

Ecotoxicological Risk of Mixtures

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Abstract

Water pollution is a global environmental challenge that nearly always involves the degradation of aquatic habitats by mixtures of chemical contaminants. Despite this practical reality, environmental regulations and resource management institutions in most countries are inadequate to the task of addressing complex and dynamic combinations of chemicals. Moreover, our scientific understanding of mixture toxicity and the assessment of corresponding risks to aquatic species and communities have not kept pace with worldwide declines in biodiversity or the introduction of thousands of new chemicals into societal use. In this chapter, we review recent research specific to mixtures in three contexts that are broadly applicable to freshwater and marine ecosystems. These include oil spills, urban non-point source pollution, and the agricultural use of modern pesticides. Each of these familiar and geographically extensive forcing pressures is threaded with uncertainty about interactions between contaminants in mixtures. We also briefly consider relevant and often overlapping environmental regulations in the United States and Europe to illustrate why a proactive consideration of chemical mixtures remains elusive in institutional ecological risk assessment. As the case examples show, however, the problem of mixtures is not intractable and targeted research can guide effective conservation and restoration strategies in a chemically complex world.

Keywords: Ecological risk assessment; Environmental monitoring; Habitat; High-throughput screening; Non-point source pollution; Oil spills; Pesticides; Stormwater; Synergism.

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Introduction

In aquatic ecosystems, exposure to mixtures of toxic chemicals resulting from human activities is the norm, and assessing the biological effects of chemical mixtures remains an enduring challenge in the field of ecotoxicology (Lydy et al., 2004; Villanueva et al., 2014). Examples of anthropogenic sources of chemical mixtures include oil spills, agricultural uses of pesticides,

wastewater discharges containing pharmaceuticals and personal care products, and urban stormwater runoff. Monitoring provides ample evidence that chemical mixtures contaminate surface water (Gilliom, 2007), ground water (Toccalino et al., 2012), and drinking water (Donald et al., 2007) in urban, agricultural, and undeveloped areas. Likewise, numerous studies document adverse effects of exposure to chemical mixtures in fish (e.g., Incardona et al., 2013), invertebrates (e.g., Bjergager et al., 2012), amphibians (e.g., Hayes et al., 2006), and aquatic communities (e.g., Relyea, 2008), even when mixture constituents are present at very low concentrations.

Given that thousands of synthetic chemicals are currently in societal use worldwide, it would be logistically impossible to empirically assess the toxicity of each possible combination to aquatic species. Moreover, the co-occurrence of chemical and nonchemical stressors such as pathogens, thermal extremes, and salinity gradients further complicates the ecotoxicology of mixtures in natural systems. This environmental complexity is increasingly necessitating the adaptation of ecological theory, particularly in the design of field studies (Relyea and Hoverman, 2006; Rohr et al., 2006).

Despite the inherent difficulties of measuring the toxicity of complex chemical mixtures, several methods have been developed including concentration-addition and independent-action models, toxic unit approaches, and probabilistic methods (Backhaus and Faust, 2012; Nowell et al., 2014). These methods are mostly limited to cases of noninteracting chemicals where the individual constituents have been identified and the concentration–response relationship of single chemicals is known. This includes, for example, toxicity estimates for pesticides with similar and dissimilar modes of action using concentration-addition and response-addition models, respectively (Belden et al., 2007; Backhaus and Faust, 2012). The accurate prediction of chemical interactions in mixtures, particularly those that result in synergistic toxicity, remains a priority in aquatic ecotoxicology (Cedergreen, 2014). Although some attempt has been made to model complex interactions (see Rodney et al., 2013), the ability to predict chemical interactions is hindered by the lack of a generally acceptable conceptual framework.

A longstanding goal in ecotoxicology is the greater inclusion of environmental realism in the design of scientific studies, in both the laboratory and the field (Chapter 6). On the “eco” side, this has included a push for research on native species (vs laboratory models) (Chapter 9), the scaling of toxicological effects from individuals to populations and communities, the use of ecological concepts to guide study design, and hypothesis-driven experimental field manipulations. On the “tox” side, there has been a corresponding push to embrace environmental complexity, including chemical mixtures, interactions between chemical and nonchemical stressors, indirect (i.e., food web-based) impacts, and harmful toxicological effect that are delayed in time. In this way, our evolving understanding of chemical mixtures has been, and will continue to be, at the forefront of advancements in aquatic ecotoxicology.

In the United States and Europe, mixtures toxicity is not routinely assessed under the regulatory authority of the federal and state governments that administer environmental laws and statutes. As such, most chemicals are regulated on a single-chemical basis, which may lead to an underestimation of actual risk facing aquatic communities exposed to chemical mixtures. A nonsystematic approach to assessing chemical mixtures toxicity may lead to different toxicity predictions, varying degrees of chemical regulation, and ultimately different interpretations of risk. In this chapter, we discuss recent research specific to mixtures in three contexts (i.e., oil spills, urban non-point source pollution, and current-use agricultural pesticides) that are reflective of anthropogenic stressors in aquatic ecosystems worldwide. Additionally, we discuss the importance and challenges of assessing chemical mixtures under the authority of certain overlapping environmental statutes.

18.1 Worldwide Oil Spills

Over the past few decades, technological improvements have generally led to a decline in the total volume of petroleum spilled from production and transportation processes into the world's oceans (Council, 2003). Nevertheless, large marine oil spills still occur with regularity. These include, for example, the 2002 *Prestige* tanker spill off the coast of Spain, the 2007 *Hebei Spirit* tanker spill off the coast of South Korea, the 2009 Montara/*West Atlas* wellhead blowout in the Timor Sea, and the 2010 Macondo/*Deepwater Horizon* disaster in the Gulf of Mexico. Moreover, with increasing overland pipeline transport of products such as diluted bitumen from oil sands extraction, accidents near freshwater habitats are likely to increase in frequency such as the pipeline spills into the Yellowstone River in 2011 and 2015. Although larger oil spills are highly publicized and intensively studied, the dramatic increase in global shipping traffic will likely lead to an increase of smaller fuel oil spills stemming from container ship accidents. There has yet to be a formal quantification of the frequency of these smaller spills, but this number clearly has increased along with container ship traffic, based on the frequency of internet news articles on container ship accidents over the past 15 years. Although much smaller in scale, these spills can have significant localized impacts on marine resources, as indicated by the effects of the 2007 Cosco Busan fuel oil spill on Pacific herring (*Clupea pallasii*) spawning grounds in San Francisco Bay (Incardona et al., 2012a). An improved understanding of the toxicity of petroleum-derived mixtures is critical for guiding spill response activities, assessing natural resource injury in the aftermath of spills, and to better support the reparation and restoration processes.

Crude oils are complex mixtures that contain thousands of different compounds as well as elemental metals. Geologically distinct crude oils differ primarily in the ratios of large and interrelated families of compounds (Stout and Wang, 2007), including a range of aliphatic and aromatic hydrocarbon classes, resins, asphaltenes, and polar compounds containing nitrogen, sulfur, or oxygen atoms. The relative composition of these compounds determines the general physicochemical properties of a given crude oil. For example, the lighter, low-sulfur crude

oils from the Northern Gulf of Mexico have proportionally less sulfur-containing aromatic heterocycles than heavier, sulfur-rich crude oils from Alaska or the Middle East (Wang et al., 2003). A major challenge for understanding mixtures toxicity relating to crude oils and their products is that mixtures are changed by the refining process, and mixtures change over time after petroleum products are released into marine and aquatic environments.

The smaller but more frequent spills of marine fuel oils present a challenge with a distinct suite of characteristics. Bunker fuels, the generic term applied to fuel stored on board ships, typically consist of a highly viscous heavy residual oil mixed with a lighter fuel (typically diesel), to facilitate pumping and flow. Because of the nature of modern refinery practices, these residual oils have chemical compositions that are distinct from unrefined (parent) crude oils (Uhler et al., 2007). By definition, residual oils are the dregs of the refining process, and have often been subjected to conversion processes such as catalytic cracking (Matar and Hatch, 2001). Consequently, many chemical and elemental components of the parent crude oil are much more highly concentrated in the remaining residual oil (Clark and Brown, 1977; Wang et al., 2003; Uhler et al., 2007). Residual oils and mixed fuel oil products have a higher percentage and total mass of relatively more water-soluble aromatic compounds and larger fractions of uncharacterized polar compounds (an “unresolved complex mixture”) that can approach 30% of the mass (Clark and Brown, 1977). Residual fuel oils also often contain a higher metals content, typically nickel and vanadium (Matar and Hatch, 2001). Generally, there is a higher degree of chemical heterogeneity among modern residual fuel oils (Uhler et al., 2007).

In the case of any marine or aquatic oil spill, be it crude or residual fuel, different taxa can be exposed to different mixtures from the same oil depending on the exposure pathway. Although there are likely tens of thousands of unique compounds in crude oil, only a small fraction of these have appreciable water solubility, with less than 1% becoming dissolved in water (Gros et al., 2014). Thus, mixtures can be extremely different if organisms are exposed to either whole particulate oil or water-dissolved components, or both. If oil is dispersed into small droplets, filter-feeding organisms can be exposed to whole oil through ingestion of droplets, for example. “Weathering” of petroleum released into the environment changes both the composition of oil that remains and the composition of dissolved mixtures derived from that oil. Weathering includes both biological processes (e.g., microbial degradation) and abiotic (physicochemical) factors such as volatilization, photodegradation, and dissolution. Dissolution weathering determines the composition of water-soluble mixtures that are available for uptake by lipid-rich organisms such as fish embryos.

Dissolution weathering represents a major challenge for understanding oil spill toxicity because it leads to a virtually constant change in mixture composition over time. This process has been best characterized in the context of a gravel/cobble shoreline contaminated by stranded oil, emulating the shoreline oiling in Prince William Sound following the 1989 *Exxon Valdez* spill (Short and Heintz, 1997). However, a similar process governs the dissolution of water-soluble compounds from an oil slick or dispersed oil droplets in open water

(Gros et al., 2014). As discussed in detail below, toxicity studies on petroleum products have focused extensively on polycyclic aromatic hydrocarbons (PAHs) because of previously known toxicity of these compounds and their general, albeit low, water solubility and bio-availability. Solubility of PAHs decreases with increasing molecular weight, as determined by both the number of rings and degree of alkyl substitution. For example, two-ringed naphthalene (molecular weight 128) has higher solubility than three-ringed phenanthrene (molecular weight 178); parent phenanthrene in turn has higher solubility than a multiply methylated C4-phenanthrene (molecular weight 234). Dissolution of PAHs as water percolates through oiled gravel follows first-order loss-rate kinetics (Short and Heintz, 1997). Consequently, oil-derived PAH mixtures are initially dominated by the most water-soluble compounds (e.g., naphthalene, parent three-ringed compounds). Over time, the mixture becomes dominated by the less water-soluble alkylated compounds (Figure 18.1).

Petroleum toxicity represents an exceptionally complicated example of the challenge of mixtures ecotoxicology. This is due to the dynamic complexity of the mixture over time, even for situations in which the toxic properties of many of the component PAHs are known. For organisms exposed to whole oil, the uncertainty is magnified by the large number of unidentified compounds in the mixture, together with known compounds with poorly characterized toxicity. Nevertheless, there has been a considerable effort in recent years to break down complex PAH mixtures into component parts to identify which compounds are driving harmful impacts to aquatic species, particularly developing fish exposed to water-soluble components of oil.

A major lesson learned from the *Exxon Valdez* oil spill is that developing fish embryos are very sensitive to low-level exposure to crude oil, which leads to a syndrome of developmental defects related to heart failure (Incardona et al., 2011a). This form of crude oil developmental toxicity was attributed primarily to the proportional content of PAHs and related families of heterocyclic nitrogen, sulfur, or oxygen compounds, such as dibenzothiophenes. Uptake by oil-exposed organisms is more likely given their higher water solubility relative to aliphatic hydrocarbons and polycyclic alkanes (Council, 2003; Carls and Meador, 2009). Early studies with embryos of pink salmon (*Oncorhynchus gorbuscha*) and Pacific herring showed that toxicity per unit mass of total dissolved PAHs increased as the mixture shifted over time from compositions dominated by two-ringed naphthalenes to three-ringed fluorenes, dibenzothiophenes, and phenanthrenes (Marty et al., 1997; Carls et al., 1999; Heintz et al., 1999). In the past decade, studies of single PAH compounds, primarily using the zebrafish model, and isolated petroleum fractions further linked developmental toxicity in fish to three-ringed compounds (Incardona et al., 2004, 2005; Adams et al., 2014a). The zebrafish studies also showed that the etiology of the petroleum developmental toxicity syndrome was disruption of embryonic cardiac function and morphogenesis (Incardona et al., 2005; Adams et al., 2014a). It is now well-established that both crude and residual oils from different geological sources disrupt heart development in a diversity of fish species (Incardona et al., 2009; Hatlen et al.,

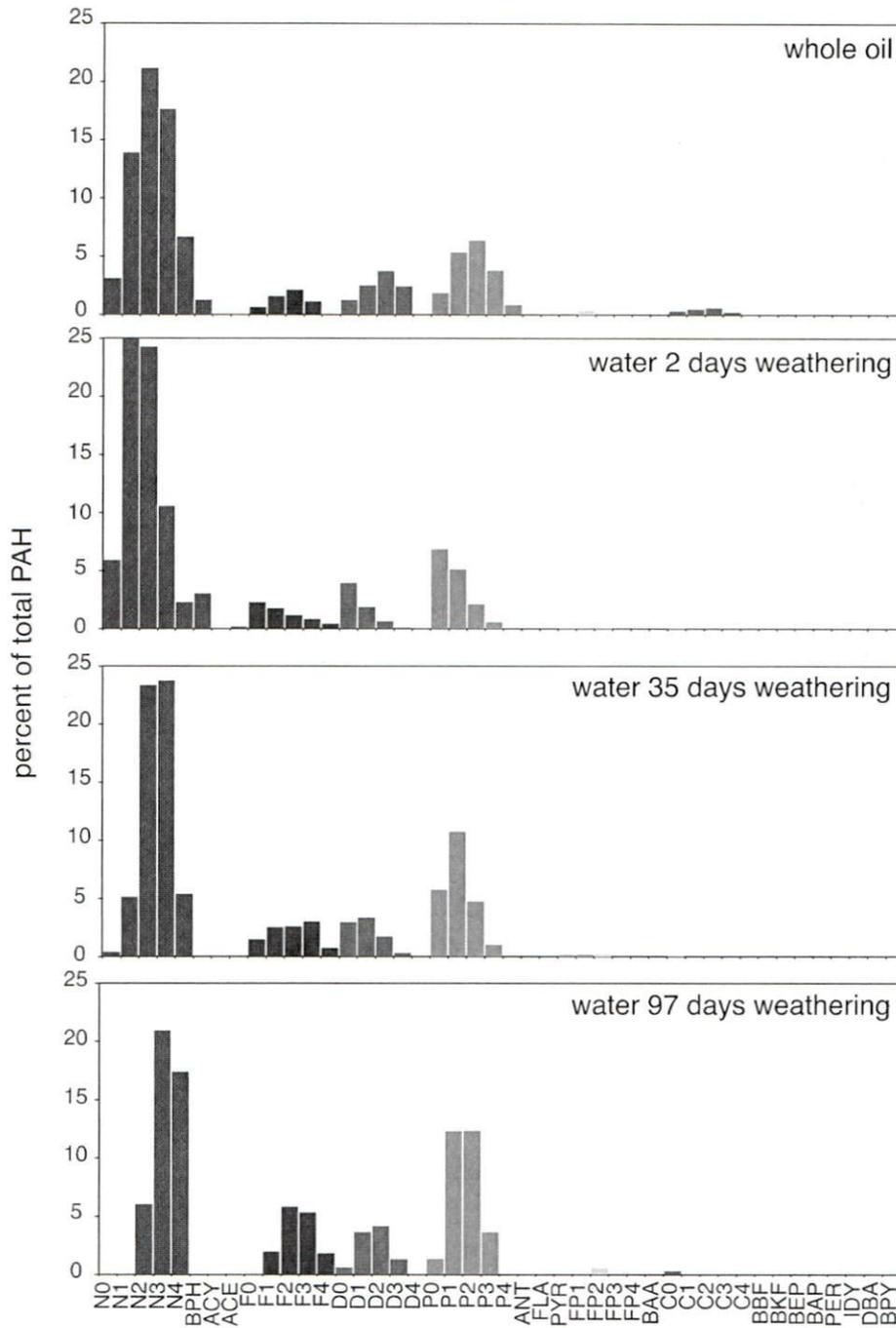


Figure 18.1

Gravel coated with Alaska North Slope Crude oil was weathered with freshwater for zebrafish embryo exposures (see Hicken et al., 2011). PAH compositions are shown for the source oil (top) and in extracts of water at the indicated days of water flow through the column (weathering). ACE, acenaphthene; ANT, anthracene; ACY, acenaphthylene; BAA, benz[a]anthracene; BAP, benzo[a]pyrene; BBF, benzo[b]fluoranthene; BEP, benzo[e]pyrene; BKF, benzo[k]fluoranthene/benzo[k]fluoranthene; BPH, biphenyl; BPY, benzo[ghi]perylene; C, chrysene; D, dibenzothiophene; DBA, dibenz[a,h]anthracene/dibenz[a,c]anthracene; F, fluorene; FLA, fluoranthene; FP, fluoranthenes/pyrenes; IDY, indeno[1,2,3-cd]pyrene; N, naphthalenes; P, phenanthrene; PER, perylene; PYR, pyrene. Parent compound is indicated by a 0 (e.g., N0), whereas numbers of additional carbons (e.g., methyl groups) for alkylated homologs are indicated as N1, N2, etc.

2010; Incardona et al., 2012a, 2013; Jung et al., 2013; Incardona et al., 2014), by a mechanism involving the blockade of potassium and calcium ion channels essential for excitation-contraction coupling in heart muscle cells (Brette et al., 2013). These studies all implicate the three-ringed PAHs (and sulfur heterocycles), although direct evidence remains to be demonstrated (e.g., receptor binding).

Studies of crude oil developmental cardiotoxicity have thus been simplified to a degree because of the common biological response to an array of different oil types with seemingly different chemistries. This is consistent with a subset of the PAHs causing most (but not all) of the toxicity. However, there are still many open questions and unresolved issues relating to crude oil toxicity and other complex PAH mixtures. First, the cardiotoxic potencies of single PAH compounds are far less than the potency of oil-derived mixtures, and there is currently no clear explanation for this. Simple models based on additivity or classical metabolic synergism are unlikely to account for the potency of PAH mixtures.

Second, the developmental toxicity of PAH mixtures becomes yet more complicated when considering habitats affected by industrialization and urbanization in which PAH mixtures include the typical petrogenic compounds plus an array of higher molecular four- to six-ringed compounds typically derived from combustion (pyrogenic PAHs). Single-compound studies have indicated a variety of mechanisms for the toxicity of the higher molecular weight PAHs, including dioxin-like cardiotoxicity mediated by the aryl hydrocarbon receptor (AHR), AHR-independent cardiotoxicity, and metabolism-dependent toxicity (Incardona et al., 2006, 2011b; Van Tiem and Di Giulio, 2011; Jayasundara et al., 2015). Thus the potency and specific effects of different petrogenic and pyrogenic PAH mixtures are likely to arise from much more complicated interactions and include contributions from not only additivity and classical metabolic synergism, but also from distinct synergies between independent toxic mechanisms converging on the cardiac system (e.g., disruption of excitation-contraction coupling by ion channel blockade combined with AHR-dependent disruption of normal cardiac gene expression).

Finally, there is very little information on the interaction between the toxicity of PAH mixtures and other environmental stressors. The potential importance of these interactions is highlighted by the markedly enhanced toxicity of PAHs (and/or other petroleum-derived compounds) by photosensitization reactions with ultraviolet radiation in sunlight (Arfsten et al., 1996; Yu, 2002; Diamond, 2003). This represents an entirely different form of lethal toxicity in transparent organisms such as fish embryos and can occur at tissue concentrations below that causing serious cardiotoxicity (Barron et al., 2003; Hatlen et al., 2010; Incardona et al., 2012b). Interactions with sunlight are particularly important for residual oils, which have a very potent phototoxicity associated with uncharacterized compounds rather than the conventionally measured PAHs (Hatlen et al., 2010; Incardona et al., 2012b). The large-scale uses of chemical dispersants in the response to the *Deepwater Horizon*—MC252 oil spill prompted a renewed focus on oil-dispersant interactions. However, most studies indicate that

dispersants primarily increase the bioavailability of oil compounds such as PAHs, rather than specifically enhancing toxicity or acting synergistically (e.g., Adams et al., 2014b). Nevertheless, few studies have addressed this issue with a high degree of rigor using physical biochemical methods appropriate for assessing interactions between hydrocarbons, dispersant surfactants, and biological membranes.

Despite major advances in the past decade described previously, much about the ecotoxicology of petroleum-derived mixtures remains unknown. This is understandable given the considerable chemical complexity of petroleum, but progress is hampered by lack of consistent policy or funding for proactive research. Almost all of our understanding of oil spills is based on assessment activities developed in response to a spill—after the fact. Future research needs to continue along the lines described here, to dissociate the biological effects of individual oil hydrocarbons as drivers for the toxic potency of dynamic mixtures. Moreover, whereas traditional toxicity testing generally relies heavily on model organisms and standardized bioassays, natural resource injury assessments usually involve wild species. At a minimum, future work should focus on (1) characterization of mixture toxicity in species native to areas where future spills are likely to occur or recur, such as the Arctic and the Gulf of Mexico; (2) interactions between toxic oil and other environmental stressors such as temperature (particularly the extreme cold of the Arctic) and sunlight; and (3) understanding how toxic effects at the level of target organs (e.g., the heart) or processes (e.g., development, growth) in individual organisms translates into larger scale ecosystem-level impacts.

18.2 *Urban Stormwater Runoff*

At present, one of the most important and complicated aquatic conservation challenges worldwide is urban stormwater runoff. For the latter part of the twentieth century, most developed countries were successful at controlling end-of-pipe or point source pollution discharges from municipal and industrial sources. However, these efforts have been less effective at reducing diffuse land-based runoff to aquatic habitats. In urban watersheds, rainfall that would normally infiltrate to soils falls instead on impervious surfaces, including the roofs of residential and commercial buildings, parking lots, streets and roads, highways, and similar types of land cover. The corresponding increase in surface runoff creates significant problems related to both water quantity (i.e., urban flooding) and water quality, the latter a consequence of stormwater mobilizing complex mixtures of toxic chemicals.

Waterways near urban areas throughout the world are almost universally afflicted with the so-called “urban stream syndrome” (Walsh et al., 2005). The syndrome is characterized by poor water quality, declines in aquatic species abundance and diversity, and the proliferation of nonnative, pollution-tolerant taxa. However, urban streams are also negatively affected by a variety of physical habitat-forming processes, including extreme flow regimes, erosion and sedimentation, loss of stream channel complexity, and loss of substrate. The relative

contributions of physical and chemical drivers to species declines are not well understood. This creates a frequent dilemma in the arena of urban habitat restoration (i.e., whether and to what extent to expend resources to improve water quality) as opposed to more conventional physical habitat restoration.

Urban stormwater is chemically complex, and numerous environmental monitoring studies have shown that motor vehicles are major sources of toxic contaminants. These originate from vehicle exhaust, the wearing of friction materials (brake pads), leachate from tires, and the leaking of lubricants, fuel additives, and other materials. Untreated runoff from roadways and parking areas therefore contains a highly diverse mixture of chemicals, only a fraction of which has been assessed for toxicity to aquatic species. These include the previously discussed PAHs from internal combustion engines, incomplete fuel combustion, oil, and grease. Urban runoff also contains metals from brake pad wear and other sources (e.g., metal roofs, other building materials), most notably copper, zinc, nickel, lead, and cadmium. For the most part, studies of stormwater quality in the built environment have focused on fluctuating concentrations of PAHs and metals (e.g., Shinya et al., 2000) as a consequence of varying rainfall patterns, antecedent dry intervals, traffic density, and various mitigation measures.

The toxicity of PAHs and metals to fish and other aquatic organisms, alone and in combination, are reasonably well understood. This is due, in part, to the fact that these contaminants pose ecological hazards in many other contexts. In the case of PAHs, for example, this includes oil spills and legacy industrial contamination from the past century. Certain PAHs known to be cardiotoxic to fish are prevalent in both crude oil and urban runoff (Incardona et al., 2011a), and decades of research on the former have informed more recent science on the latter. For example, developing zebrafish embryos and larvae exposed to stormwater-derived mixtures of PAHs (McIntyre et al., 2014) show nearly the same symptoms of cardiac dysregulation and heart failure evident in zebrafish exposed to PAHs from crude oil (Incardona et al., 2013). The ecotoxicology of metals have also been studied for many years in association with the impacts of mining, wood preservatives, antifoulant paints, and common pesticides.

Among toxics, the predominant focus on PAHs and metals in urban stormwater science (e.g., Brown and Peake, 2006) has captured only part of the picture, however. There are hundreds of additional chemicals in urban runoff, most of which are very poorly understood in terms of toxicity to aquatic species. They include chemicals used in the manufacture of tires, uncharacterized components of refined fuels and fuel additives, oxygenated hydrocarbons, surfactants, plasticizers, nanomaterials, and the like. Furthermore, the contaminant exposure scenario gets considerably more complicated when stormwater conveyance systems are connected to sewerage systems, leading to combined sewer overflow events during intense rainstorms (see example, in Figure 18.2). Where this occurs, the chemicals in impervious surface runoff are discharged to receiving waters in combination with a broader mixture of pharmaceuticals, personal care products, flame retardants, and other chemicals used in everyday household life.

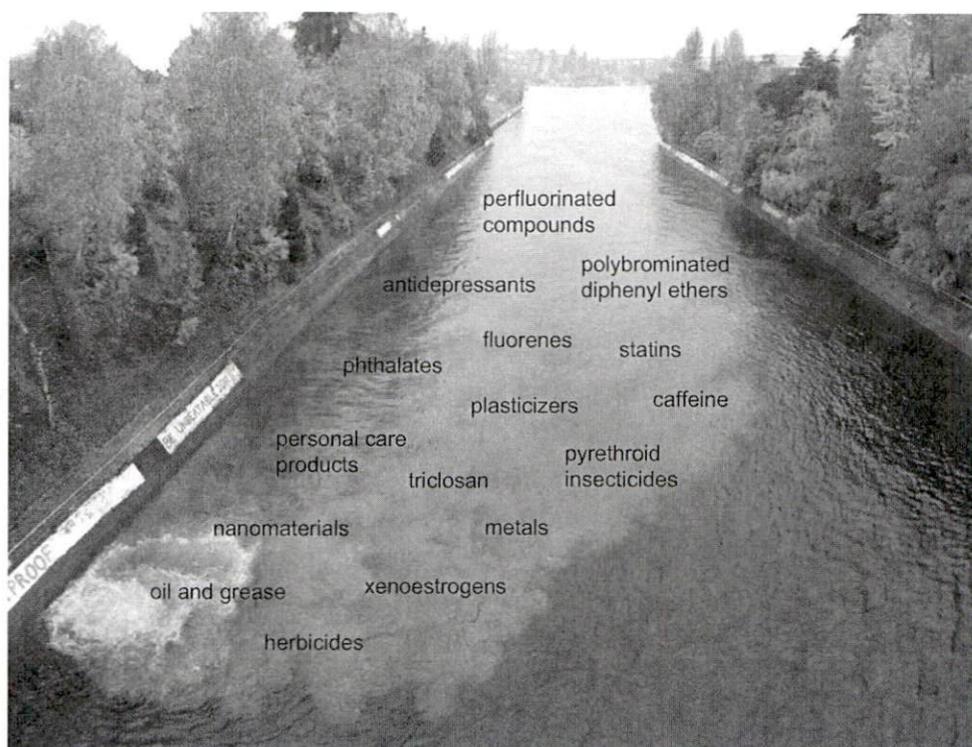


Figure 18.2

Combined inputs of stormwater and untreated sewage to an urban waterway in Seattle, Washington, during a period of high rainfall. Subsurface discharge from a combined sewer overflow (CSO) is evident in the lower left. *Photo by Blake Feist, National Oceanic and Atmospheric Administration (NOAA).*

Despite the ubiquity of urban runoff around the world, the toxic impacts to aquatic biota remain, surprisingly, poorly understood. In the United States, researchers began turning their attention to “unrecorded pollution” in the years following the passage of the 1972 Clean Water Act (e.g., Pratt et al., 1981). In the ensuing decades, urban runoff has been tested primarily using a range of different bioassays, with various toxicity identification and evaluation procedures to correlate measured contaminants with observed effects on species growth or survival (e.g., Kayhanian et al., 2008). In general, urban runoff is more acutely toxic to aquatic invertebrates than to fish, and the initial phase (the first flush) is usually more toxic than runoff collected subsequently during a storm event. More recently, modern molecular tools available for zebrafish and other species have proven useful for confirming contaminant exposure (e.g., upregulation of *cyp1a* gene expression) and for shedding light on which components of complex chemical mixtures in stormwater are driving more nuanced, sublethal toxicity (McIntyre et al., 2014).

Nevertheless, conventional bioassays have remained the state of the science in urban runoff toxicology, often using nonnative model species (but see Snodgrass et al., 2008; McIntyre et al., in press). Thus, although freshwater ecologists have widely documented the urban

stream syndrome (“eco”) and civil engineers and their colleagues have shown that urban runoff is usually toxic (“tox”), the two areas of science have not converged in recent decades to the extent that we know which chemical components are driving real-world biological decline in receiving waters. Moreover, as noted earlier, this may include uncharacterized contaminants that are simply not being monitored in the laboratory or the field.

As a case example, a research effort focused on adult coho salmon has been ongoing for more than a decade in the urban streams of Puget Sound, in the vicinity of Seattle in the northwestern United States. Coho salmon return from the ocean to coastal urban watersheds in the fall of each year, when autumn rains increase surface flows in spawning habitats. In the late 1990s, spawner surveys took note of fish behaving erratically, including surface swimming, gaping, loss of orientation, and loss of equilibrium. Affected fish died within a few hours, and in most cases females did not survive long enough to spawn (Scholz et al., 2012). Overall mortality rates have been high over the years (>50% of the total run within an urban watershed each fall), at levels very likely to limit the long-term viability of wild coho populations in Puget Sound (Spromberg and Scholz, 2011). This remains an important resource management challenge because coho are commercially important, are culturally iconic, and are designated as a species of concern under the US Endangered Species Act.

The evidence now suggests that coho spawners are succumbing to urban runoff, but the causative agents remain unidentified (Scholz et al., 2011). The severity of the recurring fish kills across urban watersheds is proportional to the amount of impervious surface and, in particular, the density of roads (Feist et al., 2011). This suggests a role for contaminants originating from motor vehicles. However, controlled exposures to artificial stormwater containing realistic mixtures of metals and PAHs do not produce the mortality syndrome in otherwise healthy adult coho (Spromberg et al., submitted). Rather, this requires exposure to actual highway runoff, which rapidly (within 4h) evokes the same suite of symptoms as observed in field surveys. As further evidence for a toxic chemical component, pretreating highway runoff with conventional bioinfiltration (a form of green stormwater infrastructure) completely reverses the lethal impacts on coho spawners (Spromberg et al., submitted). Therefore, stormwater is very likely conveying an as-yet unidentified contaminant(s) from motor vehicles to coastal drainages where it consistently kills an ecologically important migratory fish species. Importantly, it would be impossible to infer this phenomenon from conventional toxicity bioassays.

18.3 Pesticide Mixtures

According to recent estimates, approximately 5.2 billion pounds of pesticides were used globally in 2006 and 2007, with nearly 1.1 billion of those pounds applied in the United States alone (EPA, 2011). Pesticides are grouped into classes and defined by their intended biological target, chemical structure, and mode of toxic action (Fulton et al., 2014; Solomon et al., 2014). For example, organophosphates and pyrethroids are classes of neurotoxic

insecticides that are widely used to kill insect pests (Table 18.1). Pesticides are used in numerous residential and urban applications, but by far the largest volumes are used on agricultural lands (EPA, 2011) to control biological pests and weeds that threaten crop production. Pesticide mixtures, whether as coapplied individual compounds or formulated products, contaminate waterways adjacent to agricultural areas following application spray drift, surface runoff, and accidental direct applications. Agricultural pesticide products are typically sold as formulations, or mixtures of other chemicals in addition to the pesticide active ingredient eliciting the intended toxic effect. These additional ingredients serve to enhance the application or effectiveness of the pesticide, but may themselves be toxic to nontarget organisms (Stark and Walthall, 2003). In the United States, pesticide formulations include other toxic ingredients that are frequently not disclosed on product labels because they are proprietary information. Likewise, toxicity data on pesticide formulations are frequently lacking because even the most basic toxicity tests (e.g., LC_{50} s) of the formulated product are often not required during product registration.

Surface water and sediment monitoring confirms that pesticides commonly cooccur as mixtures in aquatic habitats, in both urban and agricultural areas (Kolpin et al., 2002; Stone et al., 2014). Some of the more commonly detected pesticide classes are shown in Table 18.1. Large-scale water quality syntheses across the United States (Gilliom, 2007) and Europe (Loos et al., 2009, 2013) have shown that these and other pesticides are frequently detected as complex mixtures of many chemicals. For example, aquatic monitoring by the US Geological

Table 18.1: Commonly Detected Current-Use Pesticides in Aquatic Habitats

Group	Class	Mode of Action	Example Pesticides
Insecticide	Organophosphate	Irreversible inhibition of AChE enzymes	Malathion Chlorpyrifos Diazinon
	Carbamate	Reversible inhibition of AChE enzymes	Carbaryl Methomyl
	Pyrethroid	Blocks sodium channel inactivation	Bifenthrin Permethrin Cypermethrin
	Neonicotinoid	Nicotinic ACh receptor agonist	Imidacloprid Thiametoxam
Herbicide	Phenylpyrazole	Blocks GABA-regulated chloride channel	Fipronil
	Triazine	Inhibits photosystem II	Atrazine Cyanazine Simazine
Fungicide	Phosphonoglycine	Inhibits photosystem II	Glyphosate
	Chlorophenoxy acid	Auxin growth regulator	2,4-D
	Azole	Inhibits ergosterol biosynthesis; Inhibits P450 monooxygenases	Prochloraz

AChE, acetylcholinesterase; GABA, gamma-aminobutyric acid.

Survey has shown that urban streams were contaminated with at least four pesticides 75% of the time and agricultural streams contained seven or more pesticides 50% of the time (Gilliom, 2007). Chemical concentrations in aquatic systems are inherently variable and influenced by differences in the timing, intensity, and duration of pesticide applications, as well as by site-specific meteorological and geological conditions. Nevertheless, a recent global synthesis estimates that more than 40% of agricultural land area is predicted to be at risk of contaminating nearby streams via runoff (Ippolito et al., 2015).

Considering that the United States alone has nearly 17,000 pesticide products currently registered for use, it is generally impracticable to measure the toxicity of all possible mixture combinations for different aquatic species under different exposure conditions. However, progress has been made using known principles of toxic action. The two most widely accepted mixture models are concentration-addition and response-addition, for use with mixtures of pesticides having the same and different modes of action, respectively. These methodologies rely on the assumption of no interaction (Hertzberg and MacDonell, 2002). Notably, an investigation of large mixture datasets has shown that concentration-addition is a conservative predictor of mixture toxicity, regardless of the modes of action of the mixture constituents (Belden et al., 2007). Additionally, concentration-addition has been suggested as a first-tier model of potential mixture effects (Backhaus and Faust, 2012; Beyer et al., 2014). Derivations of concentration-addition methods have been used to develop mixture assessment approaches such as toxic units (Schafer et al., 2013; Ginebreda et al., 2014) and the pesticide toxicity index (Nowell et al., 2014). For dissimilarly acting pesticides, response-addition is an appropriate model to predict mixture toxicity (Cedergreen et al., 2008). This model assumes that, although the mixture components do not influence each other's uptake or metabolism, they still affect the same toxicological endpoint (e.g., mortality, swimming behavior).

Concentration-additive models generally predict that exposure to pesticide mixtures will produce greater toxicity than exposure to individual chemicals. This depends on the toxicity of individual pesticides, and is especially apparent in mixtures that produce toxicity when the constituent pesticides are present at concentrations below their single-chemical no-effect levels. The mode of toxic action is also an important factor when considering combined biological effects. For example, the organophosphate, pyrethroid, and neonicotinoid classes of insecticides have different modes of action—acetylcholinesterase inhibition, sodium channel inactivation blockade, and nicotinic acetylcholine receptor agonism, respectively. However, all three of these neurotoxicants increase postsynaptic activity in the nervous system, and mixtures are expected to produce enhanced (additive) neurotoxicity in aquatic species (Figure 18.3). Therefore, the biological underpinnings of toxicity are important considerations when assessing pesticide mixtures of potential concern.

For fish, environmental surveillance data have shown that most environmental exposures to pesticide are likely to be sublethal. Also, because many of the more toxic modern pesticides target the nervous system, there has been a focus on fish behavior as a toxicological endpoint.

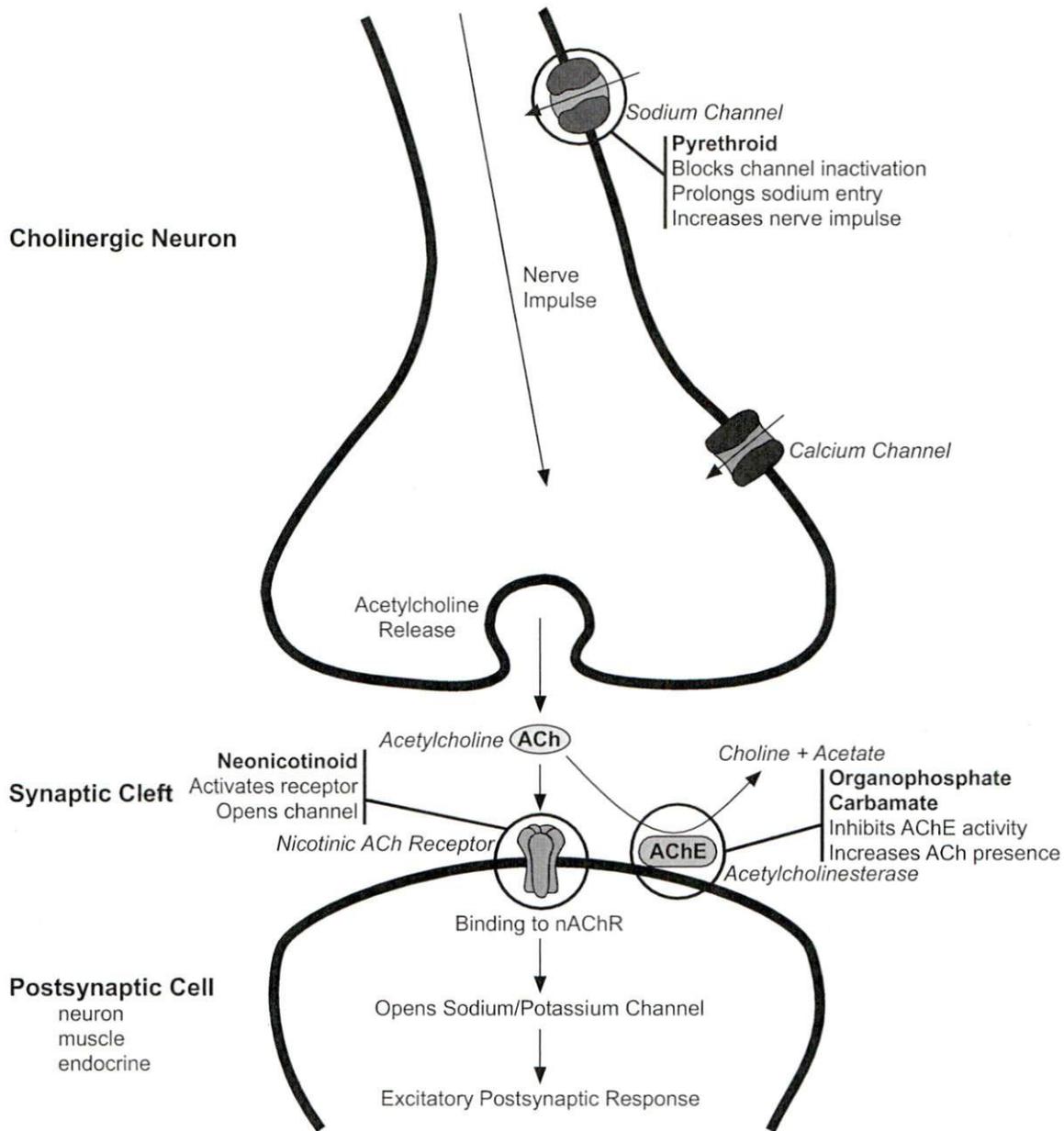


Figure 18.3

Diagram of a cholinergic nerve synapse highlighting the mode of action of organophosphate, pyrethroid, and neonicotinoid pesticides.

For example, Laetz et al. (2013) demonstrated reduced swimming speeds and decreased food strikes in juvenile salmon exposed to binary mixtures of organophosphates. Behavioral impairment was evident at low, environmentally relevant concentrations (1 ppb cumulative concentration) and was correlated with acetylcholinesterase inhibition.

From a natural resource management perspective, reducing or eliminating exposures to combinations of pesticides that interact synergistically in mixtures remains a priority. There

are currently no reliable models to predict synergism from single-chemical toxicity data, making direct experimentation the only available tool (Altenburger et al., 2012). Among mammals, coexposure to the organophosphate malathion has long been known to increase the toxicity of other organophosphates (Frawley et al., 1957; Casida et al., 1963). Using juvenile salmon, Laetz et al. (2009) showed that binary mixtures of organophosphate and carbamate insecticides are synergistic, producing greater acetylcholinesterase inhibition *in vivo* than predicted from concentration-addition. Similarly, the azole fungicide prochloraz was found to be synergistic with other fungicides (Norgaard and Cedergreen, 2010) in experiments with *Daphnia magna*. Additionally, prochloraz and esfenvalerate produced synergistic toxicity in *D. magna* (Bjergager et al., 2012). Looking at the occurrence of synergy more holistically, Cedergreen (2014) analyzed datasets from numerous mixtures experiments and showed that synergy occurred in about 7% of the cases, with organophosphates and azole fungicides almost always involved in synergistic mixtures. Therefore, data showing synergy of specific pesticide combinations, or in certain pesticide classes (i.e., organophosphates), can be valuable in predicting the ecological risk of greater-than-additive toxic impacts.

Looking to the future, there will be an increasing need to move beyond the traditional reductionist approach to estimating toxicity (i.e., single-chemical, single-species laboratory tests) to better anticipate pesticide risks to wild populations, species, and communities (Macneale et al., 2010; Scholz et al., 2012). Ecological theory has been useful in this regard (Clements and Rohr, 2009). For example, mesocosms composed of organisms in multiple trophic levels were exposed to a complex pesticide mixture and followed for 18 weeks (Hua and Relyea, 2014). The direct mortality that occurred in some taxa created complex trophic interactions. Additionally, food web theory may offer a promising framework for predicting ecosystem-level effects of pesticide mixtures (Halstead et al., 2014). Knowledge of ecological parameters such as food web relationships and species interactions may allow us to make reliable predictions of the responses of complex aquatic systems to the toxic effects of pesticide mixtures.

18.4 Regulatory Provisions for Mixtures

Most US and European environmental regulations do not explicitly provide for the evaluation of mixtures toxicity. One notable exception is the US Comprehensive Environmental Response Compensation and Liability Act of 1990, which mandates that mixtures are assessed when determining the potential risks from chemical spills and certain legacy hazardous waste sites (EPA, 2015a). Other laws that do not explicitly call for mixtures assessments may nonetheless still incorporate mixtures analyses within the scope and intent of the legislation. For example, in 2006, the European Union issued the Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH) legislation in an effort to enhance the regulation of chemical contaminants (European Union, 2006). This legislation was primarily based on assessing potential adverse effects from single-chemical toxicity assays (Syberg et al., 2009).

Accordingly, the assessment of mixture effects was not included except in certain specific cases (e.g., petroleum products). For the ensuing decade, efforts have been made to broaden REACH by incorporating mixture assessment methods (Syberg et al., 2009; Swedish Chemicals Industry, 2010; Cedergreen, 2014).

In the United States, there is currently no regulatory or policy framework that calls for an increased understanding of toxicity and environmental effects of PAHs or other petroleum hydrocarbon mixtures. Oil spills may trigger potential violations of the Clean Water Act, but violations are determined on the basis of potential for harm on total volumes of released oil, and there is no provision for different levels of harm based on mixture composition. Following the Exxon Valdez oil spill, the Oil Pollution Act of 1990 and subsequent amendments improved the natural resource damage assessment process and provided a framework for regulating the response, reparation, and restoration activities surrounding the release of a complex mixture (petroleum) into the environment. However, the activities regulated by the Oil Pollution Act of 1990 are not contingent on an understanding of which components of the spilled mixture cause environmental injury. The only water quality criteria for either individual PAHs or PAH mixtures in the United States are in the state of Alaska, and this criterion is based on a total PAH value of 10 $\mu\text{g/L}$ irrespective of the mixture composition. Regulation of petroleum-derived mixtures is further challenged by the fact that some components of oils (and fuel oils in particular) have never been toxicologically characterized.

The pesticide registration process in the United States provides a prominent example of the discrepancy between single chemical and mixture toxicity assessments. Pesticides are registered or re-registered under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), which is an economic cost-benefit statute that has no explicit mandate to assess mixture effects to nontarget organisms (EPA, 2015b). Under FIFRA, registrations of agricultural and commercial pesticides are supported by single-chemical toxicity tests. The goal is to ensure, to the extent practicable, that no unacceptable adverse ecological effects will occur based on data produced by these standardized, single-chemical assays. Unfortunately, traditional toxicity tests do not incorporate simultaneous exposure to multiple pesticides or other natural stressors. Furthermore, guidance for conducting cumulative risk assessments of pesticide has been developed for only select circumstances (i.e., organophosphate pesticides that have the same mechanism of toxic action), and for assessments of potential impacts to human health (EPA, 2002).

In the decades following the publication of *Silent Spring* (Carson, 1962), pesticides have been a societal concern for vulnerable fish and wildlife throughout the world. In the United States, the risks that the federal registration process may pose are evaluated under the Endangered Species Act (ESA). The aim of the ESA is to ensure that a federal action (i.e., registration of an individual pesticide) does not jeopardize a listed species or adversely modify their designated critical habitats. The ESA is broadly intended to consider all risks associated with a proposed federal action. Therefore, real-world environmental exposures to mixtures may be

incorporated into an ecological risk analysis. Cumulative assessment methodologies developed by the EPA (EPA, 2002) have been applied in some cases, such as threatened or endangered Pacific salmonids (NMFS, 2008, 2009, 2010).

In the United States, the National Research Council recently reviewed current methodologies used by federal agencies for conducting pesticide risk assessments under FIFRA and the ESA (National Research Council, 2013). The importance of assessing the risk of pesticide mixtures was a primary component of the report. Since the release of the report, several US federal agencies, including the National Marine Fisheries Service, the Fish and Wildlife Service, and the Environmental Protection Agency have been coordinating the development of new procedures to incorporate pesticide mixtures into the ecological risk assessment framework that underpins pesticide registrations (or re-registrations). An explicit consideration of mixtures would represent a key advancement for natural resource management because (1) exposures to pesticide mixtures are the rule rather than the exception in most aquatic habitats and (2) assessments based on individual chemicals are likely to underestimate actual risk where mixtures occur. The inclusion of pesticide mixtures analyses in both ESA and FIFRA regulatory decision-making is expected to yield more consistent and ecologically relevant determinations of risk to threatened and endangered species.

18.5 Conclusions

Chemical mixtures have been a longstanding issue in aquatic ecotoxicology. This is unlikely to change, given the ever-increasing number of anthropogenic chemicals in global societal use. Other important environmental stressors, such as shifting thermal regimes in aquatic systems related to climate change, are expected to have important modulating effects on the direct and indirect toxicity of mixtures to aquatic organisms (Schiedek et al., 2007; Hooper et al., 2013). Some fields of research, including epigenetics and genomics, offer promising advances in the detection of toxic effects in a host of nontarget aquatic species (Altenburger et al., 2012). Such advances will improve the tractability of performing more robust and systematic assessments of complex chemical mixtures. Therefore, incorporating the toxic effects of chemical mixtures into ecological risk assessments and regulatory decision-making processes will remain vitally important for the conservation and protection of imperiled aquatic species and the habitats upon which they depend.

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