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PERSPECTIVES IN AQUATIC TOXICOLOGY

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INTRODUCTION

Aquatic toxicology is the study of the effects of foreign substances on aquatic organisms from the small phytoplankton to the great whales. Many of the biochemical systems of these organisms are similar to those of terrestrial animals, such as those that respond to foreign substances such as the mixed function oxidases (1). However, the activities and conversion rates often show great variability among aquatic species, as is the case with terrestrial forms.

Beyond the biochemical systems themselves, differences exist that result from millions of years of evolution—pressures to diversify and adapt to special conditions prevailing in lakes, rivers, estuaries, and oceans. Osmoregulatory systems (mediated principally through the gills) in fish, filter feeding systems in mollusks, and chemosensory systems in a wide variety of aquatic invertebrates were among the many diverse adaptations to aquatic

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environments over the millennia. Simultaneously, biochemical and physiological processes have become modified to support these and other unique accommodations to life.

In recent years, increased inputs of anthropogenic contaminants and other stresses such as the destruction of habitats have brought about drastic changes in aquatic ecosystems. Correspondingly, increased interest has centered on: (*a*) the accumulation and toxic effects of contaminants on the survival of aquatic organisms; (*b*) the uptake and accumulation of pathogenic organisms and chemical contaminants in aquatic resources destined for human consumption; and (*c*) the release of biodegradable organic matter and nutrients, which under quiescent conditions may result in localized eutrophication, organic enrichment, and oxygen depletion (2).

In response to these and other events, aquatic toxicology has undergone significant changes in the past decade. The science that initially focused on body burdens of toxic environmental chemicals and acute bioassays, but had lethality as the principle endpoint, has expanded into studies involving metabolic conversions of carcinogens, modifications of DNA, and other distinctly biochemical processes. The classic concept that research should rightly focus on a few laboratory rodents and microorganisms has given way to recognition that aquatic species are important surrogates for toxicological research. Moreover, in an environmentally conscious society aquatic species are a concern in their own right and, as such, aquatic toxicology has an obvious place in understanding factors that govern their health and survival.

It is difficult, if not impossible, to review here the many and varied accomplishments in aquatic toxicology. Thus, we have emphasized recent developments that we believe represent significant advances in knowledge. Finally, the limited space available precludes inclusion of important areas tangential to the basic science of aquatic toxicology. For example, while we recognize the utility and necessity of developing additional and refining existing aquatic bioassay models (i.e. for natural resource damage assessment), we believe their critical review is best left to specialized treatments elsewhere.

We begin with a consideration of toxic effects—the deposition of foreign substances in aquatic habitats and their subsequent exposure, uptake, and bioconcentration. This is followed by a consideration of metabolism and the interaction of metabolic products with macromolecules and biochemical systems. Discussion then focuses on how exposure to foreign chemicals alters the health status of organisms. The highest level of biological organization, the ecosystem, is exceedingly complex and, as a result, very difficult to study. Presently, available technology for conducting laboratory studies and/or field studies of xenobiotic impacts on ecosystems is limited. Nonetheless, we conclude with a discussion of recent advances.

CONTAMINANTS IN THE AQUATIC ENVIRONMENT

Deposition of large amounts of toxicants into the aquatic environment occurs from a variety of anthropogenic sources and may involve literally thousands of substances, particularly in urban and industrial areas (3). A partial list includes most metals, chlorinated organic compounds, polycyclic aromatic hydrocarbons (PAHs), as well as various nitrogen-, oxygen-, sulfur-, and bromine-containing compounds. For example, in the water column and sediments of Puget Sound, USA, thousands of different man-made compounds exist, many of which are structurally difficult to identify. Notwithstanding, polychlorinated biphenyls (PCBs) have been measured in the sediments of Puget Sound at concentrations up to 2,000 times higher than in reference sites, and PAHs ranged from 100–1,000 times concentrations at reference sites (4–6). In addition, high levels of PCBs and PAHs have been reported on the east coast of the United States (7), the Gulf of Mexico (8), and the Great Lakes (9). In fact, globally, wherever human activities impact the aquatic environment, complex suites of man-made chemicals are usually an obvious consequence.

The initial fate of toxicants entering aquatic environments is either accumulation on the benthic and/or terrestrial substrate, distribution in the water column, or uptake by organisms. Benthic sediments often receive the most attention as they frequently act as ever-increasing reservoirs for environmental contaminants. Furthermore, sediments are continually changing in response to abiotic and biotic conditions. For example, the degradation rates of individual PAHs in marine sediment slurries, in the presence or absence of other aromatic hydrocarbons, were examined (10). Degradation and mineralization of individual PAHs (anthracene, naphthalene, or phenanthrene) was influenced by preexposure to the other PAHs and benzene in organic-rich aerobic marine sediments. Thus, an almost limitless potential for compound synergisms and antagonisms exists in eukaryotic organisms and, as such, any attempt to predict toxic effects based solely on compound concentrations and distributions is greatly compromised.

While it is intuitively obvious that aquatic organisms are often exposed to waterborne xenobiotics, only recently has the importance of a particular component of the water column, the sea-surface microlayer, been recognized (11–13). This layer, the upper 50 μM of the water column, has been shown to concentrate contaminants that significantly impact phytoneustron (14). Studies of trace metals in the microlayer, for example, revealed concentrations 10 to 1,000 times higher than those in the subsurface water only a few centimeters below (11, 13). Hence organisms that spend only a few hours or days in the microlayer, such as the larval stages of many fishes, may be predisposed to death, deformities, cancers, or other disorders, some of which might not be

manifested until a later life-stage. Thus, the limited studies available suggest the potential for far-reaching effects among organisms briefly inhabiting this upper portion of the water column and demonstrate the need for more effort to assess toxicological impacts.

The integument and gills of aquatic organisms, which differ in permeability according to species, are important sites of contaminant uptake. The presence of an efficient circulatory system in fishes, and to a much lesser extent in invertebrates, allows for the rapid distribution of contaminants crossing these barriers. Uptake of metals into gill tissues, for example, correlates with the weight-specific metabolic rate (15). Consequently, small fish tend to accumulate metals more rapidly than large ones (16). Furthermore, the rate of metal uptake is dependent on the structure, solubility, and concentration of the metal species, as well as the organism's individual physiology, which can be highly variable. For example, in studies with marine worms, mercury had the greatest rate of uptake (apparently due to higher metal permeability and not to active transport) followed by copper, silver, zinc, cadmium, and arsenic. The rate of metal uptake correlated highly with toxicity (17). Extraoral orifices, including anal and opercular cavities, provide additional sites for toxicant entry.

Thus, a variety of potential sources and routes of toxicant exposure exist in aquatic systems, some distinctly different from those in terrestrial animals. Clearly, aquatic organisms must either quickly and efficiently metabolize and eliminate these compounds or otherwise attempt to mitigate their toxic effects through, for example, immune responses or changes in behavior. Consequently, additional knowledge of the pharmacokinetics of toxicants is necessary for understanding the mechanisms by which toxicants exert their influence. Recent investigations into the kinetics of pentachlorophenol (PCP) exposure have provided us with an initial understanding of fish pharmacokinetics (18). The total amount of PCP in the fish, its concentration in water, and the total amount of metabolites (in the water, whole fish, and bile) were determined. Equations incorporating these variables, based on a two-compartment pharmacokinetic model, were generated. Furthermore, salicylamide, an inhibitor of PCP metabolism, decreased the metabolic clearance of PCP, which caused an increase in the bioconcentration factor (BCF). This allowed for generation of a clearance-volume compartmental model to characterize the accumulation and disposition of PCP in trout. Furthermore, it permitted partitioning of the BCF in terms of the underlying physiologic and biochemical processes (i.e. uptake clearance, metabolic clearance, and apparent volume of distribution). Thus, the BCF becomes important in predicting the effects of xenobiotics on aquatic organisms. High BCFs mean high body burdens can result from relatively low concentrations of a compound in the water, thereby causing toxicity to the organism or transfer to other trophic levels.

METABOLISM OF CONTAMINANTS IN AQUATIC ORGANISMS

Bioconcentration is the phenomenon by which compounds are accumulated in organisms at a higher concentration than the surrounding environment. As described below, the recent literature is replete with reports of many different compounds bioconcentrating in a variety of aquatic species. Unfortunately, detailed studies of the resultant biological effects are often lacking. In a study with fathead minnows, the bioconcentration factors for 30 common organic chemicals were determined (19). Bioconcentration factors ranged from 2.7 for tris-(2,3-dibromopropyl)phosphate to 194,000 for Aroclor 1260. Significantly, these values were based on whole body burdens; no information was obtained on individual tissues. In another study (20), rainbow trout were exposed to radiolabeled 2,4,5-trichlorophenol and pentachlorophenol. In this case, bioconcentration factors of approximately 50-, 100-, 1000-, and 10,000-fold for muscle, blood, liver, and bile, respectively, were observed. Furthermore, by neglecting the influence uptake in food, these types of experiments may, in fact, underestimate what would occur under natural conditions as the food items of feral fishes would most likely include organisms that had also accumulated contaminants (21).

Considering a phylogenetically different aquatic species, studies were conducted over a two-year period in which captive harbor seals were fed either fish from the heavily polluted Dutch Wadden Sea or fish captured in relatively "clean" areas of the Atlantic Ocean (22). The concentrations of individual PCB congeners were measured in both the fish and seal blood. Significantly higher levels of PCBs were found in both the fish from the Dutch Wadden Sea and in the blood of seals fed these fish. Furthermore, from structural studies of the PCB compounds in the blood, the authors were able to predict the degree of PCB bioconcentration in the seal. These studies add to growing evidence that toxicants that are not extensively metabolized tend to move up the food chain. Subsequently, the effect on plasma retinol and thyroid hormone concentrations of feeding PCB-contaminated fish to seals was investigated (23). Seals fed fish from the Wadden Sea had significantly lower concentrations of plasma retinol, free thyroxin, and triiodothyronine compared to seals fed fish from the "clean" areas of the Atlantic. The PCB-induced reduction in plasma retinol concentrations was reversed when seals on the Wadden Sea fish diet were switched to Atlantic Ocean fish. The authors speculate that the alterations in plasma retinol and thyroid hormone concentrations are critically involved in reproductive disorders and viral infections observed in seals and other marine animal populations in the Baltic Sea, North Sea, and Wadden Sea. These studies are valuable in that they not only document significant bioconcentration factors in the seals, but also relate contaminant concentrations to specific biological effects. Additional studies linking bioconcentration data to biological effects are needed.

Various factors influence the bioconcentration potential of an organism. For example, studies of metal uptake in four species of centrarchid fishes demonstrated that females take up more mercury than males. Presumably, these differences are related to the physiological differences (hormonal status) associated with the onset of reproduction (24). This phenomenon, however, cannot be generalized across all phyla. For example, the accumulation of PCBs in the blubber of both male dolphins and male pilot whales was reported to be twofold higher than females (25).

Temperature can also be expected to significantly influence the bioconcentration capacity of poikilotherms. Exposure of brown trout to PCBs from food and water under different temperature regimens resulted in variable bioconcentration rates (26). Greatest concentrations of PCBs occurred in fish exposed to high daily temperature cycles (7–18 °C) as opposed to constant temperature (12.9 °C) or cycling at lower temperatures (4–11 °C).

Thus, it is evident that temperature, hormonal status, and other factors (e.g. nutrition, age, etc) can significantly affect an organism's physiology and the ability to bioconcentrate xenobiotics. What is not well understood, however, are the mechanisms by which these factors regulate bioconcentration capacities. How circulating levels of hormones or altered metabolic rates (e.g. induced by temperature change) cause shifts in bioconcentration potentials needs further study.

Both laboratory and field studies indicate that environmental contaminants biomagnify; that is, exhibit higher tissue accumulations as they are transferred to higher trophic levels. Furthermore, this phenomenon is broadly apparent among aquatic species from the simplest phytoplankton and invertebrate larvae to diverse fishes and marine mammals. For example, biomagnification through two trophic levels including a marine alga and an echinoderm has been reported (27). The alga, *Fucus distichus,* was exposed to waterborne 2,6-[^{3}H]dimethylnaphthalene (2,6-DMN) and then fed to sea urchins *(Strongylocentrotus droebachiensis).* Though *F. distichus* readily accumulated 2,6-DMN from seawater, the compound was not metabolized by the alga and was transferred to the sea urchins as the parent compound. Metabolic transformation of 2,6-DMN to 3,4-dihydroxy-3,4-dihydro-2,6-dimethylnaphthalene, mercapturic acid, and 2,6-dimethylnaphthyl sulfates occurred in the sea urchins, although significant quantities of native 2,6-DMN persisted. Presumably, this end product could be then transferred to the next highest trophic level and cause possible further biomagnification.

The concentrations of PCB congeners (selected to span the range of chlorination) in the water column, sediments, and organisms representing four tropic levels in a freshwater lake were determined (28). In all cases no detectable concentrations of PCB congeners (detection limit $\approx$ 0.3 ng/L) were found in the water samples and only low levels ($\bar{X}$ = 5.35 ng PCB/g dry

weight) were found in the sediments. Mean plankton, mollusk, and crustacean concentrations were 0.41, 2.8, and 3.22 ng/g fresh weight, respectively. Finally, at the highest tropic level, the eel, the mean PCB congener concentration in whole tissue was 45.7 ng/g wet weight. The compositions of the PCB mixtures in organisms from the different trophic levels cannot be explained in terms of simple partitioning of PCB congeners between water, sediment, and organisms. Instead, it is likely that biomagnification via consumption of contaminated food contributed significantly to the total PCB concentrations.

In summary, both bioconcentration and biomagnification of xenobiotics in aquatic species are multifaceted due to the myriad of compounds, their potential synergisms and antagonisms, and routes of exposure. Based on the weight of similar mammalian studies, the resultant high body burdens of these compounds can be expected to predispose organisms to a variety of potentially deleterious biological effects. Regrettably, among aquatic species, very little is known about these processes. Consequently, future studies should attempt to relate body burden(s) of xenobiotics and their biotransformation products to specific toxicological effects.

Of the many metabolic reactions occurring in aquatic species, biotransformation reactions of organic compounds have received major emphasis. Oxidative conversions, such as those catalyzed by cytochrome P-450-dependent mixed-function oxidases (MFO), are often the initial and most common reactions. Available information about MFO in aquatic species has been extensively reviewed (1, 29–32). Most studies of Phase I biotransformation reactions are either descriptions of MFO activities in various aquatic species and tissues, or investigations into the effects of varying physical and chemical parameters on MFO activities (30–44). Most studies have centered on fish liver, though extrahepatic MFO activities have been detected in gill, intestine, and heart tissue (45).

Exogenous P-450 induction by 3-methylcholanthrene (3-MC), phenobarbital (PB), pregnenolone-16α-carbonitrile (PCN), and isosafrole (ISF) among mammalian species is well documented. Likewise, among fishes 3-MC, PCN, and ISF activities have been identified in several species. Although conflicting reports exist throughout the literature, close re-examination (46, 47) of earlier studies (48, 49), and the most rigorous recent data (50, 51) have revealed no PB-like cytochrome P-450 induction in fish. This may be due to the lack of structural genes similar to the P-450 isozymes present in mammalian systems, or to differences in gene regulation (1, 31). For example, in an elegant study 21-day old rainbow trout embryos were initiated with either the carcinogens aflatoxin B_1 or N-methyl-N'-nitro-N-nitrosoguanidine and subsequently promoted with a variety of compounds including PB (52). Of the five promoters tested (PB, beta-naphthoflavone, p,p'-dichlorodiphenyltrichlorethane, indole-3-carbinol, 17-beta-estradiol), only PB did not affect

tumorigenesis in either initiation group. Finally, single proteins from fish liver microsomes have been identified that cross-react with mammalian antibodies to PB-inducible P-450 proteins in rat (1). Likewise, antibodies to scup P-450B were found to react with the same rat proteins. The authors suggest that defining the relationships between these anti-P-450 cross-reacting proteins in fish and mammals may ultimately explain the lack of PB response in fish as well as aid in identifying the mechanisms of PB induction.

Among marine invertebrates, the bivalve mollusks have received considerable attention regarding their ability to perform Phase I reactions. There is evidence for the presence of both cytochrome P-450 (53, 54) and flavin-containing monooxygenase (FMO) (55–57) activities in several bivalve species. Recent studies of the Pacific oyster (57) demonstrated significant FMO activity in visceral mass and gill tissue. Indirect evidence of a P-450 system was suggested through observation of NADPH-cytochrome c reductase activity. In addition, NADPH-independent benzo(a)pyrene hydroxylase activity in both oyster visceral mass and gill microsomes suggested a co-oxidation pathway involving a one-electron transfer of oxygen from a lipid hydroperoxide.

All the major Phase II conjugation reactions reported in mammalian species are also present in aquatic species and have been carefully reviewed (58).

Evidence for glyocsylation of the phenolic hydoxyl groups of xenobiotics to form glucuronic acid has been provided from examination of the bile and urine of several xenobiotic-exposed fishes including goldfish, rainbow trout, and salmon (58). In addition, the formation of glucuronide conjugates of the dihydrodiol derivatives of benzo(a)pyrene [B(a)P] and naphthalene in the bile of benthic flatfish following administration of the parent compound has been confirmed (59, 61). Furthermore, glucosides have been found as metabolites of naphthalene in tissue extracts of starry flounder (60), rock sole (60), and coho salmon (62). Among invertebrates, dihydrodiol and phenol conjugates susceptible to β-glucuronidase hydrolysis have been reported in shrimp (63) and lobster (64). Furthermore, excretion of glucosides has been demonstrated in sea urchins (65).

Although sulfated conjugates of organic contaminants have been reported in both aquatic invertebrates and fishes, among invertebrates they appear to be major metabolites (27, 63, 65). Furthermore, among aquatic organisms there is no evidence for hydrolysis of sulfate conjugates back to precursor parent compounds as occurs in some mammals (58).

Mercapturic acids and premercapturic acids (R-cys conjugates) have been reported in several aquatic species (58). Sea urchins fed 2,6-dimethylnaphthalene excreted these compounds (27) as did winter flounder injected with the glutathione (GSH) conjugates of styrene oxide (66). The preliminary steps in mercapturic acid formation, conjugations with glutathione, have, however,

garnered the most attention in recent years. To date, all invertebrate and vertebrate species examined form glutathione conjugates. As expected, significant attention is directed toward the glutathione S-transferases (GST). This enzyme system has been purified from aquatic vertebrates (67–69) and has been found to have similar molecular weights (approximately 25,000 daltons) to GSTs isolated from mammalian species.

GSH content was recently compared among hepatocytes of feral English sole collected from polluted and clean water (70). GSH levels in hepatocytes (0.8–3.2 nmol/mg protein) from contaminated waters were only slightly elevated above clean water values. The authors argue that fishes with low GSH levels may be especially susceptible to chemically induced hepatocarcinogenesis, which is prevalent among English sole inhabiting these waters (3–6). Another species of benthic flatfish, the starry flounder, inhabiting the same waters, has a tumor incidence an order of magnitude lower than that of the English sole (71). It has been reported that the starry flounder had 300% higher GST levels than the English sole (71, 72). It has also been suggested that elevated GST activity may aid in protecting the starry flounder from tumor induction (72). However, GST activities were recently measured in Norwegian flounder collected from three polluted sites, with varying degrees of contamination, and a reference site (73). Elevations in GST activity were observed in fish collected in the polluted sites; however, the only significant differences in activities between the three contaminated sites ran counter to the pollution gradient (i.e. higher GST activity in specimens from sites with lower contamination).

Generally, acetylation reactions in aquatic species have not attracted much attention. The acetylation of amines has been documented in both aquatic vertebrates [dogfish (74), rainbow trout (75), carp (76)] and aquatic invertebrates [snail (77), sea urchin (65, 78) and gum boot chiton (65)]. The most frequently studied animal has been the rainbow trout and xenobiotics metabolized by trout include sulfanilamide, sulfadimidine, 3-trifluoromethyl-4-nitrophenol, 4-nitroaniline, 2-amino-4-phenylthiazole and ethyl m-aminobenzoic acid (58).

The carboxylic acid groups of aquatic contaminants can be conjugated with amino acids prior to excretion. Whereas glycine has been the most commonly studied amino acid in mammalian species, taurine conjugates are the only rigorously identified metabolites documented thus far among aquatic animals (58).

The studies described above are important in that they provide a foundation for obtaining a clearer understanding of the biochemistry of xenobiotic processes in aquatic organisms. It is of obvious scientific interest when associations between compound exposure and activation of a particular biotransformation enzyme are made. Unfortunately, however, most studies of biotrans-

formations have not been linked to biological effects, such as the development of tumors, decreased reproductive success, and alterations in behavior.

EFFECTS OF CONTAMINANTS ON AQUATIC ORGANISMS

Contaminant uptake from the water column is especially significant in marine fishes. These animals typically ingest copious amounts of seawater in connection with their osmoregulatory requirements, whereas freshwater fish drink very little. The effects of water pollutants on osmoregulation in fishes was recently reviewed (15). Most studies have centered on various metals, detergents, ammonia, petroleum, and chlorine. With the exception of acids, the mechanism of osmoregulatory disruption for these compounds involves inhibition of the Na-K-ATPase enzymes in the gill, and possibly the gut and kidney (e.g. 79, 80).

To date, most studies of chemically induced osmoregulatory effects have employed sublethal doses for periods of less than seven days and report statistically significant alterations in plasma or serum ion compositions; however, whether or how these differences relate to altered health status has seldom been addressed. Nevertheless, coho salmon exposed to copper for up to 6 months exhibited reduced survival in seawater and reduced migration as well an inhibition of gill Na-K-ATPase activity (81). Whether osmoregulatory disfunction alone accounted for these effects is unclear. Additional studies are warranted of toxicant effects on osmoregulation, with particular attention directed toward elucidation of specific deleterious biological effects. Of particular interest would be xenobiotic-induced physiological responses of euryhaline fishes that move freely between seawater and freshwater and that reverse the active movement of ions across gill membranes, alter the amount and composition of urine, then excrete and modify epithelial permeability, all in a matter of hours.

Recently, the ability of toxicants to adduct to nucleic acids has received considerable attention. Feral fish (82–87), laboratory fish (88, 89), and fish cell lines (83, 90) exposed to toxicants have all been examined for DNA adducts. DNA was isolated from the livers of brown bullheads collected from sites with high levels of sediment-bound PAHs and from control specimens held in clean water aquaria (87). Aromatic DNA-carcinogen adducts were quantified using ^{32}P-postlabeling techniques. The DNA of fish from the polluted areas invariably contained adducts not present in control specimens. Subsequently, the persistence of specific adducts was demonstrated in both English sole and winter flounder from Puget Sound and Boston Harbor, respectively, and in English sole exposed to extracts of contaminated sedi-

ments. The authors concluded that metabolites of aromatic compounds, such as PAHs, were preferentially adducted to hepatic DNA of fish residing in industrialized areas. Not only has adduct formation been associated with xenobiotic exposure, but the identification of a novel DNA adduct in feral English sole liver tumors was also recently described (85). DNA from neoplastic hepatic tissues of feral fish environmentally exposed to carcinogens was shown to contain the guanine-derived lesion 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua). Concentrations of the lesion ranged from 0.97 nmol/mg to 5.11 nmol/mg DNA. FapyGua was not detected in non-neoplastic tissue and has not been previously reported to occur in the DNA of any other living systems. These findings report the hypothesis that reactive oxygen species damage DNA in living systems and thus play an important role in the formation of neoplastic tissues.

Recently, five species of marine and freshwater fish from both unpolluted (minimal anthropogenic activity) and polluted waters were analyzed for DNA adducts using ^{32}-P-postlabeling techniques (91). Each species, regardless of where it was collected, appeared to contain four to nine qualitatively similar DNA adducts when visualized with thin-layer chromatography (TLC). Unfortunately, individual adducts were not quantified beyond estimating percentage contributions of individual adducts to total adduct levels on TLC. Furthermore, the major adduct patterns on TLC appeared to be species-specific; that is, the adduct pattern for a species was distinctly different from all other species. Finally, total adduct levels in individual species (typically $\approx$14–27 attomol adducts/μg DNA) did not differ significantly between fish from polluted and unpolluted waters. The authors suggested that the $^{32-}$P-postlabeling technique may not be sensitive enough (1 adduct/10^{10} nucleotides) to detect adducts caused by pollution alone. Furthermore, they concluded that the majority of DNA modifications in fish are caused by "natural factors" rather than man-made chemicals. However, their unpolluted site, although substantially less polluted than the polluted sites, was nonetheless somewhat impacted by anthropogenic activities. Water quality was defined by the load of industrial and domestic wastes, which was expressed in population equivalents (p.e.) (polluted water = 2,400,000 p.e.; unpolluted water 5,000 p.e.). Furthermore, the average benzo[a]pyrene monooxygenase activity (pmol/benzo[a]pyrene hydroxide/mg protein/min) in livers of fish inhabiting these waters was also used (polluted site = 22 − 35 ± 17; unpolluted site 3.4 − 6 ± 1.8). In fact, the detection of adducts in the unpolluted site may, in reality, be additional evidence for the exquisite sensitivity of the $^{32-}$P-postlabeling technique. This latter interpretation is consistent with previous studies (87) showing that the DNA from the liver of brown bullheads collected from the polluted Buffalo and Detroit Rivers exhibited several DNA adducts not found in the livers of contaminant-

exposed aquarium fish. With the information presently available, it is difficult to evaluate the significance of the ^{32}P-postlabeling studies to aquatic toxicology.

The significant worldwide discharge of metals into aquatic ecosystems has directed considerable attention toward the metallothioneins (MT) of aquatic vertebrates. Metals bind to these low-molecular weight proteins and, within limits, appear to render the metals less toxic. The liver is frequently the tissue of choice for studying metallothioneins in fish due to its tendency to accumulate metals (15). In a manner similar to mammals, copper-acclimated coho salmon synthesized MT at levels proportional to exposure concentrations (92). Furthermore, MT levels persisted as transfer of acclimated fish to copper-free water resulted in no significant reduction in MT concentration after 4 weeks. Elevated MT levels (269 ± 23 nmol/g liver) occurred in feral fish residing in water contaminated with high levels of copper, zinc, and cadmium [fiftieth percentile heavy metal concentrations (μg/L); Cu 9, Zn 170, Cd 0.7] (93). MT levels decreased linearly with decreasing metal concentrations to 58 ± 14 nmol/g liver in four sampling sites [fiftieth percentile heavy metal concentrations (μg/L); Cu 1, Zn <5, Cd <0.5].

Structural and functional alterations in cellular organelles have provided a means of identifying and characterizing responses of cells to environmental contaminants (94). Furthermore, the identification of such changes increases the potential for understanding the mechanism(s) of a particular toxicant's effects as subcellular changes in organelles will usually precede an integrated cellular, organ, or whole-animal response. An organelle receiving considerable attention among aquatic species is the lysosome (95). Studies with marine mollusks have demonstrated that, similar to mammals, lysosomes react to xenobiotics by increasing or decreasing lysosomal contents (96), the rate of membrane fusion events (95–97), or membrane permeability (95, 98, 99). The significance of these studies to aquatic toxicology is that they represent an attempt to study mechanisms by which toxicants exert an effect. These types of studies go beyond quantifying the uptake and retention of xenobiotics or describing effects on the organismal level (e.g. disease, cancers, etc). Presumably, these discrete alterations on lysosome structure and/or function may be transformed to effects on the animal, population, or ecosystem. Future studies should focus on some of these questions.

Considerable attention is being directed toward the development of both primary and continuous cell cultures from aquatic species. These in vitro model systems retain many characteristics of the in vivo counterparts, yet are easier to maintain and manipulate. Furthermore, the high degree of variability, often due to exogenous factors such as pH, temperature, hormonal status, and age in whole-organism models is avoided or controlled. These systems have the same limitations as mammalian ones in that a cell line is merely the

clonal expansion of a single cell and a primary culture is derived from a single organism. Furthermore, isolated cells, although derived from an organ, cannot be expected to retain all the characteristics of that organ in culture. Thus, consideration must be given in relating the results of these in vitro studies to those from whole organisms. In 1980, 61 fish cell lines representing 36 fish species had been described (100). Presently, over 150 fish and marine mammal, including seal, dolphin, and whale, cell lines have been described and many more are rapidly becoming available. Few aquatic invertebrate cell lines are available, however, probably because their development has not received as much attention as have vertebrates, but also because intrinsic difficulties have proved intractable. The importance of diseases in relation to environmental contaminants in these species may well cause a resurgent growth in the next decade.

Primary cell cultures are often utilized for toxicological studies of aquatic species. As expected, the detoxifying function of the liver frequently makes primary hepatocyte cultures the tissue of choice. A recent review (101) considered the status of isolated fish hepatocytes and their efficacy as model systems for research in toxicology. Although relatively few toxicological studies have utilized primary hepatocyte cultures, this approach was deemed to have considerable potential. The rationale was that, in a manner analogous to mammalian models, subsequent studies will allow for the determination of the mechanism(s) of action of xenobiotics, the role of metabolism in mediating toxicity, and the toxicity of complex mixtures to aquatic species. For example, trout liver cells were utilized to establish short-term primary cultures exhibiting cell-to-cell communication (102). With transmission electron microscopy, the in vitro formation of biliary ductules, plasma membrane specializations forming junctional complexes, and canaliculi characteristic of normal trout liver were visualized. Thus, in vitro cell populations exhibiting morphological relationships resembling in situ conditions can now be used to study the effects of toxicants on cellular interactions in fish liver.

The understanding of the relationship between toxic chemicals and disease among aquatic organisms has benefited from both laboratory and field studies. Rainbow trout were exposed to either 2,3,7,8-tetrachlorodibenzo-p-dioxin or Aroclor 1254 and then challenged with infectious hematopoietic necrosis virus (103). Following virus challenge, virally induced histopathological lesions were more severe and occurred more frequently in toxicant-exposed fish as compared to controls. Likewise, the effects of two marine pollutants, hexachlorobenzene and pentachlorophenol (PCP) on the immune system of a marine polychaete have been determined (104). Although both bioconcentrated and caused an elevation in a bactericidal glycoprotein induction, PCP exposure also caused a reduction in the numbers of amebocytes capable of forming rosettes with formaldehyde-treated rabbit erythrocytes.

These and similar studies have demonstrated that animals compromised by toxicant exposure appear to have reduced resistance to parasites and diseases. Further studies are necessary before any definitive conclusions can be drawn.

Numerous surveys have been published of the distribution of various types of disease among aquatic species in areas heavily impacted with aquatic pollution. We refer the interested reader to the published proceedings of the symposium Toxic Chemicals and Aquatic Life: Research and Management (105–110). These proceedings contain detailed reviews of the relationship of aquatic pollution and prevalences of disease from a variety of areas including the west coast of North America (8, 105), North Atlantic coast including Chesapeake Bay (106), southeastern North America (107), Europe (108), and Japan (109). Also included in this volume is a review of shellfish diseases in relation to toxic chemicals (110). In total, these studies document the global distribution of aquatic diseases and their relationship to environmental contamination. Furthermore, from these studies at least one generality emerges. Fin erosion has been reported to occur in most sites high in anthropogenic pollution (5–7, 105–111). In fact, when more detailed studies (reviewed in 106, 107) are conducted, populations with high incidences of fin erosion often exhibit a variety of microbial diseases including parasitic infections, lymphocystis, herpes-like viruses, baculovirus, and vibriosis. One study investigated infestations of a colonial ciliate (*Epistylis* sp.) on freshwater and marine fishes in habitats rich in organic wastes (112). The ectoparasite was found to be more heavily infested on skin of host fishes from contaminated waters than from clean waters. Moreover, a secondary systemic bacterial infection *(Aeromonas hydrophila),* which caused hemorrhaging lesions, occurred with greater frequency in parasitized fishes residing in the polluted waters. Likewise, a histozoic myxosporidan protozoan *(Myxobolus lintoni),* capable of causing protruding growths in *Cyprinodon variegatus,* normally occurs in stressed populations (112). Infections have been reported to occur in polluted areas such as Galveston Bay (113) and the coasts of Mississippi and Louisiana (107, 112). Perhaps the etiology of fin erosion may include depression of immune response function due to contamination and a concurrent increased susceptibility to pathogens in the environment. Furthermore, as observed (7), the ever-increasing numbers of contaminants discharged into aquatic environments often make it impossible to assign specific causes to a particular agent or combination of agents.

Perhaps one of the most rapidly advancing areas of aquatic toxicology is chemical carcinogenesis. A critical review of the literature pertaining to cancerous diseases in aquatic animals was provided in 1986 (114). Subsequently, as detailed below, some of the most valuable recent studies have come from laboratories embracing the newly developed techniques of molecular biology.

Although target organs for neoplasia in fishes, (e.g. liver, stomach, and kidney) are functionally similar to mammalian systems, there are significant structural differences. For example, the frequently studied rainbow trout liver does not contain the repeating mammalian mosaic of portal and central veins (115, 116). Furthermore, fish liver often contains exocrine pancreatic tissue that functions independently of the liver. Moreover, many invertebrates contain a hepatopancreas that is functionally an integrated organ (117). An extensive characterization has been completed of the histopathologic spectrum of idiopathic lesions found in the liver of feral English sole from Puget Sound, Washington (118). A variety of lesions were described, including putative preneoplastic lesions and hepatocellular and biliary neoplasms. Morphologically, these lesions resemble those induced by hepatocarcinogens in laboratory exposures of fish and rodents. Statistical analysis of the patterns of co-occurrence of these lesions suggests the existence of morphologically identifiable steps, indicative of progression toward hepatic neoplasms in a manner analogous to rodent models. Furthermore, this was the first study in which it was possible to demonstrate close morphological congruity between a set of idiopathic hepatic lesions in a feral population and an established series of hepatic lesions inducible in laboratory rodent models following hepatocarcinogen administration.

A valuable resource for studies of cancer in lower vertebrates is the Registry of Tumors of Lower Animals, established in 1965 to facilitate study of neoplasms and related disorders in invertebrates and poikilothermic vertebrates (119, 120). The registry is a collaborative endeavor between the National Cancer Institute and the Smithsonian Institution.

In the late 1970s, the rainbow trout embryo exposure model was developed for studies of hepatocarcinogenesis (121–124). Trout embryos, at the eyed-stage of development, were exposed to chemical carcinogens via the surrounding water. The major advantages of this type of exposure regimen include passive uniform exposure, minimum carcinogen requirement, safety, convenience, and substantiation of the adequacy of a single exposure for tumor-induction studies. The utility of the model, as suggested by the authors, was for tumor promotion and inhibition studies, embryonal carcinogenesis studies, and as a combined carcinogenesis and teratogenesis model (124). Subsequently, many such studies have been successfully conducted by the authors themselves, as well as by others utilizing rainbow trout or modifying the system for use with other fishes. A significant drawback to the model was the inability to expose embryos to an adequate dose of highly water-insoluble carcinogens. To alleviate this obstacle, the trout embryo carcinogenesis assay was modified by injecting small volumes of carcinogen directly into the perivitelline space of the trout egg (125). This model was further refined (126) so that carcinogens solubilized in DMSO were injected directly into the yolk

of the embryo. Significantly, because of these modifications, the various laboratories were then able to induce tumors in fish utilizing compounds such as B(a)P. A major weakness remained, however, in that a high mortality rate was experienced with the trout microinjection technique. This problem was recently circumvented by shifting the time of microinjection to the sac-fry stage of development (127). In these studies induction of liver tumors was achieved without apparent loss of sensitivity to the carcinogenic effects of aflatoxin B_1. Finally, this methodology has been successfully modified for the injection of chemicals into small fishes (128).

A second model receiving considerable attention has been the small aquarium fish (129, 130). Of the dozen or so species utilized, the most popular are the Japanese medaka *(Oryzias latipes),* guppy *(Poecilia reticulata),* zebra fish *(Branchydanio rerio),* and the platty fish and swordtails *(Xiphophorus maculatus, Xiphophorus helleri).* The time to sexual maturity is rapid and 3–5 generations can be reared in a single year. Their small size (3–5 cm when sexually mature) allows large numbers of fish to be maintained throughout their entire life cycle in a small laboratory. For example, in our laboratories we routinely raise medaka at densities of 1–2 fish per liter of water. Carcinogen exposure can be accomplished in a number of ways including diet, skin painting, embryo microinjection, repeated short-term (hours) or long-term continuous exposures, and controlled field exposures. Tumor induction can occur in as little as 5 weeks, depending on the species and compound, and the entire animal can be mounted on a paraffin block or frozen for later sectioning and histopathological/histochemical analysis. A major limitation of the small aquarium fish model, however, is the minimal amount of tissue available for biochemical and molecular study.

In recent years tumors have been induced in most major tissues of small aquarium fishes with a variety of compounds. The histological progression of several of the neoplasms have been studied and found to be remarkably similar to human neoplasms. For example, continuous exposure to diethylnitrosamine (DEN) caused ductular neoplasms of the exocrine pancreas of rivulus *(Rivulus marmoratus)* (131) and brief (2 hr) exposure to methylazoxymethanol acetate (MAMA) caused acinar cell neoplasms in the guppy *(Poecilia reticulata)* (132). Likewise, various types of liver neoplasms common to mammals, including trabecular hepatocellular carcinomas, poorly differentiated hepatocellular carcinomas, and cholangiocellular carcinomas, have been induced in medaka with DEN (133). Intraocular neoplasms have been induced by methylazoxymethanol acetate exposure in medaka (134). Although classified as medulloepitheliomas, components of these neoplasms have remarkable similarity to retinoblastoma, a malignancy of the eye occurring in young children. Given the current interest in this disease, its accompanying suppressor genes, and the apparent genetic predisposition for

cancer in humans (135), studies are currently underway to determine if medaka could serve as a suitable vertebrate model for studying chemical induction and pathogenesis of retinoblastoma. Finally, the induction and pathology of fibrosarcomas and rhabdomyosarcomas has been reported in xiphophorine fishes following exposure to N-methyl-N-nitrosourea (136). Thus, their many similarities to human and laboratory animal cancers make tumors of small aquarium fishes excellent companion models for the study of cancer.

Recently, a number of studies with fish have indicated that the expression of certain oncogenes can be correlated with the increased incidence of neoplasia among fish inhabiting polluted waters (137, 138).

Small aquarium fishes of the genus *Xiphophorus,* including platyfish and swordtails, have been developed as an excellent laboratory model for the study of oncogenes and suppressor genes. Furthermore, they should effectively serve as a model for similar studies of other impacted populations in polluted environments (139–143). Wild fish contain a normal gene (*Tu*) and regulatory or suppressor genes (*R*) for *Tu* that protect fish from neoplasia. The *Tu* gene is apparently responsible for critical events in early development but its expression must be regulated in later life to prevent the development of melanoma. Elegant hybridization experiments with wild populations in the laboratory allowed for elimination of the *R* systems permitting expression of *Tu* and the resultant neoplasia.

It has been argued (144) that comparisons of oncogene sequences from divergent species are an important step toward understanding the evolution and function of cellular oncogenes in neoplasia. For example, the c-*myc* gene was isolated from a rainbow trout genomic library by hybridization to v-*myc* under relaxed conditions and a subclone was partially sequenced (145). The rainbow trout c-*myc* gene displayed extensive homology to exons II and III of the c-*myc* gene of chicken and human. However, c-*myc* did not hybridize with the DNA from a number of fish tumors including lymphosarcomas from pike, and hepatocellular carcinomas from medaka and white perch. Since amplification and rearrangement of the *myc* gene was not detected in these tumors, the blots were rehybridized to other viral oncogenes including v-*abl,* N-*myc,* H-*ras,* v-*ets, v-myb,* v-*erb*-A and v-*erb*-B. No evidence of amplification or rearrangement of these genes was apparent (144).

Studies of carcinogenesis in fishes continue to grow in importance as it becomes apparent that novel aspects of the biology of fish can be utilized to increase our understanding of oncogenesis among all species. For example, the critical life stages (embryo) can be manipulated much easier than rodent models. Furthermore, with species such as the rainbow trout, 1,000s of genetically similar offspring can be produced in a single spawn, allowing for elaborate testing regimens as well as abundant tissue for study. Clones of

homozygous diploid fish offer the opportunity to use groups of fishes, with minimal biological variability, to establish quantitative dose-response relationships in carcinogenicity testing (146). For the most part, besides humans, fishes residing in polluted environments are the only vertebrate species that exhibit high incidences of neoplasia. Consequently, toxicological studies can be performed that are epidemiologic in nature, involving invasive sampling techniques. Understanding the causes and mechanisms regulating oncogenesis in feral populations, however, will continue to be the most demanding. Wild populations are exposed to complex suites of anthropogenic chemicals, as well as subject to the influences of metabolites generated from synergistic, antagonist, and other reactions. Future studies must strive to forge links between exposure, metabolism, and the biochemical mechanisms responsible for neoplastic changes in tissues. These studies will provide another avenue for understanding carcinogenesis.

Behavioral toxicology continues to receive modest attention among aquatic toxicologists. As suggested by the studies discussed below, inappropriate behavioral responses may contribute to the premature demise of organisms or populations with a potentially equally devastating effect on the ecosystem. The initial visible response of organisms to environmental perturbations is often a change in their behavior. Behavioral responses can be adaptive, representing an attempt by the animal to mitigate the effects of environmental disturbances (e.g. avoidance behavior) or maladaptive, in which a significant departure from the behavioral norm occurs (e.g. alterations in courtship behaviors resulting in failure to reproduce; 147). Maladaptive behaviors, while often not immediately life-threatening, can be particularly insidious as the potential for long-term individual or species survival is reduced.

The ontogeny of behavior was recently examined (148, 149) in the coho salmon following embryonic exposure to B(A)P. While hatching success was not compromised, significant differences in the time to hatching and pattern of hatching occurred. Fish exposed prior to organogenesis hatched earlier than controls and fish exposed during the organogenesis hatched later than controls. Furthermore, the successful emergence of coho salmon alevins from artificial redds was significantly reduced by about 40%, as was the ability of these fish to perform the normal upstream orientation and swimming behaviors characteristic of the control fish. However, no differences in weight, total length, or fin development were observed. Exposed fish, regardless of time of exposure, foraged significantly more (28% to 44%) than controls. Previous studies (150) had demonstrated that B(A)P primarily concentrated in the central nervous system (CNS) and eye tissues of fish and that continuous exposure of trout to B(a)P caused ocular, neural, and skeletal defects (151). Therefore, impairment of prey capture was expected because of the role of the CNS and vision in performing this behavior.

Similar impairment of the early-life feeding behaviors of largemouth bass following pentachlorophenol exposure has been documented (152). Fish reared in concentrations of 67μg PCP/L and greater performed significantly fewer feeding acts (orientations, bites) and had a lower rate of prey capture than did control fish.

Studies have been conducted on the effects of oil and oil spills on birds (153, 154). The histopathology of Cassin's auklets following external application of Santa Barbara crude oil and beached common murres recovered from an accidental oil spill of bunker C fuel oil was examined (153). In both cases, birds exhibited hepatocellular dissociation and hemosiderosis, renal tubular necrosis, and hemolytic anemia. In a subsequent study (154), breeding wedge-tailed shearwaters were exposed to oil (0.1–2.0 ml) via external application to the breast plumage or orally 30 days prior to egg laying. Application of oil resulted in reduced lay, lower hatching success, and reduced breeding success. Birds dosed orally exhibited similar effects but to a lesser extent. These recent studies may conclusively demonstrate that even small amounts of oil are capable of profound pathological and behavioral effects and suggest serious consequences for the population due to reduced reproductive success. Finally, these studies are reminiscent of nearly four decades of work that elucidated the deleterious effects of DDT and PCBs on fish-eating birds (e.g. 155–159). While not reviewed here, these earlier studies may be of use in the design and testing of hypotheses of oil-related impacts on bird populations.

CONTAMINANT EFFECTS AT THE ECOSYSTEM LEVEL

Studies of the highest level of organization, the ecosystem, are especially difficult because of our general inability to measure the multitude of conflicting variables. While the effect of a chemical contaminant on a particular species might ultimately be ascertained by methods discussed above, it is much more difficult, if not impossible, to determine what effect(s) will occur at the ecosystem level.

In response to these problems, studies of microcosms and the increasingly more complex meso- and macrocosms have provided valuable insight into contaminant effects on aquatic ecosystems. These models are of sufficient size and complexity that they closely mimic the natural environment and are easily duplicated. Microcosms are analogous to the agronomist's small field plot and form a bridge between the laboratory and the natural environment (160). Furthermore, in contrast to traditional laboratory studies, it is possible to study effects on a wider range of organisms as well as secondary effects such as predator-prey interactions, algal blooms, and dissolved oxygen concentrations.

The recent literature contains many examples of the effects of individual compounds or mixtures on microcosms. Nonetheless, the most relevant studies involve either simultaneous assays of toxic effects on single species and microcosms or comparisons between microcosms and toxic discharges into the natural environment. For example, single-species algal assays, synthetic microcosms, and experimental ponds exposed to similar concentrations of the herbicide atrazine were studied (161). A comparison of concentrations that reduced algal activity or biomass to 50% of control values (EC50) was determined. For the eight algal species examined, the mean species EC50 values for ^{14}C uptake ranged from 37 to 308 μg/L, which was very similar to the values obtained from the microcosms (103–159 μg/L) and experimental ponds (100μg/L). The authors concluded, based on the similarity of results, that a combination of single-species assays and synthetic microcosms provided a reasonable estimate of atrazine concentrations capable of producing similar effects in natural communities.

The responses of macroinvertebrate/protozoan microcosms exposed to a complex industrial effluent (principle components included chlorides, ammonia, phenols, lead, and copper) have been compared with observed effluent effects in a receiving stream. The microcosms were developed on artificial substrates anchored to stream bottoms for 30 days. Of the 35 macroinvertebrate taxa examined, only mayflies were adversely affected by the effluent. Four mayfly taxa showed significant reductions in population density at 1.0% treatment and four additional taxa were reduced at 10%. Furthermore, organisms from another taxa, the chironomides, exhibited significantly higher population densities at the 0.1% effluent concentration level and above. Subsequent field studies confirmed that data from the microcosms could predict which indigenous species would be lost and which would be stimulated at particular effluent levels in the receiving stream (162). Mayflies were either absent or had greatly reduced densities at stream sampling sites where effluent concentration ranged from 3.5 to 14.1% and *Paratanytarus* sp., comprising over 95% of the Chironomidae, were found at significantly higher densities downstream of the effluent outfall. The authors speculated that effluent stimulation of their peripytic food sources was responsible for the increased chironomid densities.

The lipid composition of the digestive glands of bivalves was recently used for comparison of field and mesocosm responses to environmental contaminants (163). Compared to a reference site, elevations in both lipid content and lipid:protein ratios of the digestive glands of *Mytilus edulis* were observed at three highly contaminated sights. Furthermore, populations of *Carcinus maenas* showed similar elevations, but only at a single highly contaminated site. Conversely, in mesocosm experiments only *M. edulis* from the highest dose basin exhibited elevated lipid content and lipid:protein ratios. Further-

more, *C. maenas* in the medium dose basin showed a decrease in both parameters. The authors attributed the differential response of field and mesocosm populations to the differences in metabolic capacity for detoxification brought on by differences in trophic transfer.

PERSPECTIVES FOR THE FUTURE

Studies on the effects of toxic substances on aquatic life have increased substantially in the past decade. There has also been an increasing emphasis on understanding mechanisms governing toxic effects at the cellular and subcellular level. Studies of this type are essential for the future because large gaps in our understanding of mechanistic changes and the processes that govern them often preclude linking exposures to the manifestation of biological events, such as cancer and other disease conditions.

Additional challenges remain that are both intellectually stimulating and of coinsiderable scientific value. These relate to establishing association between exposure, uptake and metabolism, and biological effects in individual organisms. The more imponderable, yet important, problem of determining and understanding effects on populations will undoubtedly bc a slow and difficult process. Beyond this goal lies the even more perplexing problem of determining and understanding the dynamic interactions of ecosystems and attendant toxicant-induced alterations. Clearly, this is one of the greatest and most formidable challenges facing the aquatic toxicologist. Right now, the "tools" allowing us to probe the workings of ecosystems are both primitive and elusive. Moreover, the greater issue of elucidating xenobiotic-induced effects on these complex systems is mostly a distant but important goal. Finally, the development of predictive models, to aid our understanding at all levels of organization, holds great promise (21, 164–167). However, as present models are refined and additional ones developed, an equivalent effort must be placed in the validation of these models.

Overall, the next decade should prove to be one of great accomplishment if fundamental advances are made in filling the gaps in knowledge described above. However, the achievement of this objective will certainly depend on progress made in developing techniques to elucidate the nature of xenobiotic interactions at all levels of aquatic biological organization, ranging from essentially molecular events to alterations in ecosystems. Whichever course is charted, the future holds hope that substantive advances in knowledge will indeed take place in the ever-developing field of aquatic toxicology.

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