assessment paradigms originally constructed to assess exposures to acute toxicants and carcinogens that tended toward persistence. Resolving this issue, understanding the degree by which chronic and sublethal effects alter populations, and identifying

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contaminants most responsible for adverse effects is of primary importance in accurately evaluating chemical safety.

Here, we discuss one limitation in our understanding of CEC fate: transformation products of bioactive compounds, like pharmaceuticals, generated during natural attenuation or engineered treatment. In particular, we focus on endocrine-active steroid hormones because of their widespread use as pharmaceuticals, the extent of available pharmacology knowledge, their demonstrated potency, and their link to sublethal effects in aquatic organisms. Consequently, we, and others, view steroidal hormones and their environmental fate and effects as high priority research areas.[1] Furthermore, the combination of their potency and structure-dependent bioactivity makes them an excellent case study to examine conservation of bioactivity through environmental transformations. Bioactive transformation products can be viewed as environmental designer drugs, built in nature by some of the same (bio)chemical processes exploited by pharmaceutical manufacturers for drug discovery and capable of evading many commonly employed analytical techniques. Most importantly, their formation poses formidable challenges to how we evaluate contaminant fate, particularly with respect to their attenuation. Thus, our objective is to illustrate underexplored or underappreciated emerging issues and uncertainties related to transformation products. We also aim to highlight potential areas where unintended consequences related to the formation of such environmental designer drugs in natural and engineered aquatic systems might arise in the future as a means of better understanding and managing contaminant risks.

Receptor Agonists and Environmental Bioactivity. In the U.S., we manufacture over 21 000 human pharmaceutical products using at least 1200 unique small molecule drugs.[2] Over 10% of these drugs target nuclear receptors such as estrogen (ER), androgen (AR), progesterone (PR), or glucocorticoid receptors (GR), and include many synthetic steroids far more potent than their endogenous analogs (17β-estradiol, testosterone, progesterone, or cortisol). Besides human pharmaceuticals, potent steroids are widely used in agriculture,[3] and all vertebrates naturally excrete endogenous steroids with inherent bioactivities.[4] Plant steroids such as phytoestrogens, can also occur widely in aquatic environments, including WWTP effluent.[5] Thus, once accounting for metabolites and transformation products, there are likely hundreds of unique steroids discharged to the aquatic environment at some concentration, even if only a few of them are measured directly.

In addition to their primary receptor targets, steroids, their metabolites and transformation products also can have nontarget, inadvertent interactions (i.e., "side effects") with a host of molecular receptors, enzymes and biochemical pathways. Even binding specific receptors as ligands can alter the activation of other receptors through cross-talk mechanisms, or impairment of hormone biosynthesis through feedback pathways. Therefore, we really should not be surprised by reports that anthropogenic impacts on receiving waters can include inadvertent up- or down-regulation of receptor-mediated pathways in aquatic organisms exposed to these complex mixtures, even at trace levels.[6−10] Numerous side effects typically result from pharmaceutical use in humans, implying that we should expect bioactive contaminants to exhibit side effects and nontarget interactions in aquatic organisms as well.

When examining the literature for field studies, many examples exist where bioassays indicate widespread or frequent detection of receptor agonists, yet concurrent chemical analysis only weakly links these biological responses to specific causative agents.[9,11−14] Unexpected and often unexplained receptor activities are observed for ER, AR, and, more recently, GR. For example, Stavreva et al.[14] observed GR activity in 27% and AR activity in 35% of surface waters across 14 states in the eastern U.S., with chemical analysis unable to account for most of the observed bioactivity. In another study, widespread and persistent GR activity in wastewater and wastewater-impacted receiving waters, even at substantially higher levels than ER or AR activity,[15] could not be explained by chemical analysis of 41 known glucocorticoids. Such discrepancies are even greater when considering causality in animal end points and undesirable endocrine-mediated effects like intersex fish, including masculinized or feminized fish or vitellogenin induction in male fish observed in some receiving waters.[16−20]

Until we can explain such observations, we will remain uncertain about the true relationship between contaminant exposure and ecological risk, as well as best management practices to mitigate such effects.

A Potential Role for Transformation Products in Instances of Unexplained Bioactivity. Linking biological responses observed at molecular, cellular, organismal, or population scales to causative agents has long been a significant problem in ecotoxicology. Contaminants occur in complex mixtures whose composition varies spatially and temporally, and we now appreciate that transformation products of bioactive compounds can themselves retain bioactivity. For example, of the ∼380 pesticide analytes in a recent survey of five Swiss rivers, ∼30% of the detected compounds were transformation products, and assessment of mixture toxicity was needed to accurately define ecological risk.[21] While the vast majority of transformation products are likely benign, there are multiple scenarios for product formation and some are more effective than others at mitigating risk (Figure 1). The simplest case, and most desirable for mitigation, occurs when loss of parent concentration is accompanied by formation of known and innocuous products, thus closing the system's mass balance (Figure 1a). Here, transformation results in concurrent bioactivity removal, and there is a clear degree of reaction progress where the risks associated with contaminant exposure attain acceptable levels. We note that even without requisite product identification, a direct correlation between parent decay and proportional removal of bioactivity is the default assumption in current fate models and risk assessment. Alternatively, transformation products may be identifiable, thereby closing the system mass balance, yet these products retain some degree of bioactivity (Figure 1b). In this case, understanding product bioactivity is critical. Depending on the specific bioactivity (e.g., 10%, 50% or 100% relative to the parent) and receptor end point, the risk associated with this system is only partially, or even not, mitigated by parent transformation. Currently, this case requires complementary analytical and toxicological methodologies to appropriately assess risk. Finally, a third scenario (Figure 1c) is most challenging, and may be more common than we currently appreciate, especially when the potential for interactions with multiple biological end points is considered. Here, not only is some degree of bioactivity retained but the specific products responsible for the response remain unexamined or unidentified. These cases are often characterized by dynamic

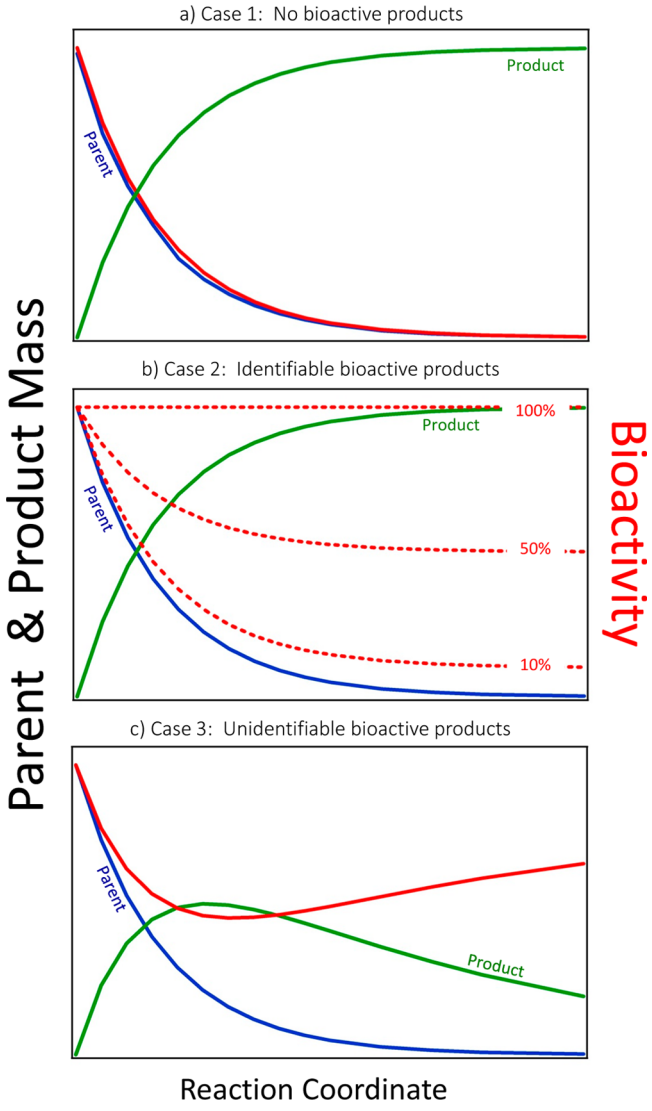


Figure 1. Potential scenarios for how bioactivity can change with reaction progress during environmental transformations. Plots show mass of the parent compound (blue) and identifiable product (green) on the left vertical axis, while changes in bioactivity (red) are plotted on the right vertical axis. (a) Case 1 represents the transformation of a parent compound into a known, identifiable, and nonbioactive product such that bioactivity scales with parent compound concentration. (b) In Case 2, the product is known and identifiable, but also bioactive. Dashed red lines consider how bioactivity would change as a function of reaction progress when the known product exhibits bioactivity that is 10%, 50% or 100% of the parent compound's bioactivity. (c) Case 3 considers the case in which bioactivity diverges from trends in the measured concentration of parent and product (i.e., the red bioactivity line increases while concentrations of the parent and the identifiable product decrease). In this case, unknown (i.e., unidentified) products must be responsible for the persistent bioactivity.

concentration profiles that diverge from trends in bioactivity measured with in vitro assays. This scenario probably describes at least some of the aforementioned examples[14,15] where GR activity has been detected via bioassays yet specific causative agents cannot be identified by targeted analyses. As another example, Stalter et al.[22] observed an increase in developmental retardation for rainbow trout after ozonation of a membrane-filtered, municipal wastewater, which they attributed to the formation of toxic but unknown transformation products.

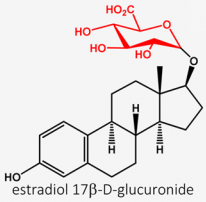
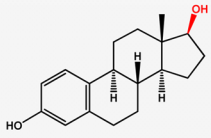
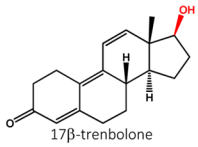
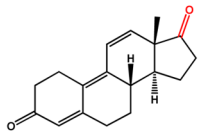
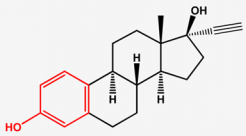
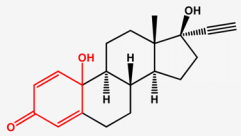
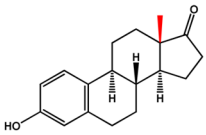
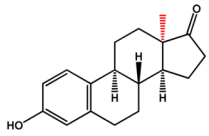
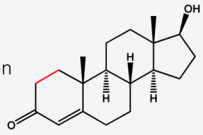
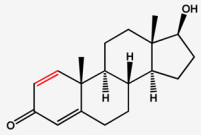
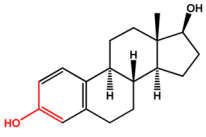
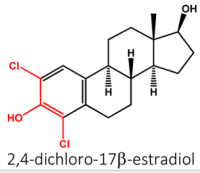
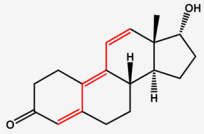
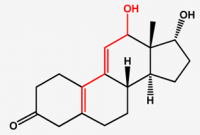
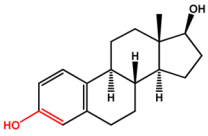
One risk factor only rarely evaluated is the potential for cross receptor reactivity where environmental transformations alter the biological target of the product relative to its parent. Highly specific receptor interactions (i.e., ER, AR, PR, GR) are derived from rather subtle but localized structural differences between steroids. Because the tetracyclic steroid backbone is common to all of these ligands, simple transformations can alter binding between these receptors.[23,24] For example, some synthetic progestins like levonorgestrel, norethindrone, and gestodene are also strong androgens,[24] although this androgenic characteristic would usually not be assessed by study designs focused on progestogenic characteristics. Steroids also can act concurrently as agonists and antagonists for multiple receptors.[25] This complexity complicates accurate characterization of environmental risk because single step transformations of progestins, for example, can yield glucocorticoids or androgens (e.g., progesterone dealkylation forms androstenedione) and androgen aromatization yields estrogens. Dose of the parent and/or transformation product can also be important; some steroids may bind to a specific receptor at low doses, but multiple receptors at higher doses. In addition, there are significant cross-talk mechanisms between receptors and central nervous system targets that impact endogenous steroid biosynthesis, clearance and feedback loops. The common use of a single receptor end point to assess bioactivity upon transformation may therefore underestimate risk in cases where parents and products bind to different nuclear receptors. Similarly, cross-receptor interactions provide a potential explanation for nonlinear uncertainty in some bioanalytical approaches, as multiple receptor interactions potentially all modulate a single adverse outcome such as impaired reproduction.

Examples of Bioactive Transformation Products. Potent synthetic steroids are examples where only slight structural modifications relative to their endogenous analogs can induce drastically higher potency and enable interactions with multiple receptors, including responses not intuitively obvious from structural inspection alone. Ethinyl estradiol (EE2) is perhaps the best known example of a synthetic steroid. It differs from its endogenous analog 17β-estradiol (E2) only by a C17 ethyne group, yet is up to 27-fold more potent than E2 in fish.[26] As another example, the bioactivity of trenbolone, an anabolic growth promoter, is not solely defined by its androgenic properties, but because it also exhibits 37% higher binding affinity to PR than progesterone and also elicits anti-GR activity.[27]

Accordingly, transformations in natural and engineered systems that only slightly alter contaminant structures can imply retained bioactivity and thus not fully mitigate exposure. Table 1 demonstrates some steroid transformations yielding products of known, measured or probable bioactivities.[28−46] As first examples, there is substantial precedent for glucuronide deconjugation in biologically active environments, which regenerates the active parent from an inactive, polar metabolite.[28] Similarly, keto-hydroxy interconversions are common (e.g., testosterone to androstenedione) and yield bioactive, and in some cases more potent, products (e.g., formation of 17β-estradiol from estrone).[28−30]

Further examination of the literature reveals additional laboratory reports of single-step transformations that also retain receptor binding activity. Methyl inversion during photolysis of estrone produces lumiestrone, which is 40% as estrogenic as its parent.[37] Photolysis of trenbolone metabolites yields photohydration products that not only exhibit unique

Table 1. Reported environmental transformations with known or suspected bioactive steroidal products

Reaction	Parent	Product	Description and Sources
Deconjugation	 estradiol 17β-D-glucuronide	 17β-estradiol	Steroids are predominantly excreted from humans and animals in the form of biologically less active glucuronide and sulfate conjugates. Glucuronide conjugates exhibit shorter lifetimes in wastewater treatment systems than sulfate conjugates, with deconjugation occurring by enzymatic hydrolysis.[28]
Hydroxy-Keto Interconversion	 17β-trenbolone	 trendione	A common biological transformation often reported in natural and engineered systems, this pathway results in the interconversion of 17β-estradiol to estrone,[28] testosterone to androstenedione,[29] or, as shown here, the interconversion of different metabolites of trenbolone acetate.[30]
Oxidation	 ethinyl estradiol	 17α-ethinyl-1,4-estradiene-10,17β-diol-3-one	Partial oxidation of ethinyl estradiol using a homogeneous catalytic system (Fe(III)-TAML/H_2O_2) yields epimers of a C10 hydroxylated derivative with estrogenicity greater than the parent.[31] Notably, the same product has been reported for the biotransformation,[32,33] ozonation,[34] and photocatalytic degradation (UV/TiO_2)[35] of ethinyl estradiol.
Epimerization	 estrone	 lumiestrone	Direct photolysis of estrone results in formation of lumiestrone as its major product via photochemical inversion of the C13 methyl group.[36,37] Various *in vitro* assays[36,37] suggest lumiestrone, which is also more photopersistent than estrone, retains estrogenic activity.
Dehydrogenation	 testosterone	 boldenone	Boldenone, or 1-dehydrotestosterone, is the major product for the aerobic bacterial transformation of testosterone across environmentally relevant conditions.[29] Boldenone is also a potent, long-acting anabolic steroid used in veterinary applications.[38]
Chlorination	 17β-estradiol	 2,4-dichloro-17β-estradiol	Of seven products identified from the chlorination of 17β-estradiol, persistent ER activity was attributed to the presence of 2,4-dichloro-17β-estradiol and 2,4-dichloroestrone (also observed). Although not identified, synthetic monochloroestradiol analogs, which were presumed reactive intermediates, exhibited ER activity comparable to estrone.[39]
Hydration	 17α-trenbolone	 12-hydroxy-17α-trenbolone	Direct photolysis of trenbolone acetate metabolites proceeds via photohydration, yielding products that not only exhibit bioactivity unique relative to their parents[40] but also are metastable and decompose to regenerate their parents in the dark.[41] While photohydration occurs for testosterone and androstenedione,[42] reversible photohydration appears limited to dienones (e.g., dienogest) and trienones.
Dimerization	 17β-estradiol	 17β-estradiol dimer	Oligomeric products have been reported during the oxidative enzymatic (e.g., laccase[43] and ligninase[44]) transformation of estrone, 17β-estradiol, and ethinyl estradiol, even at environmental (i.e., ng/L) concentrations. Although poorly researched, steroidal dimers can exhibit unique character relative to monomers,[45] and some propose their role as receptor antagonists by simultaneously binding adjacent receptors.[46] Environmental decoupling also can regenerate bioactive parent steroids.

bioactivity,[40] but also can undergo subsequent dehydration to regenerate the potent parent steroid at high yield in the dark.[41] Yang et al.[29] observed 1,2 dehydrogenation of testosterone by manure-derived bacteria to yield the major product boldenone (i.e., "1-dehydrotestosterone"), an anabolic steroid with AR binding affinity 56% greater than testosterone.[27] Some additional reaction pathways are summarized in Table 1, all of which involve minor to modest structural alterations to yield products with likely bioactivity. In fact, as best exemplified by testosterone (Figure 2), there exist many structural modifications, including some reasonably expected to be environmentally relevant, that retain or create functional groups

dx.doi.org/10.1021/es503425w | *Environ. Sci. Technol.* 2014, 48, 11737−11745

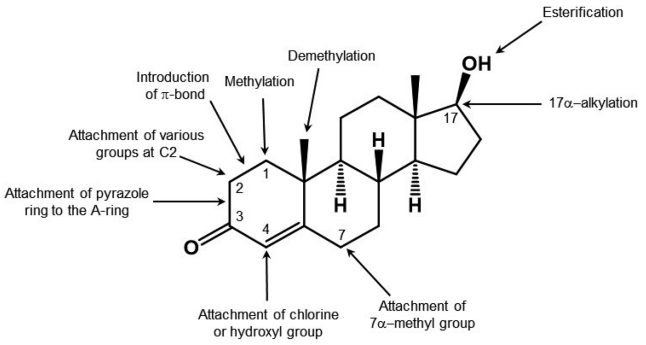


Figure 2. Structural modifications of testosterone known to increase the anabolic potency in the steroidal products (used with permission from Kicman and Gower[47]).

responsible for receptor interactions, even increasing properties like anabolic activity.[47]

Thus, we cannot reasonably expect that all transformation processes will be equally effective at eradicating bioactivity, complicating risk assessment for instances of persistent bioactivity upon parent removal. Certain "soft" transformations inducing only slight structural modifications may be surprisingly ineffective when a molecule's bioactivity is linked to more than just highly localized functional groups. Such soft transformations appear most common for natural attenuation (e.g., solar photolysis, biodegradation), but may also occur in some engineered treatment systems. These types of systems can be conceptualized as "reagent-limited", typically lacking the photo- or biochemical energy to induce drastic structural modifications, much less mineralization. In contrast, "hard" transformations are those inducing dramatic structural modifications that extend beyond localized moieties to encompass changes across the molecule's entirety (e.g., ring cleavage in steroids), although several sequential reactions may be needed to attain extensive structural modification. Such transformations are more typical during chemical oxidation processes where oxidants are often dosed beyond the initial demand to achieve a residual (or excess) concentration.

Conceptual Steps Forward. We propose that accurate assessment of risk mitigation during transformations of bioactive contaminants needs to consider whether any transformation products remain within the "pharmacophore." Overington et al.[2] defined the pharmacophore as the "ensemble of steric and electronic features that is necessary to ensure optimal interactions with a specific biological target structure and to trigger (or block) its biological response." This concept implies that a collection of ligand features collectively explains the potential for and magnitude of any interaction with biological targets. Each mode of action or molecular target has its own pharmacophore (e.g., each nuclear receptor represents a pharmacophore for steroids). Contaminant interactions with different molecular targets (i.e., "side effects") also imply that promiscuity of pharmacophores is possible and even likely, so we should not assume that contaminant bioactivity is necessarily isolated to any single biological end point. Complete characterization of bioactivity may therefore have to consider not only pharmacophore retention, but also the evaluation of distinct and overlapping pharmacophores.

Thus, the potential for adverse ecological risks arises when contaminants embody a pharmacophore and subsequent transformations are best characterized by their potential to destroy (or alternatively preserve) that pharmacophore. It is a major assumption that transformation processes always reduce bioactivity by removing key structural attributes of the pharmacophore. While this is often true and should remain the initial assumption, those cases of retained or even increased overall bioactivity in products indicate that a wider context is necessary. Currently, most fate research and also aspects of the regulatory process define assessment of environmental fate largely in terms of "removal", or the quantitative reduction in contaminant concentration in any particular system.[48] Removal alone is an imperfect surrogate for the characterization of environmental fate and ecological risk; it has no implicit link to any pharmacophore. Thus, we should expect there will be cases where it overestimates reductions in ecological risk.[49] Similarly, ecological risk assessments utilizing persistence in weight of evidence evaluations may be missing what is most important: a quantitative link to bioactivity and hazard for both parent structures and environmental transformation products.

Possible Solutions and Research Opportunities. Despite extensive effort, the overall relevance of much research focused upon defining environmental exposures and persistence often remains very unclear because it lacks strong links to biological hazard. This has historically been addressed via Whole Effluent Toxicity testing as required by U.S. EPA's Clean Water Act, where biological testing of wastewater effluents is used as a surrogate for potential environmental impacts.[50] If toxicity is observed, then a toxicity identification evaluation (TIE), a biologically guided fractionation of complex water samples to determine causality, is employed. Unfortunately, TIE bioassays as suggested by the U.S. EPA[51] utilize exclusively acute toxicity end points that do not account for any subtle biochemical effects associated with endocrine disrupting chemicals. The U.S. EPA did establish the Endocrine Disruptor Screening Program (EDSP) after amendments to the Safe Drinking Water Act and Food Quality Protection Act.[52] Although this comprehensive program was developed primarily to screen commercial chemicals for potential endocrine impacts, it is painfully slow largely because of the reliance on animal testing. Notably, the EPA has recently considered the inclusion of more high-throughput in vitro screening assays as part of the "EDSP of the 21st Century (EDSP21)".[53] To the best of the authors' knowledge, neither of these programs holistically considers transformation products.

Moving forward, it will be imperative to link molecular initiating events to chronic and sublethal effects in aquatic organisms. Such has been the emphasis of the U.S. EPA and the Organization for Economic Cooperation and Development (OECD), which promote Adverse Outcome Pathway (AOP) paradigms for toxicity testing.[54] This approach employs high throughput in vitro receptor based bioassays to screen chemicals for specific AOPs which, if observed, provide a tiered process for additional whole animal testing linking molecular initiating events to population impacts. Utilizing numerous databases such as TOXCAST, data also can be used for in silico models with the ultimate goal of reducing animal testing.[52] While AOPs provide the biological linkages between exposure and adverse effect, there still remains a clear need for exposure assessments within this process.

In this context, we note that most exposure scenarios in receiving waters are best defined as a complex mixture of parent compounds, metabolites and products whose activities may span several different receptor end points at a range of different potencies. However, few ecotoxicology study designs focus on mixture effects or explicitly consider the potential conservation

of bioactivity through environmental transformations. Aquatic organisms near wastewater discharges will be exposed mostly to parent compounds and wastewater-derived transformation products, while downstream organisms will encounter not only persistent parents, but also many environmental transformation products generated via natural attenuation. Furthermore, water quality is vastly different and dynamic around the globe; for instance, seasonal differences can dramatically change the fate characteristics of a particular chemical within a watershed. Until we can capture the complexity and dynamics of these systems with inclusive bioactivity-based characterizations that provide links to biological relevance, our understanding of the ecological implications of contaminant discharge remains incomplete.

Sophisticated chemical analyses such as high-resolution mass spectrometry and compound specific isotopic analysis will certainly play an important role in improved fate characterization for many contaminants, particularly with respect to directed characterization of transformation products.[55] For example, using high-resolution mass spectrometry with bioassay directed fractionation, causative agents can probably be identified. However, while comprehensive, this technique is costly and technically advanced, can be limited by the need for pure standards, and struggles with complex environmental matrices.

Computational approaches should prove especially valuable for achieving high throughput screening, both from the perspective of predicted environmental fate and also for possible biological characteristics of products as a complete first pass in silico assessment. Currently, computational fate approaches are most mature for biotransformation, where tools like the University of Minnesota's Biocatalysis/Biodegradation Database[56] can predict probable transformation pathways. The use of electronic theory to predict transformation products[33] also could be expanded and validated to better characterize contaminant fate. Outputs from computational fate modeling can subsequently be integrated with in silico tools such as molecular docking models to investigate conserved bioactivity within products and mixtures quickly and relatively easily. Molecular docking tools and high throughput screening methods are improving rapidly and can have excellent accuracy for evaluating nuclear receptor interactions and screening multiple end points.[57] Applying these techniques, originally developed for drug discovery, to environmental research should be especially promising, although these approaches are limited to known receptor end points and do miss complex interactions. Notably, such tools have been proposed for AOP frameworks to provide in silico linkages between fate, exposure, and threshold end points, which can be directly utilized in risk assessments.

We therefore advocate an integrated and collaborative approach relying on complementary chemical analysis, bioanalytical tools, and predictive computational approaches to help understand environmental mixtures and improve TIEs for the cases lacking identification of causative agents in bioactive samples. What is the next logical step when sophisticated chemical analyses cannot identify causative agents? Based upon themes well developed in the drug discovery literature, we would argue that causality is probably best explained by structures similar to known high affinity ligands because a high affinity backbone structure (i.e., the steroidal rings) provides the basis of a pharmacophore. Beginning computational assessments with known high affinity ligands as parents may intrinsically provide guidance for subsequent directed analysis of bioactive products. Such a tool has been developed for the ER by U.S. EPA and is known as the ER Expert System (ERES). Using an inventory of 893 entries, the model has been proposed as a prioritization mechanism for Tier 1 testing through the EDSP.[58]

This integrated approach will also help to develop criteria for identifying high risk contaminants and prioritizing pollutant classes and functionalities likely to generate bioactive products. Such prioritization schemes can be based upon traditional aspects including parent compound potency but also incorporate reactivity to account for product risks. This may improve the analysis of complex wastewater or agricultural runoff mixtures by providing analytical targets for products and metabolites. Classically, we have viewed limited persistence and rapid reaction rates as desirable fate outcomes, yet there will be instances where these characteristics will be disadvantageous when they create persistent, bioactive products that are difficult to detect via directed analyses. To prioritize transformations, our focus should be placed on identifying potent compounds highly susceptible to single step environmental transformations with reasonably persistent and bioactive products. We should also alter our experimental and modeling approaches to reconsider product stability for high risk contaminants. Most experimental data gathered using fixed conditions and relatively short time scales provide little insight of long-term product fate, particularly when subsequently exposed to dynamic environments with redox gradients, diurnal (light/dark) cycles, and diverse reactive entities in both natural and engineered systems. For example, while crude, there is much to be gained by subjecting product mixtures to stark contrasts, such as comparing stability at low pH versus high pH, aerobic versus anoxic redox conditions, light versus dark systems, or in the presence of reagents used in treatment (e.g., free chlorine). These relatively simple diagnostics would help to define the spectrum of reactivity expected for product mixtures across diverse aquatic systems.

We would be remiss not to consider the role of engineered treatment in controlling the risks associated with bioactive transformation products, specifically because a significant portion of trace organic loading to the environment occurs through wastewater effluent subjected to physical, biological, and/or chemical processes. Transformation products are expected in treated effluent; many organic chemicals are relatively recalcitrant to biological treatment or simply transformed into more stable structures, and organic contaminants are rarely mineralized during disinfection and oxidation processes. For example, most water is disinfected with chlorine, yet the degree to which chlorine can oxidize organic contaminants is highly dependent on water quality and operational conditions. The extent of transformation in chlorine systems can be critical with respect to formation of bioactive products. A notable example is Hu et al.,[39] who observed estrogenic products after 17β-estradiol chlorination and identified several chlorinated derivatives (e.g., 2,4-dichloro-17β-estradiol and 2,4-dichloro-estrone) likely responsible for persistent bioactivity. Considering alternative oxidants, studies indicate that while ozone can be highly effective for estrogenicity attenuation in wastewater, the resulting by-products can be toxic, though also biodegradable and thus relatively easily removed.[22] In fact, it is only with chemical oxidation via advanced oxidation processes that the majority of trace organics appear well attenuated, although the dissolved

organic carbon is only meagerly reduced.[59] Collectively, research to date demonstrates that both weak and powerful oxidants, as well as UV light, can lead to the formation of transformation products that retain, accentuate, or create bioactivity during water treatment. Considering the increasing interest in the use of ozonation and other forms of oxidation for chemical contaminant attenuation, more research is warranted to identify, biologically characterize, and attenuate the resulting products. Ultimately, these data should contribute to the life cycle assessment of various treatment trains to better balance the overall financial and energy costs versus actual improved protection of environmental and public health.

We note that although we have focused our discussion here upon transformations of endogenous and synthetic steroids, we believe concepts of conserved structure and conserved bioactivity apply more generally to other contaminant classes. For example, microbial transformation of nonylphenol polyethoxylates results in enrichment of highly estrogenic isomers.[60] Other potent compounds whose transformation products might merit prioritization or at least closer scrutiny include chemotherapeutic agents, naturally occurring toxins, and allelochemicals. In particular, many pharmaceuticals can occur as enantiomers: mirror image, nonsuperimposable isomers with surprisingly distinct bioactivity. Treatment processes can selectively transform one enantiomer or potentially result in interconversion,[61] with both cases likely to result in conserved bioactivity in effluent. Finally, rather than conserved bioactivity there exist several examples where transformation of seemingly benign substances produces more bioactive or even toxic transformation products. Brinkmann et al.[62] recently demonstrated the development of ER binding capabilities for hydroxylated biotransformation products of heterocyclic aromatic hydrocarbons whose parent structures have no intrinsic ER activity. Similar are reports[63] of genotoxic and mutagenic transformation products arising from use of medium pressure UV (polychromatic UV) in the presence of NOM and nitrate.

In summary, it is clear that environmental fate approaches originally designed for persistent or acute toxicants may not be equally effective for reactive contaminants that induce chronic or sublethal effects. Persistence itself is not an adverse outcome, merely a risk factor, and the critical issue for prioritizing contaminant occurrence is a strong relationship between bioactivity and potential adverse effects on organisms. As a community, we may need to refine our approach to evaluating environmental fate toward a greater focus on biological hazard to account for inherent challenges posed by potent bioactive contaminants and their transformation products. Moreover, we should not think of bioactive contaminants, including steroids and many other pharmaceuticals, as lone islets of bioactivity in a surrounding sea of benign structures. Rather, they are islands among larger archipelagos of bioactivity linked together by structural commonalities. Environmental transformation processes will in some cases allow for stealthy movements between these islands, and this possibility should not be neglected because these movements provide pathways to unintended consequences. Many factors ultimately affected the choice of which individual bioactive structure was selected for the commercial products that are eventually released into the environment, but we also must consider their surrounding families when we consider environmental hazard. Utilization of effects driven bioanalytical techniques is probably the most cautious and insightful approach to characterization of this complex terrain.

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Notes

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