CHAPTER 5

A LAYPERSON'S PRIMER ON MULTIPLE STRESSORS

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Abstract: This article introduces the concept of multiple stressors. It has been written for the layperson, in terms that do not require a strong scientific background. It has been written to facilitate scientists' communication with the public and funding agencies about multiple stressors. This article briefly explains several major classes of contaminants whose global dispersal and long-term persistence in the environment might cause them to contribute to multiple stressors. Highlighted is our lack of understanding about the potential interactions among multiple stressors and the need for much additional research. Interactions are explained through a simple example of various plausible responses that an organism might exhibit when exposed to both cadmium and radiation. Our current approach for determining human and ecological risks from contaminants is explained such that the reader is aware of why multiple stressor research is needed. This article stresses the need for a coordinated, multinational, multidisciplinary research plan for multiple stressors.

Keywords: interactions; mixed stressors; multiple contaminants; effects

Introduction

Late in 1997, Dr Larry Zobel, the medical director for the 3 M Corporation, was puzzled by some laboratory analyses he had requested (Fisher, 2005). The analyses were on workers that produced a 3 M chemical, perfluorooctane sulfonate (PFOS). PFOS is a chemical used to make SCOTCHGUARD, and is also found in products as dissimilar as GORETEX, TEFLON, power plant pipe linings and jet engine gaskets. PFOS allows two other disparate

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chemicals to bond together; a property that gives it wide commercial appeal. What was puzzling Dr Zobel was not that tiny amounts were showing up in the workers' blood samples, which was to be expected, but that the chemical was showing up in the clean blood samples from control individuals. To try to resolve the issue, 3 M contacted biological supply companies and purchased pooled samples from blood donors that represented some 760 random locations within the USA. PFOS was in every sample. This was perplexing. Dr Zobel then went to the Red Cross and asked for samples from 600 more blood donors throughout the United States. Same result, PFOS was in every sample. He then turned to Europe, where the chemical had never been manufactured, and obtained samples from Belgium, the Netherlands and Germany. Same result, PFOS was in every sample. Dr Zobel's lab went on to test over 1,500 more samples, including some 600 children. They found PFOS in every sample but two, with levels in some children scoring above those found in the 3 M workers. Alarmed, 3 M notified the US Environmental Protection Agency of its findings, and 2 years later, 3 M announced it would cease production of PFOS. University researchers, alerted to the problem, began to look for the chemical in non-human samples. They found it everywhere they looked. PFOS was in polar bears of the Canadian Arctic, the blood of Inuit's in Alaska, cormorants in the Sea of Japan. It seems that everything contained trace quantities of PFOS. In the course of a single human generation, we contaminated virtually all of earth's biological systems with PFOS.

Dr Zobel's story illustrates how small and interconnected our world truly is. It is astonishing to imagine all of the biological mechanisms, the physical routes of transport, and the chemical's environmental resilience required to disperse so thoroughly in such a relatively short amount of time. And yet, PFOS is not unique. Mixtures of chemicals are ubiquitous in the air we breathe, the food we eat and the water we drink. Polychlorinated biphenyls (PCBs), pesticides, endocrine disruptors, heavy metals, radiation….the list goes on and on (Muir et al., 2005). Surprisingly, our methods of determining acceptable levels of contaminants and of calculating a pollutant's risk to humans and the environment do a very poor job of considering contaminant mixtures; instead, we largely study contaminants as if they occurred in isolation (Cory-Slechta, 2005). The long-term human and ecological risks from chronic exposures to contaminant mixtures are not known. A lack of knowledge about complex mixtures of pollutants is among the major challenges facing the environmental sciences (Eggen et al., 2004).

This primer (i) introduces the concept of multiple stressors and briefly explores some major classes of pollutants that have the potential to be within complex mixtures; (ii) provides an overview of how human and ecological risk analyses evaluate pollutants and highlights the difficulties of studying

multiple stressors; (iii) explains the concept of interactions among multiple stressors and the need for a multinational, consolidated research effort to understand them.

Some Major Players

We live in a chemically sophisticated world. Better living through chemistry is the reality. Humans are masters at combining chemicals in magical ways to produce goods that truly enrich our lives. The price we pay, however, is that complex mixtures of metals, nicotine, and benzene are found in our blood; PCBs, PAHs and POPs settle in our fat; we inhale pesticides that cling to our house dust; fire retardants are found in breast milk; endocrine disruptors are excreted in our urine (Duncan, 2006). All of this occurs while the ice melts in the arctic from global warming. Exposure to multiple stressors is the rule, not the exception.

The global dispersal of PFOS is not unique; as is evident by the occurrence of several contaminants in what was previously thought to be pristine habitats. The arctic environment is a good example (Bard, 1999; AMAP, 2002; Macdonald et al., 2005; Muir et al., 2005). Although thought to be isolated from industrial processes known to cause pollution, mixtures of contaminants are showing up and impacting arctic wildlife. Eagles, sea otters and Steller sea lions in the Aleutian Islands have elevated levels of the pesticide DDT; sea ducks, walrus and caribou have high levels of cadmium; killer whales in the North Pacific are now considered to be the most contaminated mammals on earth (Ayotte et al., 1995). Polar bears with higher concentrations of PCBs have altered immune responses that are likely to increase the animals' susceptibility to infections (Muir et al., 2005). Research on Greenland bears reveal increasing concentrations of DDT, polybrominated diphenyl ethers (PBDE flame retardants), and a banned insecticide called chlordane. The contaminants appear to be influencing reproductive organs. Testis and ovary length, and length of the baculum, a bone that supports a bear's penis, decreased significantly with increasing concentrations of the contaminants (Sonne et al., 2006).

Some of the major classes of contaminants that persist in the environment, readily disperse, and have been shown to be components of mixed contaminants include:

- Persistent organic pollutants (POPs); including industrial chemicals (e.g., PCBs, brominated flame retardants), byproducts of industrial processes (e.g., dioxins and furans, hexachlorobenzene), and pesticides (e.g., DDT, chlordane, atrazine)
- Metals; especially cadmium and mercury, both of which are released by fossil fuel combustion, waste incineration, and in various mining and metallurgical processes

- Radionuclides (e.g., cesium-137, strontium-90, plutonium); primarily from past atmospheric testing of nuclear weapons, accidents such as Chernobyl, releases from nuclear fuel reprocessing plants in Europe, and the dumping and storage of nuclear waste
- Petroleum hydrocarbons; either originating locally as a result of spills and discharges from shipping, pipelines, oil and gas drilling, or transported long distances via the atmosphere

These pollutants tend to have strong geological stability, accumulate in fatty tissues, have relatively long biological half-times within organisms, and increase in concentration at higher levels of the food chains (Wormley et al., 2004). Generally, they have been shown to produce:

- Reproductive effects; reduced ability to conceive and carry offspring, reduce sperm count, and/or feminization of males.
- Immunological effects; decreased ability to fight off disease
- Neurological and developmental effects; reduced growth and permanent impairment of brain function
- Mutations; that lead to cancer or genomic instability

A World of Contaminants

It appears that in mastering the use of chemicals to improve our lives, we have also mastered the fouling of our own nest; indeed, the nests of all living organisms are impacted by our better living through chemistry. And there are a lot of chemicals. As of August 2005, over 26 million substances had been indexed by the American Chemical Society's Abstracts Service (CAS, 2005). One-third of these (nearly 9 million) were commercially available; however, only 240,000 are regulated by government bodies worldwide (Daughton, 2005). Some 82,000 chemicals are registered for commercial use in the USA alone, and an estimated 2,000 new ones are introduced annually for use in everyday items such as foods, personal care products, prescription drugs, household cleaners, and lawn care products (Duncan, 2006). About 10% of these chemicals are recognized as carcinogens, but only a quarter of the 82,000 chemicals in use in the U.S. have ever been tested for toxicity (Suk and Olden, 2005; Duncan, 2006).

The EPA receives approximately 100 applications per month from companies seeking to introduce new chemicals on the market. With each application, the manufacturer supplies information on production volume, use and environmental release rates; but not a word on toxicity, unless the manufacturer

happens to have such data. Critical information such as the chemical's effects, physical properties, and health impacts must come from EPA files or public databases. The burden rests with the EPA to prove a problematic chemical should be restricted. Perhaps it comes as no surprise that since 1979, the EPA has forced restrictions on just nine applications. 82,000 chemicals results in a daunting research task when trying to determine potential effects from their innumerable possible combinations. In vivo testing of all the various combinations of mixtures, at all the conceivable dose levels is impossible from an ethical, economical or pragmatic perspective (Cassee et al., 1998).

Rather than requiring a government agency to test for toxicity, the European Union is taking a different approach (Duncan, 2006). Last year they gave initial approval to a measure called REACH—Registration, Evaluation, and Authorization of Chemicals—which would require companies to prove the substances they market or use are safe, or that the benefits outweigh any risks. The chemical industry and the US government oppose the REACH concept.

Interactions

All organisms are exposed to a diverse mix of chemicals, pollutants, and stressors. However, our regulations for deeming when risks are acceptable come largely from assessment protocols based on the unrealistic assumption that pollutants occur in isolation from each other. This approach prevents us from properly evaluating mixtures of stressors; particularly, it prevents a determination of the potential interactions among pollutants or stressors. It is with these interactions that 1+1 can indeed = 3.

Interactions, in this context, can be explained by a brief discussion of the plausible outcomes that an organism might experience when exposed to both radiation and the metal contaminant, cadmium. Radiation is a well-known mutagenic that damages DNA. Ionizing radiation induces DNA strand breaks. The most potent type of radiation-induced DNA lesion is double-strand breaks (DSBs; reviewed in Ward, 1995). When cells detect DSBs they arrest their cell cycle and attempt to reestablish chromosome integrity. If the cell damage is extensive, a cell may program itself to die via apoptosis, perhaps because the cost of repair is too great or to avoid the risk of mis-repair and propagation of damage to subsequent cell generations (reviewed in Zhivotovsky and Kroemer, 2004). Some of the damage caused by radiation is due to the ionization of water within the body and the formation of free radicals. Free radicals are molecular species with unpaired electrons. Free radicals are very reactive and can damage DNA by oxidative reactions. Mammals have evolved very effective methods of repairing DNA damage; and humans often assist the repair process by consuming antioxidants

(e.g. vitamins C and E, green tea, red wine) that provide additional defense against free radicals.

Cadmium (Cd) is a metal. When animals are exposed to metals they increase the production of a protein called metallothionein (MT), which attaches to the metal, making it less toxic. MT also has antioxidant characteristics that reduce the impact of free radicals. Additionally, Cd is known to inhibit DNA repair.

What might the organism's response be, then, if these two pollutants act together? Responses could simply be additive, no interaction. Radiation causes damage, Cd causes damage, the damages are additive (1+1 = 2). If, however, Cd induces the upregulation of MT, and if the MT acts as an antioxidant, then it is plausible that the antioxidants produced from Cd exposure also scavenge the free radicals produced by the radiation. In this scenario, the Cd would provide a protective effect, with the interaction being antagonistic (1 + 1 = 1, or perhaps 0). Alternatively, if Cd inhibits DNA repair, it is plausible that Cd would augment the radiation damage by reducing the efficiency of DNA repair mechanisms. In this case a synergistic effect could occur (1 + 1 = 3, or 5, or 35).

Thus several mechanistic reasons exist for interesting interactions to occur when radiation and metals co-occur, and yet, only by researching these contaminants together would you ever be able to discern if any interaction, good or bad, occurs. The two contaminants seldom exist in isolation. Indeed, metals co-occur with radioactive contaminants 99% of the time at the contaminated sites managed by the US EPA's Superfund program (Table 1). This example illustrates the relevance and importance of studying chemicals as they occur in nature – as mixtures.

Sometimes interactions among pollutants can cause effects to significantly magnify. Yang (2004) examined the effects of Kepone, a fire ant pesticide, and carbon tetrachloride in rats. Carbon tetrachloride is used in the production of

TABLE 1. Percent occurrence of the top five contaminant groups occurring on sites in association with radioactive contamination, averaged across all EPA regions. This analysis was done using a database of sites listed as "currently on the final National Priority List" from the Agency for Toxic Substances and Disease Registry (ATSDR). Thus, of all the sites containing radionuclide contamination, 99% also contained metals and 77% also contained VOC

Contaminant	Metals[1]	VOC[2]	Inorganic[3]	PAH[4]	Pesticides[5]
% occurrence with rad contamination	99%	77%	73%	67%	54%

[1]Lead, Arsenic, Cadmium and Zinc; [2]Volatile Organic Compounds (e.g., Acetone, Benzene, Toluene); [3]Inorganic compounds (e.g., Asbestos, Cyanide, Sulfuric acid, Sulfate); [4]Polyaromatic Hydrocarbons (e.g., Fluorine, Anthracene, Diethyl phthalate); [5]Heptachlor, DDT, and Dieldrin.

refrigeration fluid, propellants for aerosol cans, as a pesticide, as a cleaning fluid, in fire extinguishers, and in spot removers. When both chemicals were administered at low, environmentally relevant doses the two synergistically interacted such that effects were magnified 67-fold.

Interactive effects are not only caused from exposure to multiple contaminants, the phenomenon occurs due to exposure to multiple stressors, and stress can come from a myriad of sources. Stress is an unavoidable aspect of life for all populations in differing degrees and manifestations, and thus an inevitable contributor to risk (Cory-Slechta, 2005). Two recent examples concern amphibians, which have been undergoing worldwide population declines. Relyea (2003) examined interactions when amphibians were exposed to carbaryl, a pesticide, in the presence of predators. Pesticide concentrations from short-term acute exposures that would normally not adversely affect growth or survival proved lethal when the exposure occurred in the presence of predatory stress. The chemical stressor was magnified many fold by the non-chemical stressor of the predator cue. Likewise, Teplitsky et al. (2005) reported a greatly enhanced stressor action of the fungicide fenpropimorph to tadpoles when they were developing in the presence of a predator. The combined action of the predatory stress cue and the low-level fungicide resulted in delayed and smaller maturation beyond exposure to either stressor alone.

With 82,000 chemicals in the environment, it becomes quite plausible that they might not all act independently, but instead impacts to organisms could be influenced by exposure to multiple stressors. The interaction of two or more chemicals is determined in part by which mode/mechanism of toxic action is operative, and points to the necessity of doing research at environmentally relevant dose levels (McCarty and Borgert, 2006). The order of exposure also complicates analyses. The response produced by an exposure to chemical A then B may be different from B then A. Additionally, all environmental contaminants are changed to metabolites or conjugates in the body, and these new products may also have biological activity that may or may not be similar to the parent compound. Thus even a single compound may become a functional mixture (McCarty and Borgert, 2006).

Determining Risks and Acceptable Concentrations

How are risks actually determined? Generally, one pollutant at a time! We study mercury, in isolation. We study cadmium, in isolation; we study yet another four-letter-coded, organic contaminant, in isolation. For each, we develop a dose-response curve, from which we determine a no-observable effect level (NOEL); apply safety factors that account for uncertainty, and then derive exposure limits and permissible levels (Dourson and Patterson,

2003; Cormier et al., 2003; Suter et al., 2004). [For a synopsis on the evolution of the ecological risk assessment framework in the USA see Suter et al. (2003) and Suter (2006)]. We then repeat the process for the next contaminant. Each contaminant studied in isolation, and thus with no possibility of detecting interactive effects.

The focus on individual chemical agents has been a significant first step in toxicological/environmental studies. Studying chemicals in isolation provides necessary information on the pollutants mode of action, or the mechanism whereby it causes an effect. However, it means that we lack adequate data, methods and models to assess risks realistically for most mixtures to which people and the environment are routinely exposed (Suk and Olden, 2005).

In 1996, the US EPA was directed to include chemical mixtures in its assessment of risk for pesticides that have a common mode of action. Because the mixtures are limited to those that have the same mode of action, the consequent effect is often one of additivity for mixtures. Thus to date, mixtures of chemicals have been dealt with legislatively by largely restricting them to classes that are chemically related and using an additive approach to risks (Cory-Slechta, 2005). Under these conditions additivity should not be surprising, given that the approach may be little different from simply increasing the dose of a representative agent acting under the same mode of action. Merely to use an effect summation approach, however, has proven to often underestimate risks. For example, Silva et al. (2002) found that simple summation of the individual effects of eight weak estrogenic chemicals, each administered below the No Observable Effect Concentration (NOEC), underestimated observed effects by a factor of 20.

EPA's most recent guidance for mixtures (US EPA, 2000) is for human health risk characterization. It does not recommend any single approach for mixtures, but provides a number of options for the practitioner to consider. Two other US agencies stressing mixture research are the National Institutes of Environmental Health Sciences (NIEHS) and the Agency for Toxic Substances and Disease Registry (ATSDR). Two guidance documents on the assessment of chemical mixtures have been produced by the ATSDR (US DHHS, 2004a, 2004b), and NIEHS is supporting mixture research (Suk and Olden, 2005). The ATSDR has recently undertaken the development of a series of "Interaction Profiles" for substances most commonly found at EPA Superfund Sites. To compensate for current lack of knowledge, ATSDR applies an additional safety factor of 10 for mixtures of non-cancerous chemicals and 100 for cancerous chemicals.

These significant knowledge gaps represent major complications thwarting both academic investigations of and regulatory approaches to the toxicity of chemical mixtures. For example, ATSDR and EPA recommend using data from similar mixtures as surrogates for the mixture of concern if data

are lacking. However, despite the promulgation of guidance for assessing mixtures, clear criteria have yet to be developed for determining when two mixtures are sufficiently similar to use one as a toxicological surrogate for the other. Indeed, there is no generally accepted classification scheme for categorizing toxicological effects or modes/mechanisms of toxic action (McCarty and Borgert, 2006).

All agencies involved recognize that substantial enhancements to experimental and risk assessment methods are needed. It is generally believed that improvements can be achieved by using organism-based uptake, distribution, and elimination modeling, coupled with data from well-defined, model-based in vivo and in vitro experiments analyzed with improved statistical and mathematical protocols. Good examples of modeling approaches to assess the ecological effects from multiple stressors while considering spatial and temporal parameters are provided by Hope (2005) and Nacci et al. (2005), while McCarty and Borger (2006) provide an excellent review of chemical mixtures. Mixed-exposure research will require the development and refinement of mathematical and physiological models that can be used to estimate the effects of stressors on whole body systems. In addition to a historical perspective of assessing the effects of chemical mixtures, Yang et al. (2004) highlight the need for physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) modeling approaches.

To be successful, substantial improvements are needed in our knowledge of biological mechanisms of toxicity, chemical structure function relationships, and dose-response relationships. Such knowledge may in turn lead to the development of biological screening tools and improve our ability to model exposure-effect relationships. Anderson et al. (2006) provide an example of integrating several approaches to contaminant responses. Their method involves quantifying molecular, biochemical, and cellular responses in individual organisms collected from stressed and less-stressed sites, and along gradients within the sites, in conjunction with chemical, organism, and population measures. Ultimately, they use a dynamic-energy-budget model to analyze growth of individuals and potential impacts to the population. However, in the short term the traditional approach of calculating hazard indices and summing cancer risk estimates is likely to remain the predominant from of mixture risk assessment.

Path Forward

Understanding the interactions among chemical mixtures and multiple stressors is one of the most perplexing and difficult areas of science within toxicology and risk assessment (Suk and Olden, 2005). A multinational, multidisciplinary strategic research plan is needed for chemical mixtures that

is coordinated, comprehensive and cogent (Suk and Olden, 2005). There has been a lack of federal leadership related to research on chemical mixtures, a situation that has caused chronic funding problems and hindered the development of a broad-based mutually agreed-on and clearly articulated strategic research plan (Sexton et al., 1995). Consequently, despite significant scientific advances, the field of chemical mixture research can generally be characterized as uncoordinated, unsystematic and under funded; problems that are exacerbated by the complexity of mixture-related exposures (Sexton et al., 1995).

The recommendations of Sexton et al. in 1995 are still appropriate. Toxicologic research should proceed along three parallel and complementary tracks: (i) studies of basic interaction mechanism using simple combinations of important chemicals, with the express objective of developing and refining mechanistically based mathematical models; (ii) studies of the toxicity of high-priority, environmentally relevant mixtures with the express objective of reducing critical scientific uncertainty in health risk assessment; and (iii) studies that examine both constituent interactions and whole-mixture toxicity in simplified artificial mixtures (e.g., the 10 most important chemicals impacting the Arctic or Superfund sites).

Any comprehensive framework that seeks to predict and explain the effects of chemical mixtures must take into account the following (McCarty and Borgert, 2006): the mechanisms of toxicity of the component chemicals, the potential points at which these mechanisms interact, the dose-dependence of both the mechanisms of toxicity and the mechanism of interaction, be designed to be used at various levels of biological organization, and account for species-specific difference in both toxicity and interaction.

Intact animals are probably the only model adequate for evaluating mixed stressors (other than chemicals), such as physical stressors (e.g., extreme cold or heat, exercise), personal factors (e.g., nutritional deficiencies, aging, etc.), hormonal changes (e.g., co-exposure to endocrine disruptors, pregnancy), biological stressors (e.g., infectious agents), and psychological stressors. Intact animals are also required to study reproductive (e.g., fertility, teratological), postnatal development and growth phenomena.

Fish, especially zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*), have several features that make them useful models for evaluating mixed stressors. Fish are accepted model vertebrates for studying genetics, developmental biology, toxicology and human disease (reviews: Shima and Mitani, 2004; Hill et al., 2005). The main advantages of using zebrafish and medaka as models over other fish are their small size, ease of husbandry, and prolific breeding capacity. Unlike other fish, such as salmon and trout, the small size of zebrafish and medaka (approximately 2.5–3 cm long) permits reduced breeding space and reduced husbandry cost. These characteristics are important for the large-scale experiments needed to evaluate the effects of contaminants

when exposures are at low, environmentally relevant concentrations. In addition, zebrafish and medaka development have been well characterized (Kimmel et al., 1995; Iwamatsu, 2004). Equally important, their eggs have a transparent membrane which allows aberrations to be easily observed, even during early development prior to hatch (Teuschler et al., 2005). A "see-through" strain of medaka has a transparent body in the adult stage as well, such that organ abnormalities can be observed in living adults (Wakamatsu et al., 2001). This mutant, and transgenic mutants that express green fluorescent proteins (GFP), have been used for several toxicity studies (e.g. Hano et al., 2005; Kashiwada, 2006). Such advantages, in addition to the availability of genome information, make these fish models ideal candidates for addressing the difficult questions surrounding multistressors.

The dispersal of so many pollutants beyond national boundaries suggests that solutions will require a long-term development plan of global perspective (McCarty and Borgert, 2006). Research on chemical mixtures should be a broad, multidisciplinary approach that goes beyond the traditional boundaries between academic disciplines, and beyond the traditional boundaries of independent nations. Collaborative funding and calls for joint proposals among several nations will result in the most rapid and efficient research on this most difficult of problems. Until we better understand the potential interactions from chronic exposure to multiple stressors, Suk and Olden (2005) recommend invoking the Precautionary Principle and erring on the side of caution.

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